ANALYSIS OF SEX DETERMINATION IN NILE TILAPIA (*Oreochromis niloticus* L.): A MOLECULAR GENETICS APPROACH

A thesis submitted for the Degree of Doctor of Philosophy

By

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Dedicated

To

SAYEMA SAYEED

My beloved wife and best friend

My Mother and Mother in law
and
My Father and Father in law

DECLARATION

I declare that this thesis has been composed by myself based on my own research. It has neither been accepted nor submitted for any other degrees. All information from other sources has been properly acknowledged.

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ABSTRACT

Seven families of XX and YY homozygous *Oreochromis niloticus* were produced by mitotic gynogenesis from XY neofemales and their genetic status was verified by multilocus DNA fingerprinting and progeny testing.

Two of these gynogenetic families and their corresponding diploid controls were used with 64 AFLP primer combinations in different levels of screening (XX/YY grand pool; XX/YY family pool; XX/YY gynogenetics and XX/XY control individuals) to search for sex-linked or sex-specific markers. Grand pool screening did not reveal any sex-linked markers. Subsequent family pool and individual level screening identified four sex-linked AFLP markers from two primer combinations, three Y-linked (*Oni*Y425, *Oni*Y382, *Oni*Y227) and one X-linked (*Oni*X420). Two of these (*Oni*X420, *Oni*Y425) were shown to be allelic. Single locus PCR markers were developed for all of those markers. Linkage analysis of these markers and the sex locus within the source families revealed tight linkage, with estimated map distances of 13cM, 17cM and 20cM for *Oni*Y382, *Oni*Y227 and *Oni*X420/*Oni*Y425 respectively. However, these sex-linked AFLP markers failed to consistently identify sex in unrelated individuals.

To develop an effective system for parentage analysis in normal and gynogenetic progeny, AFLPs and multiplexed polymorphic microsatellite loci were investigated. Both were found to be effective, but microsatellites were more appropriate since they are codominant and some loci showed high gene-centromere recombination rates, suitable for discriminating meiotic from mitotic gynogenetics, while AFLPs are dominant markers.

Spontaneous diploidization of the maternal chromosome set (SDM) was observed in

gynogenetic progeny of one XY neofemale. Maternal inheritance and ploidy status were verified by multilocus DNA fingerprinting and chromosome karyotyping.

Close genetic linkage between the red gene and an autosomal sex-reversal gene(s) in gynogenetic progeny and influences of autosomal sex-reversal gene(s) producing males in a fully inbred XX clonal line were previously reported in *O. niloticus*. To test if the same autosomal sex-reversal locus was responsible in both cases, a series of test crosses was carried out involving XX clonal neomale(s) and homozygous red females. The results indicated the involvement of more than one autosomal sex-reversal locus, one of which is linked to red body colour.

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LIST OF ABBREVIATIONS

ABI	applied biosystems
ADA	adenosine diamine
AFLP	amplified fragment length polymorphism
AP-PCR	arbitrary primed PCR
APS	ammonium persulfate
ATP	adenosine tri-phosphate
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumen
BSA	bulked segregant analysis
CA	California
CAPS	cleaved amplified polymorphisms
cDNA	complimentary DNA
cM	centiMorgan
Co	cobalt
Cs	cisium
DAF	DNA amplification fingerprinting
dATP	2'-deoxyadenosine 5'triphosphate
dCTP	2'-deoxycitidine 5'triphosphate
ddNTP	dideoxynucleoside 5'triphosphate
df	degrees of freedom
dGTP	2'-deoxyguanosine 5'triphosphate
DM	doublesex/mab-3
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside 5'triphosphate
DOP-PCR	degenerate oligonucleotide primed PCR

dTTP	2'-deoxythymidine 5'triphosphate
EDTA	ethylenediamine tetraacetic acid
EMBL	European molecular biology laboratory
ESD	environmental sex determination
FAM	fluorescein phosphoramidite NHS ester
FAO	food and agricultural organization
FISH	fluorescent *in situ* hybridization
g	gram/gravitational force
GMT	genetically male tilapia
GSD	genetic sex determination
HEX	hexachloro-fluorescein phosphoramidite NHS ester
HMG	high mobility group
hr	hour
IAM	infinite allele mutation
Inc.	Incorporation
indel	insertion deletion
IoA	Institute of Aquaculture
IPTG	isopropyl-β-D thiogalactopyranoside
JOE	carboxy-4', 5'-dichlore-2',7' dimethoxyfluorescein
kb	kilo bases
l	litre
LB	luria bertini
LOD	logarithm of likelihood
m	metre
M1	male 1
M2	male 2
Mb	mega bases
Mbq	mega bequerel
MFR	modified Fish ringers
min	minute
MIS	Mullerian inhibiting substance

ml	millilitre
mm	millimetre
mM	millimolar
MT	methyl testosterone
mtDNA	mirochondrial DNA
NCBI	national center for biotechnological information
NED	N-carboxyethyl- fluorescein phosphoramidite NHS ester
ng	nano gram
NHS	N-hydroxysuccinimide ester
nm	nano metre
nt	nucleotide
NY	New York
Obc	outbred clone
Oni	*Oreochromis niloticus*
pb	polar body
PCR	polymerase chain reaction
PE	perkin elmer
pg	pico gram
PIL	personal license
PIT	passive integrated transponder
PPL	project license
RAPD	random amplified polymorphic DNA
RC	relative to control
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNAse A	ribonuclease A
RT-PCR	reverse transcriptase PCR
SC	synaptonemal complex
SD	standard deviation
SDL-2	sex determining locus-2
SDM	spontaneous diploidization of maternal chromosome

SDS	sodium lauryl sulphate
sec	second
SF1	steroidogenic factor 1
SIPs	Sry interacting proteins
SLAM	sex-linked AFLP marker
SNP	single nucleotide polymorphism
SOX	SRY like HMG-box
spPCR	single primer PCR
SRY	sex determining region on Y chromosome
SSC	sodium chloride sodium citrate
SSCP	single stranded conformation polymorphism
SSM	slipped strand mispairing
STE	sodium tris EDTA
STS	sequence tagged site
TAE	tris acetic acid EDTA
Taq	*Thermus aquaticus*
TBE	tris boric acid EDTA
TE	tris EDTA
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TEN	tris EDTA sodium chloride
TET	Tetrachloro fluorescein phosphoramidite NHS ester
TPE	tris phosphoric acid EDTA
TSD	temperature sex determination
UK	United Kingdom
UNH	university of New Hampshire
USA	United States of America
UV	ultra violet
V	volt
V.	version
v/v	volume/volume
VNTR	variable number of tandem repeats

W	watt
w/v	weight/volume
WT1	Wilm's tumor 1
X-GAL	5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside
YAC	yeast artificial chromosome
$\chi 2$	chi-square
°C	degree centigrade
µg	micro gram
µl	micro litre
µM	micro molar
µW	micro watt

Chapter 1

General Introduction

1.1. Sex determination

Sex can be defined as the property or quality by which organisms are classified as female or male on the basis of their reproductive organs and functions (American Heritage Computer Dictionary ®, 2002). Sex is expressed in two processes: sex determination and sex differentiation. Sex determination is responsible for the mechanisms that direct sex differentiation, while sex differentiation is responsible for the development of distinct types of gonads, testes or ovaries from the undifferentiated or bipotential gonad (Hayes, 1998). In dioecious species, those with two morphologically distinct sexes, reproduction must be preceded by sex differentiation. For those species that lack the ability to alter their sex during life, sex differentiation occurs when the developing zygote or embryo commits to development as one or the other sex. This makes sex differentiation one of the most crucial developmental decisions.

The numerous available information on the mechanisms of sex determination has identified two broad categories: genetic sex determination (GSD) and environmental sex determination (ESD), into which the majority of species fall. GSD is a mechanism by which genes directly determine whether the bipotential gonads differentiate into testes or ovaries without external (environmental) influences, such as in humans. Whereas, in ESD, environmental factors experienced by either the developing zygote or embryo determine the sex of individuals, such as the incubation temperature of eggs determine the sex of individuals in some fish, amphibians, lizards, many turtles and in all crocodillians (Bull, 1985; Hayes, 1998). Thus, in ESD each individual has potential to develop into females (ovarian differentiation) or males (testicular differentiation).

Therefore, in ESD the sex determination may be influenced by environmental factors, however, sex differentiation is likely to be controlled by genetic mechanisms (Hayes, 1998).

1.1.1. Genetic sex determination (GSD)

GSD is characterized by a consistent genetic difference between the sexes, unlike the ESD in which there can be no such difference (Bull, 1985). The direction of sex differentiation in GSD is controlled genetically, such as one sex has a gene or set of genes that the other lacks. In GSD, sex-determining genes initiate the sequence of events that result in gonadal differentiation rather than direct involvement in the process of sex differentiation (Hayes, 1998).

The GSD system is principally based on the evidence of presence or absence of heteromorphic sex chromosomes. In these cases, sex determining genes are located on a chromosome which has evolved in such a way that it can be morphologically distinguishable (heteromorphic) from its homologues by cytological analysis (such as X and Y chromosome in humans). The evolution of sex chromosomes often involves the loss or degeneration of genetic material resulting in heteromorphic homologues (such as the Y chromosome is smaller than the X chromosome in humans; Graves, 1998, 2002). The best understood of the various GSD systems are those found in the free-living nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and in humans. In both *D. melanogaster* and humans, normal individuals with two X chromosomes (XX) develop into females, and those with a single X chromosome and a single Y chromosome (XY) develop into males (Bridges, 1925; Ford *et al.*, 1959; Jacobs and

Strong, 1959; Welshons and Russell, 1959). A similar situation exists in *C. elegans*, except that there is no male-specific Y chromosome, with XX individuals developing into hermaphrodites and X0 individuals developing into males (Hodgkin, 1987).

However, there is an important difference between the mechanism of sex determination in humans and that found in both *D. melanogaster* and *C. elegans*. In humans, it is the presence or absence of the small, heterochromatic, Y chromosome, which determines sex (Ford *et al.*, 1959; Jacobs and Strong, 1959; Welshons and Russell, 1959). The presence of a normal Y chromosome acts to force the embryonic gonads to differentiate into testes, which subsequently results in male development (Hacker *et al*., 1995). This form of sex determination is referred to as an XX/XY system, and is widespread in mammals. In contrast to this form of XX/XY system, sex in both *D. melanogaster* and *C. elegans* is determined by the ratio of X chromosomes to sets of autosomes (Parkhurst and Meneely, 1994). In these species, individuals with a single X chromosome and two sets of autosomes (X/A=0.5) normally develop into males. Those with two X chromosomes and two sets of autosomes (X/A=1) normally develop into females in *D. melanogaster* or into hermaphrodites in *C. elegans*. This form of sex determination is referred to as XX/X0 system. Thus, the *D. melanogaster* Y chromosome is unrelated to sex determination, although it is required for male fertility (Bridges, 1925; Parkhurst and Meneely, 1994). Thus, the presence of a Y chromosome is, in itself, not diagnostic of a XX/XY sex determination system, where sex is determined by the presence or absence of the Y chromosome.

XX/XY systems of sex determination are widespread in placental mammals and are closely related to the mechanism of sex determination that is found in marsupial

mammals. A qualitatively similar situation is also found in birds, except that avian sex determination is referred to as a WZ/ZZ system. In this system, the presence of the W chromosome results in female development, with ZZ individuals developing into males. Thus, both XX/XY and WZ/ZZ systems, sex is determined by the presence or absence of a single chromosome.

In addition to the XX/XY, WZ/ZZ and XX/X0 systems of sex determination outlined above, there are a number of other mechanisms by which GSD can occur, although they are comparatively rare among metazoans. Perhaps the most widespread of these other mechanisms is haplo-diploidy, in which females develop from fertilized eggs and males from unfertilized eggs. This method of sex determination has been found in several insect groups (Bull, 1983), monogeneans, rotifers (Parkhurst and Meneely, 1994), and the oxyurid nematodes (Adamson, 1989) and is believed to have evolved on only twelve separate occasions. Further examples of GSD mechanisms include the multi-factor systems that have been identified in certain species of fish. A good example of such a system is provided by the southern platyfish, *Xiphophorus maculatus*, in which sex determination is controlled by three commonly occurring factors. These factors are termed W, X and Y and result in three female genotypes (WX, XX and WY) and two male genotypes (XY and YY) (Belamy and Queal, 1951; Gordon, 1952).

1.1.1.1. Sex determining genes

In mammals one main gene is involved in the sex determination of almost all species. The primary sex determining gene SRY (sex determining region, Y chromosome), located on the Y chromosome (Sinclair *et al*., 1990; Koopman *et al*., 1991), has been

identified in all mammals except two species of vole that lack a Y chromosome (Just *et al*., 1995a,b). These species are unique in that the male sex is determined without SRY and without a Y chromosome. Current research indicates that certain sex determination regulatory genes have evolved rapidly, whereas others are fairly conserved (Marin and Baker, 1998; Graves, 1998, 2002). For example, information for *Drosophila* indicates that part of the sex determining pathway (dsx and tra-2) has been controlled by exactly the same hierarchy for at least 60 million years, whereas genes involved in other parts of the pathway have been added recently (sxl) (Marin and Baker, 1998). A similar pattern has developed in vertebrates; SRY is a recent addition whereas downstream genes such as Sox9 have been involved for much longer time periods (Marin and Baker, 1998; Koopman, 1999).

The SRY gene encodes a testis-specific transcript that has a conserved DNA-binding motif in the nuclear high-mobility group protein, HMG (Sinclair *et al*., 1990). Other HMG box proteins of this group have been isolated by virtue of their homology with SRY, and those with greater than 60% homology with the SRY HMG box are referred to as SOX genes (reviewed by Parker *et al*., 1999).

There are reports on genes homologous to SRY in birds, reptiles and fish, but no sex specificity has been found in those groups (Wachtel and Tiersch, 1994). Moreover, sequences corresponding to the conserved motif of SRY have been detected in 23 other non-mammalian species representing four vertebrate classes (Tiersch *et al*., 1991). An SRY type gene has also been detected which is expressed in the pituitary gland of rainbow trout *Oncorhynchus mykiss*, again with no sex specificity identified (Ito *et al*., 1995).

The involvement of SOX9 in mammalian sex determination has already been established (Parker *et al*., 1999). Male-specific expression of Sox9 (SOX9 equivalent in human) has also been reported in chicken and turtle genital ridges. The expression of Sox9 has also been reported in rainbow trout testis (Takamatsu *et al*., 1997). The mode of action of Sox9 is still unclear, but it seems that Sox9 is closer to the origin of the common sex determining system than SRY (Nomura *et al*., 1995).

1.1.2. Environmental sex determination (ESD)

ESD is mainly observed in lower vertebrates such as in some fish, amphibians and lizards, many turtles and all crocodillians. Several environmental factors have been found to influence ESD including temperature (temperature dependent sex determination, TSD), pH and social environment. Although there were several reports on pH-dependent sex determination (Heiligengerg, 1965; Rubin, 1985; Römer and Beisenherz, 1996) and social environment-dependent sex determination (Shapiro, 1979; Fricke and Fricke, 1977; Francis and Barlow, 1993), TSD has been investigated more extensively. TSD has been demonstrated in three of the five major reptile lineages, being universal in the crocodilians, common in turtles and rare among lizards (Bull, 1983). So far, three different relationships between egg incubation temperature and sex ratio have been found in reptiles. Two lizard species and one species of alligator produce females at low temperatures and males at high ones, while in many turtle species this situation is reversed. There are also a number of turtle species and a species of crocodile in which males are only produced at intermediate temperatures, with females produced at both high and low temperatures (Bull, 1985). In amphibians, high temperature results in

100% males in all frogs studied, but either all males or females depending on the species in salamanders. Despite these clear temperature effects, it is not likely that temperature is important in normal sex determination in amphibians, because all these effects were obtained by exposure to temperatures that are not normally experienced by the species (reviewed by Hayes, 1998). Further examples of ESD systems have been identified in a number of teleost fish species (Francis and Barlow, 1993). ESD systems have also been identified in several tilapia species, which will be discussed later in this Chapter.

In contrast to the wealth of genetic information about GSD, relatively little is known about the genes involved in ESD. However, it has recently been observed that incubation temperature in reptiles appears to act on genes coding for steroidogenic enzymes and sex steroid hormone receptors (Crews, 1996). In addition, it has been observed that the sex ratio of turtles can be manipulated both by temperature and by sex steroid hormones. This suggests that incubation temperature in reptiles serves as the physiological equivalent of the sex steroid hormones of mammals (Crews, 1996).

1.2. Sex determination and differentiation in fish

1.2.1. Sex determination

Primarily, fish exhibit three different modes of reproduction such as, 1) gonochorism or the existence of separate sexes, 2) hermaphroditism (same individual displaying both sexes) and 3) unisexuality (all female-species) (Price, 1984; Chourrout, 1988). Gonochoristic fish display a wide variety of sex determination systems. Broadly, three different models of sex determination can be applied to fish: chromosomal, polygenic and genotype-environmental interaction sex determination (GSD-ESD).

In chromosomal sex determination, a pair of chromosomes called heterochromosomes (sex chromosomes) accumulates most of the major genes responsible for sexual development (Bull, 1983; Tave, 1993). Morphologically differentiated heterochromosomes are rare in fish (Yamazaki, 1983; Price, 1984; Tave, 1993; Solari, 1994). However, on the basis of cytogenetic analysis and from the data of the experimental crosses, eight chromosomal systems of sex determination have been proposed in fish (Tave, 1993; Piferrer, 2001).

In polygenic sex determination (also called polyfactorial), sex-determining genes are present in autosomes (Hunter and Donaldson, 1983; Price, 1984; Kallman, 1984; Chourrout, 1988). It has been proposed that in polygenic sex determination, the sex of the embryo will be the result of the combined relative strength of the male and female factors present in the chromosome complement inherited from each parent (Avtalion and Don, 1990). Fish with polygenic sex determination are characterized by sex ratios deviating from the 1:1 male:female sex ratio which is typical of species with a pure chromosomal sex determination (for review see Piferrer, 2001) which could also be true in fish with ESD.

In genotype-environmental interactions, sex determination is under the control of both genetic and environmental factors. For example, some populations of the Atlantic silverside *Menidia menidia*, sex is determined by the incubation temperature during a critical phase of the larval development (Conover and Fleisher, 1986; Conover and Heins, 1987). Recently, mixed GSD-ESD has been reported in several fish species (for a review see Baroiller *et al.,* 1999).

1.2.2. Sex differentiation

Sex differentiation is the developmental events that allow the expression of genotypic sex into the appropriate phenotypic sex. Sex differentiation is observed by histological differentiation of the gonads and occurs first in the females and later in males. In some species such as in the genus *Oreochromis*, morphological (anatomical) differentiation of gonads precedes cytological differentiation (Nakamura and Takahashi, 1973), while in other species such as in medaka, the situation is the opposite to that of the genus *Oreochromis*, where cytological differentiation precedes morphological differentiation (Satoh and Egami, 1972).

It has been established that in fish, sex steroids act as natural sex inducers (Yamamoto, 1969). During sex differentiation, sex steroids act mainly as morphogenic factors. However, later in the life cycle, during maturation they act mainly as activational factors (reviewed by Piferrer, 2001). Recent investigations also suggest that the effects of environmental factors on the resulting phenotype are exerted by modifying aromatase enzyme activity (Pieau *et al.,* 1994; Kwon *,* 2000).

1.3. Genetic sex determination (GSD)

Various approaches have been applied to elucidate GSD in a number of aquacultural and laboratory species. The major approaches are summarized below.

1.3.1. Chromosomal analysis

Cytological observation of sex chromosomes permits a rapid and direct way to assess sex determination. Cytologically, sex chromosome differentiation may be manifested in several ways. In the mitotic stage, the regular occurrence in one sex of one fewer

chromosome than in the other sex, or the recognition of a dissimilar or heteromorphic pair by size or shape may be taken as evidence of heterogamety. More precise evaluation is possible by studying the meiotic chromosomes by analysing the nature of chromosome pairing prior to metaphase of first meiosis. Thus a monovalent in each cell chromosome complement due to the absence of a chromosome may be seen, or slightly dissimilar pairs may be recognised when they are in close pairing even if not obvious in mitotic chromosomes. Additionally differential coiling or condensation of a chromosome or chromosome pair relative to the rest of the chromosomes may also be viewed as evidence of sex chromosome specialisation. All these phenomena have been reported in fish (Purdom, 1993). In earlier reports, sex chromosome symbolisation was often confusing and to eliminate the confusion, a convention of sex chromosome symbolisation has been proposed (Table 1.1).

Table 1.1. Summary of present conventions on sex chromosome symbolisation (adapted from Purdom, 1993).

Symbol	Convention
X	The basic symbol for a sex chromosome
Y	The convention now for a male determining homologue generating the XX female and XY male convention
W	The convention now for a female determining homologue to X
Z	To replace X where the W symbol is used generating the WZ female ZZ male convention
O	The absence of a sex chromosome of any type

To date, morphologically differentiated sex chromosomes have been reported to occur in a total of 32 species of fish, which is 4.0% of the total number of species analysed cytologically (Almeida-Toledo *et al*., 2000) Considering the sex chromosomes, fish are characterised by a remarkable variability of sex determination systems. Eight

different types of sex chromosomal systems have already been described in fish (Tave, 1993) involving both morphologically undifferentiated and differentiated homologues in simple or multiple systems with male or female heterogamety (Table 1.2).

The XX/XY system has been detected as the most common system in fish. In the XX/XY sex-determining system, the sex chromosomes in females are identical (XX), while those in males are a mismatched pair (XY). In the XX/XY sex-determining system, the Y chromosome is the chromosome that determines sex, i.e. males in this system determine the sex in the offspring (Table 1.2) (Tave, 1993).

Table 1.2. Examples of chromosomal sex determination systems in fish (modified from Tave, 1993)

Sex determination system	Species	Reference
XX/XY	*Oreochromis niloticus*	Jalabert *et al.,* 1974
	Cyprinus carpio	Nagy *et al.,* 1981
ZZ/ZW	*Oreochromis aureus*	Guerrero, 1975
	Oreochromis hornorum	Chen, 1969
WXY	*Xiphophorus maculatus*	Gordon, 1946
XX/XO	*Sternoptyx diaphana*	Chen, 1969
ZO/ZZ	*Colisa lalia*	Rishi, 1976
$X_1X_1X_2X_2/X_1X_2Y$	*Awaous aeneofuscus*	Pezold, 1984
ZZ/ZW_1W_2	*Apareiodon affinis*	Filho *et al.,* 1980
XY_1Y_2/XX	*Hoplias* sp.	Bertollo *et al.,* 1983

The WZ sex-determining system is the opposite of XY sex-determining system. In this, sex chromosomes in males form an identical pair while in females it is a mismatch pair. Therefore, in this system, males are ZZ and females are WZ. In this system the W chromosome of the female determines the sex of the offspring (Table 1.2) (Tave, 1993).

The WXY system, as found in *Xiphophorus maculatus*, is a complicated variant of the XX/XY sex-determining system. In this system the W chromosome is a modified X chromosome which can suppress the male-determining ability of the Y chromosome

(Nakamura *et al.,* 1984). Thus, XY and YY fish are males, while in contrast XX, WX and WY fish are females. Therefore, either of the parents can determine the sex of the offspring, depending on its sex chromosomes.

Multiple sex chromosomes are also observed in fish (Table 1.2). These are different variations of either the XX/XY or the WZ/ZZ sex determining system. *Awaous aeneofuscus* has multiple X chromosomes ($X_1X_1X_2X_2$). However it is the male which determines the sex of the offspring by having a Y chromosome (X_1X_2Y). In the species *Apareiodon affinis*, males are homogametic with ZZ and females are heterogametic with multiple W chromosomes (ZW_1W_2) and, therefore, it is the female which determines the sex of the offspring in this system (Tave, 1993).

In genus *Hoplias*, males are heterogametic with multiple Y chromosomes (XY_1Y_2) and females are homogametic without any multiple sex chromosomes (XX). Thus in this system offspring which receive the father's Y_1Y_2 will become males and therefore males are the decisive partner in this system (Tave, 1993).

The XX/XO and ZO/ZZ sex determining systems are also variants of XX/XY and WZ/ZZ sex determining systems respectively. Females are homogametic XX and males are heterogametic without any sex chromosomes in the XX/XO sex-determining system. In this sex determining system, the male determines the sex of the offspring. The offspring which receive an X chromosome become females, while the others which receive no sex chromosome become males. In the ZO/ZZ sex-determining system, females are heterogametic ZO and males are homogametic ZZ. The female in this case determines the sex of her offspring: offspring which receive a Z become males, while the others which receive no sex chromosome become females. It is usual that regardless

of the sex determining system, the heterogametic sex determines the sex in offspring (Tave, 1993).

In the species with sex-determining systems which have multiple sex chromosomes, such as $X_1X_1X_2X_2/X_1X_2Y$, ZW_1W_2/ZZ, XY_1Y_2/XX, XX/XO and ZO/ZZ, the number of chromosomes is not consistent within the species. Females have one more chromosome than males in the $X_1X_1X_2X_2/X_1X_2Y$, ZW_1W_2/ZZ and XX/XO sex determining systems, while males have one more chromosome than females in the XY_1Y_2/XX and ZO/ZZ sex determining systems (Tave, 1993).

In addition to sex chromosomal sex determination, autosomal sex determining genes influence the sex determination in many fish species (Kosswig, 1964; Avtalion and Hammerman, 1978; Majumdar and McAndrew, 1983; Shelton *et al.,* 1983; Mair *et al.,* 1991a,b; Wohlfarth and Wedekind, 1991). Such as in the swordtail (*Xiphophorus hellerii*) and blue poecilia a number of male or female autosomal sex determining genes are responsible for the sex determination (Kosswig, 1964).

1.3.2. Interspecific hybridization

Interspecific hybridization has been used in a number of fish species primarily as a means of improving production traits (such as growth rate, survival, disease resistance) as well as to manipulate sex ratios (reviewed by Hulata, 2001). The principal purpose of hybridization in aquaculture is purely based on the fact that by hybridization, populations with desirable traits can be introduced for aquaculture. However, on some occasions hybridization has produced monosex populations, which could be an indicator of the sex determining system operating in the parent species. The most famous example

of hybridization is the inter-specific hybridization between *O. niloticus* and *O. aureus* (Hickling, 1960; Pruginin *et al*., 1975; Majumdar and McAndrew, 1983).

1.3.3. Sex reversal

Since it was shown by Yamamoto (1969) that steroid hormones can reverse the sex of fish, sex reversal with steroid hormones has been used to produce monosex populations for culture, as well as to elucidate sex determination in a number of fish species (reviewed by Tave, 1993; Penman and McAndrew, 2000; Piferrer, 2001). For example, evidence of female homogamety in salmonids and channel catfish has been revealed by hormonal sex reversal (Johnstone *et al*., 1978; Davis *et al*., 1990).

1.3.4. Chromosome set manipulations

Techniques of chromosome set manipulations, particularly gynogenesis and androgenesis have been used to study sex determination in a number of species (reviewed by Tave, 1993; Penman and McAndrew, 2000; Arai, 2001; Hulata, 2001).

Gynogenesis is a form of all maternal origin, while androgenesis is the form of all paternal origin. The technique of induced gynogenesis involves the fertilization of eggs with genetically inactivated sperm (mostly by ultraviolet irradiation) and subsequent diploidization by the suppression of the second meiotic division (meiotic gynogenesis) or suppression of first mitotic division by physical shocks (either heat, cold or pressure) or chemical treatments. In contrast, the technique of induced androgenesis involves the fertilization of genetically inactivated eggs (mostly by UV or ^{60}Co) and subsequent diploidization by suppressing the first cleavage or by fertilizing the genetically inactivated eggs with sperms from tetraploid males (Thorgaard *et al.,* 1990).

The principle behind the use of gynogenesis or androgenesis to elucidate sex determination lies in the nature of techniques; the gynogenetic or androgenetic progeny inherit the sex determining genes from only one parent. Therefore, by analysing the sex ratio of gynogenetic progeny, the sex determination mechanism of a particular species can be elucidated. If the female is homogametic, gynogenesis will produce only females, while if the female is heterogametic, gynogenesis will produce equal numbers of males and females. Similarly, in the case of homogametic males, androgenesis will produced all male progeny, while in the case of heterogametic males, equal number of males and females are produced. Although, these results do not provide solid evidence of which sex determining system is operating. However, considering the XX/XY and WZ/ZZ systems as the most common, occurrence of all female progeny from gynogenesis or androgenesis usually suggest the XX/XY and the WZ/ZZ systems of sex determination. Gynogenesis is discussed further in Chapter 2 of this thesis.

1.3.5. Sex-linked and sex-specific markers and genes

From the previous descriptions of sex determination in fish, it is quite clear that although genes on sex-determining chromosomes are involved in sex determination in most of the species investigated, other factors, such as environment and autosomal genes, are of great influence in sex determination in many fish species (Tave, 1993; Devlin *et al.,* 2001). Therefore the control of sex determination in fish is quite labile and subject to change over reasonably short evolutionary time frames (Devlin *et al.,* 2001). In some closely related species, even in the same genus, the existence of different genetic systems has been reported, such as in *Oreochromis* species. Because of the

plasticity of sex determination in fish, sex chromosomes may change often throughout the course of evolution, thus minimising the time available to accumulate unique DNA sequences associated with sex determining loci (Devlin *et al.*, 2001). Sometimes qualitative phenotypes may also be controlled by genes located on one of the sex chromosomes. In this situation, phenotypes are controlled by sex-linked genes and the inheritance of sex-linked phenotypes is observed in a species with heterogametic sex.

Table 1.3 shows examples of different types of sex-linked and sex-specific markers identified in fish. These loci responsible for phenotype, fitness traits, protein and variable DNA sequence are located on the sex chromosomes. However, recombination between these markers and the sex determination locus has been observed in most cases, suggesting that they are not tightly linked to the sex determining locus of sex chromosomes (Devlin *et al.,* 2001).

Table 1.3. Types of sex-linked and sex-specific markers identified in fish.

Type of markers	Species investigated	Association with sex	References
Phenotypic	eastern mosquitofish (*Gambusia holbrooki*)	Sex-linked	Angus, 1989
	Xiphophorus sp.	Sex-linked	Morizot *et al.,* 1991
	medaka (*Oryzias latipes)*	Sex-linked	Wada *et al.,* 1998
	guppy (*Poecilia reticulata)*	Sex-linked	Khoo *et al.,* 1999
Fitness traits	*Xiphophorus maculatus*	Sex-linked	Kallman and Borkoski, 1978
	Xiphophorus maculatus	Sex-linked	Schreibman and Kallman, 1978
	Xiphophorus sp.	Sex-linked	Ahuja *et al.,* 1979
	guppy (*Poecilia reticulata)*	Sex-linked	Fujio and MacAranas, 1989
	Xiphophorus nigrensis	Sex-linked	Morris *et al.,* 1992
Protein	Arctic char (*Salvelinus alpinus*)	Sex-linked	Nyman *et al.,* 1984
	threespined stickleback (*Gasterosteus aculeatus*)	Sex-linked	Withler *et al.,* 1986
	guppy (*Poecilia reticulata)*	Sex-linked	Fujio and MacAranas, 1989
	hybrid of brook trout and Arctic char	Sex-linked	May *et al.,* 1989
	rainbow trout (*Oncorhynchus mykiss*)	Sex-linked	Allendorf *et al.,* 1994
	channel catfish (*Ictalurus punctatus*)	Sex-linked	Liu *et al.,* 1996
DNA	*Xiphophorus* sp.	Sex-specific	Schartl, 1988, 1992; Zechel *et al.,* 1988; Weis and Schartl, 1998; Coughlan *et al.,* 1999
	medaka (*Oryzias latipes)*	Sex-linked	Matsuda *et al.,* 1997, 1998, 1999
	brown trout (*Salmo trutta*)	Sex-linked	Prodohl *et al.,* 1994
	Leporinus elongatus	Sex-specific	Nakayama *et al.,* 1994
	rainbow trout (*Oncorhynchus mykiss*)	Sex-linked	Singh *et al.,* 1994; Iturra *et al.,* 1998; Devlin *et al.,* 1998; Young *et al.,* 1998
	lake trout (*Salvelinus namaycush*)	Sex-linked	Reed *et al.,* 1995; Reed and Phillips, 1995
	threespined stickleback (*Gasterosteus aculeatus*)	Sex-linked	Griffiths *et al.,* 2000
	chinook salmon (*Oncorhynchus tshawytscha*)	Sex-linked	Devlin *et al.,* 1991, 1994, 1998; Clifton and Rodriguez, 1997; Stein *et al.,* 2001
	Oncorhyncus species (coho, chinook amago and masu)	Sex-linked	Du *et al.,* 1993; Forbes *et al.,* 1994; Devlin *et al.*, 1991, 1994, 1998; Nakayama *et al.,* 1998; Zhang *et al.,* 2001
	lake trout (*Salvelinus namaycush*)	Sex-linked	Reed *et al*., 1995; Reed and Phillips, 1995
	African catfish (*Clarias gariepinus*)	Sex-specific	Kovács *et al*., 2001

It has been observed that DNA rearrangement has occurred in many plants and animal species in sex chromosomes, particularly adjacent to the sex determination loci. These DNA arrangements normally include deletions, insertions, inversions, transpositions (of chromosomal fragments or transposable elements), or amplification of

repetitive sequences. Thus a significant proportion of sex chromosomes comprise such DNA sequences which provide unique DNA markers (Scutt *et al.,* 1997; Devlin *et al.,* 1998; Tilford *et al.,* 2001). However, in the case of fish, it is very difficult to isolate sex-linked DNA sequences, because in this group, sex chromosome evolution is at an early stage, which is a factor limiting accumulation of sufficient molecular differences (Devlin *et al*., 2001). Nucleolus organiser ribosomal gene sequences (rDNA) or 5S rDNA have been identified on sex chromosomes of some fish species (Li and Gold, 1991; Pendas *et al.,* 1994; Moran *et al.,* 1996; Khuda-Buksh and Datta, 1997; Reed and Phillips, 1997). In some cases, repetitive sequences of the sex chromosomes were also found to be associated with the telomeric sequences or simple sequence repeats, such as, $(GACA)_n$ or $(GATA)_n$ (Ewulonu, 1987; Nanda *et al.,* 1990).

The testis determining factor, SRY (sex determining region, Y chromosome), has been well characterized in mammals (Sinclair *et al.*, 1990) and is proposed to be the first gene acting in the mammalian sex determination cascade (Koopman *et al.*, 1991). Mammalian SRY was found to show similarities to the high mobility group proteins (HMG) that are architectural factors involved in chromatin structure (Graves, 2002). There are more than 20 genes that belong to the same family as SRY. They are commonly known as SOX (SRY-like HMG-box containing) genes. Several of these SOX genes (such as SOX3 and SOX9) in mammals have a conserved role in testis determination (Bowles *et al.*, 2000). However, the sex determination functions of this gene appear to be limited to mammals as genes homologous to SRY have been identified in birds, reptiles and some species of fish, but none were found to be sex-

specific (Devlin *et al.*, 1991, 2001; Nanda *et al.*, 1992; Wachtel and Tiersch, 1994; Fukuda *et al*., 1995; Ito *et al.*, 1995).

Another family of genes involved in sex determination in invertebrates is also found to be involved in vertebrate sex determination. This gene family encodes proteins that contain DM-domains (a motif identified by homology between genes involved in sex determination in insects (Dsx) and nematodes (mab3)) (Raymond *et al*., 1998). One member of this gene family, DMRT1 has been found to contain an additional male-specific motif homologous to the male specific *Drosophila* Dsx, and showed testis specific expression in several vertebrate groups including tilapia, rainbow trout and medaka (Raymond *et al*., 1998; Guan *et al*., 2000; Marchand *et al*., 2000; Brunner *et al*., 2001). DMRT1 homologues in tilapia (tDMRT1) were found to show testis-specific expression, while another DM homologue (tDMO) lacked a male-specific motif and was shown to express only in ovary (Guan *et al.*, 2000). In rainbow trout, DMRT1 (rtDMRT1) also showed more than ten fold higher level of expression in testis than ovary (Marchand *et al*., 2000). These experiments suggested that DMRT1 genes in these species are located somewhat downstream in the sex determination path way, like Dsx and mab3 in invertebrates (Marin and Baker, 1998). Although, DMRT1 expression in tilapia, rainbow trout and medaka is not sex-linked (Guan *et al*., 2000; Marchand *et al*., 2000; Brunner *et al*., 2001), other members of the DM gene family could play important roles in sex determination in fish, and thus are good candidates for finding genes involved in sex determination in this group of vertebrates.

1.3.5.1. Molecular techniques for sex identification in fish

We have come a long way in our understanding of sexual dimorphism since 355 BC, when Aristotle suggested that the difference between the two sexes was due to the heat of semen at the time of copulation: hot semen generating males, whereas cold semen made females. We now know a little more about the molecular events of sex determination.

The use of molecular markers for the identification of sex is only possible in GSD systems. If an organism has a well established GSD, it is possible to search for a sex-linked genetic marker (Griffiths, 2000). Various methods can be applied to find sex specific or sex-linked molecular markers in organisms with GSD. Hadrys *et al.* (1992) suggested that sex-linked markers may be easily identified, even in complex and relatively unknown genomes, by random amplification of polymorphic DNA sequences (RAPDs) using single primers of random sequence composition in PCR. However, Griffiths (2000) proposed the following three options:

i) **Use a known sex-linked marker**: this begins with literature review or by searching EMBL or GenBank databases. To start with it is better to search for a gene, or if this is not possible, try to look for sex-linked DNA from the organism in question or in a closely related species. The test procedure can be selected on the basis of laboratory facilities such as PCR or DNA hybridization, once candidates have been found.

ii) **Look for own sex-linked sequences**: it is known that the Y chromosome in humans represents only 2-3% of the genome, and only a fraction of this is sex-specific. This fraction is much smaller in many other organisms, so sex-linked

markers are very difficult to find. One search technique is to compare an array of DNA fragments in males and females that are selected using the same technique. This requires genome screening for species-specific sex-linked sequence using suitable molecular techniques such as, use of random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs) or subtractive hybridization.

iii) **Find a known repetitive sequence that is sex-linked**: both micro- and minisatellites are likely to be concentrated on the Y chromosome. Therefore, using Southern blot, known male and female organisms can be screened with synthesized microsatellite probes such as (CT)n and (GATA)n, (which is found to be useful in snakes (Jones and Singh, 1985). However, hybridization sometimes may produce sex-specific fragments as large as 20-60 kb (Longmire *et al*., 1993) and the technique is also slow by comparison with PCR-based techniques.

For sex identification using DNA-based molecular techniques, the most commonly used techniques are RAPD (randomly amplified polymorphic DNA), AFLPs (amplified fragment length polymorphisms) and subtractive hybridization. Other PCR-and hybridization-based techniques, such as the use of highly polymorphic microsatellite loci, spPCR (single-primer polymerase chain reaction) and hybridization with minisatellite probes have also been applied (Jones and Singh, 1985; Devlin *et al.*, 1991; Griffiths and Tiwari, 1993; ; Longmire *et al.*, 1993; Lessells and Mateman, 1998; Griffiths and Orr, 1999; Griffiths *et al.*, 2000; Reamon-Büttner *et al.*, 1998; Reamon-Büttner and Jung, 2000; Kovács *et al*., 2001). Fluorescent *in situ* hybridization (FISH)

of sex-chromosome specific probes has also been applied to localise the sex specific region in sex chromosomes (Nanda *et al.*, 2000; Harvey *et al.*, in press).

The techniques mentioned earlier have been applied in several species of plants and animals with various degrees of success. One problem is that in many cases markers obtained in one species prove to not be sex-linked in another, even in closely related species. For example, sex-linked AFLP markers isolated in the three-spined stickleback failed to identify sex in the nine-spined stickleback (Griffiths *et al.*, 2000). The authors suggested this is a consequence of the evolution of sex determination systems via the evolution of sex chromosomes throughout phyla, and the adaptation to different specialised mechanisms for reproduction. However, the advantage of identifying sex specific molecular markers is that they are reliable, quick and can allow the identification of sex at early stages of development.

Sex-linked or sex-specific DNA markers (both PCR-based and sex-chromosome specific probes) have been reported in salmonids (Devlin *et al.*, 1991, 1994; 1998; Clifton and Rodriguez, 1997; Devlin *et al*., 2001), in three-spined stickleback (Griffiths *et al.*, 2000), in platyfish (Coughlan *et al.*, 1999; Nanda *et al.*, 2000) and in African catfish (Kovács *et al*., 2001). A growth hormone pseudogene (*GH-Y*) sequence was found to be sex-linked in four *Oncorhynchus* species (Du *et al*., 1993; Forbes *et al*. 1994; Nakayama *et al*., 1998; Devlin *et al.*, 2001; Zhang *et al.*, 2001).

1.3.6. Environmental sex determination (ESD)

Like other vertebrates, fish also display ESD. Several environmental factors have been found to influence sex determination in several fish species such as pH, social

structure and prevailing temperature during early development (reviewed by Baroiller *et al*., 1999). However, the most studied environment factor is temperature, the mechanism being known as temperature-dependent sex determination (TSD). The influence of pH or the combined influence of pH and temperature has been reported in a number of fish species. For example acidic pH (6.2) during the early development was found to produce 100% males in *Xiphophorus helleri*, while slightly basic pH (7.8) was found to produce almost all female (98%) progeny (Rubin, 1985). Similar results have also been reported in *Poecilia melanogaster* (Römer and Beisenherz, 1996). In several species of the genus *Apistogramma*, maleness was observed to be inversely proportional to pH (Rubin, 1985; Römer and Beisenherz, 1996). The social structure has also been reported to influence in some fish species, such as in the paradise fish *Macropodus opercualris*, where individual isolation favours maleness while grouping induces female differentiation (Francis, 1984).

TSD in fish is better documented than other forms of ESD, and has been reported in many fish species including Atlantic silverside *Menidia menidia* (Conover and Kynrad, 1981; Conover and Fleisher, 1986), *Odontesthes bonariensis* (Strüssmann *et al.*, 1996a, 1997), sockeye salmon *Oncorhyncus nerka* (Craig *et al.,* 1996), channel catfish *Ictalurus punctatus* (Patiño *et al.* 1996), *Apistogramma* sp. (Römer and Béisebherz, 1996), European seabass *Dicentrarchus labrax* (Blázquez *et al*., 1998), hirame *Paralichthys olivaceus* (Yamamoto, 1999; Kitano *et al*., 1999). Table 1.4 shows examples of TSD in several fish species. It has been observed that both low and high temperature produced male and female in several species with the exception in hirame, *Paralichthys olivaceus*, where both high and low temperatures produce males (Yamamoto, 1999). In those fish

species, which are prone to TSD, strong interactions between genotype and ESD, particularly TSD have been suggested (reviewed by Baroiller *et al.*, 1999). The ESD in tilapia will be discussed further in a later section of this thesis.

Table 1.4. Examples of TSD in fish.

Species	Temperature	Sex	Reference
Hoplosternum littorale	High	Male	Hostache *et al.*, 1995: cited by Baroiller *et al.*, 1999
Poecilia lucida	High	Male	Schultz, 1993
Poecilia melanogaster	High	Male	Römer and Beisenherz, 1996
Hoplosternum littorale	Low	Female	Hostache *et al.*, 1995: cited by Baroiller *et al.*, 1999
Poecilia lucida	Low	Female	Schultz, 1993
Poecilia melanogaster	Low	Female	Römer and Beisenherz, 1996
Menidia menidia	Low	Female	Conover and Kynard, 1981; Conover and Heins, 1987
Pataginina hatcheri	Low	Female	Strüssmann *et al.*, 1996b
Odontesthes bonariensis	High	Male	Strüssmann *et al.*, 1996a, 1997
Odontesthes bonariensis	Low	Female	Strüssmann *et al.*, 1996a
Paralichthys olivaceus	High	Male	Yamamoto, 1999
Paralichthys olivaceus	Low	Male	Yamamoto, 1999
Dicentrarchus labrax	High	Female	Blázquez *et al.*, 1998
Dicentrarchus labrax	Low	Male	Blázquez *et al.*, 1998

1.4. Sex determination and importance of sex control in tilapia culture

McAndrew (2000) defined 'tilapia' as "a common name for large number of species within the cichlid tribe Tilapiini, particularly the species in the three genera *Tilapia*, *Sarotherodon* and *Oreochromis*, especially the larger species most commonly caught in wild fisheries or those used in aquaculture." As mentioned by the tilapia taxonomist Trewavas (1983), the term 'tilapia' was first coined by A. Smith in 1840 from the Bushman word for 'fish'. For more than a century, around a hundred tilapiine species have attracted the interest of evolutionary biologists, ethologists, ecologists and geneticists as well as many fish farmers around the world, which makes them 'global' fish (Beveridge and McAndrew, 2000).

Several species of the Tilapiini tribe began to gain popularity as cultured species during and after the second World War. However, a record has been found which shows evidence of farming of *Oreochromis niloticus* over 4000 years ago (reviewed by McAndrew, 2000). Records on tilapia culture showed that only eight to nine species of tilapia have significant potential in aquaculture (Schoenen, 1982; Pullin, 1983). Among them two species of the genus Tilapia (*T. zillii* and *T. rendalli*) and four species of the genus *Oreochromis* (*O. niloticus*, *O. mossambicus*, *O. aureus* and *O. andersonii*) are widely used in aquaculture. However, the Nile tilapia, *O. niloticus* is by far the most important species in the group in freshwater tropical aquaculture (FAO, 1999).

Recently, tilapia have been reported to be cultured in more than 100 tropical and sub-tropical countries of the world. The popularity of tilapia culture derives from their definite physiological advantages. Tilapias can tolerate a wide range of environmental conditions, such as high salinity, low oxygen and overcrowding conditions, in addition to their obvious high growth rate on natural and cheap artificial feed. Shorter reproductive generation and ability to breed in captive conditions are also added qualities behind its popularity. However, with all these positive qualities, tilapias also have two major drawbacks: their habit of prolific breeding and predation (cannibalism) in the culture ponds, which hinders aquaculture production in many tropical countries. In the tropics, tilapia can become sexually mature and begin to reproduce at an age of five months, sometimes even earlier (Tave, 1993). This reproductive efficiency of tilapias results in subsequent unwanted reproduction leading to overpopulation which in turn reduces the quality of the products as well as profits. To overcome this situation, the culture of single sex tilapia, preferably male because of a higher growth rate, has been

adopted in many of the commercial production systems (Penman and McAndrew, 2000; Edwards *et al*., 2000).

Oreochromis niloticus is a warm water species and can tolerate a wide range of variations in water quality (Balarin and Haller 1982a, b). It is omnivorous and mainly feeds on phytoplankton, several species of zooplankton and macrophytes and is also well adapted to supplementary feed. Their feeding regime varies considerably depending on their size, age, availability of feed and the presence or absence of competing species within their habitat. Fry feed on small organisms and detritus, and are thought to be cannibalistic (Philippart and Ruwet, 1982; Ajuzie and Nwokorie, 1994; Macintosh and Little, 1995).

The Nile tilapia is a polygamic fish that normally matures within 6 months and is capable of breeding when very small (below 40 g). The average fecundity of females ranges from several hundreds to 2000 per batch depending upon the age and the sizes of the females. Naturally they display the communal breeding based on a "lek" (meaning arena) system, where the males build and defend territories within a spawning area, which is visited by receptive females. Under favourable culture conditions, their breeding is continuous but fluctuating. The reproductive cycles of the females are not synchronized, although each female may breed up to 12 times a year (Macintosh and Little, 1995).

Various techniques have been tested to prevent reproduction or to eliminate fry, including manual separation of the sexes, predator-mediated control, hybridization, triploidy and monosex male production through either steroid treatment or genetic manipulation of the sex determination system (Mair *et al*., 1997; Little and Hulata, 2000;

Penman and McAndrew, 2000). The technique of production of genetically male tilapia (GMT) has been developed and has been adopted in some commercial operations (Penman and McAndrew, 2000). Due to the influence of autosomal genes and influences of environmental factors, production of 100% males in the GMT programmes is very difficult. Moreover, this technique requires extensive progeny testing of fish in order to determine their genotype, a time-consuming and technically demanding process. Therefore, for an efficient GMT programme we need an efficient and reliable technique by which sex can be identified accurately and at early stages in development. Use of molecular markers for the X and Y chromosomes would be the best option for the successful GMT production.

1.4.1. GSD in tilapia

Apparently, most tilapia display GSD and it is normally described by a simple monofactorial model. Sex determination under this model is controlled by the sex determining genes present on the sex chromosomes. Both male (XX female, XY male) and female (WZ female, ZZ male) heterogamety have been found in tilapia (Hickling, 1960; Chen, 1969), with *Oreochromis mossambicus* and *O. niloticus* having female homogamety and *O. aureus*, *O. macrochir*, *O. urolepsis hornorum* having female heterogamety. However, this monofactorial model fails to explain many unexpected sex ratios observed during various breeding experiments.

A number of different approaches have been applied to elucidate GSD in tilapia. These include studying the sex ratios of intra- and inter-specific crosses, back crosses (Pruginin *et al.*, 1975; Hulata *et al.*, 1983; Shelton *et al.*, 1983; Majumdar and McAndrew, 1983; Penman, 1989; Mair *et al.*, 1991a,b; Tuan *et al.*, 1999), sex reversal

of fry to male or female by hormone treatment and subsequent progeny testing (Hopkins *et al.*, 1979; Mair *et al.*, 1987,1991a,b; Gilling *et al.*, 1996), chromosome complement manipulation by gynogenesis, androgenesis and polyploidy (Penman *et al.*,1987a,b; Penman, 1989; Mair *et al.*, 1991a,b; Sarder *et al.*, 1999), karyotyping and differential staining (Nijjhar *et al.*, 1983; Majumdar and McAndrew, 1986; Carrasco *et al.*, 1999; Campos-Ramos *et al.*, 2001). These various techniques, particularly chromosome set manipulation and hormonal sex reversal have proved particularly useful in the study of GSD as they allow the production of individuals with genotypes not found naturally, which then have application in GMT production. For example, in three commercially important *Oreochromis* species, techniques of induced gynogenesis and androgenesis have been applied to elucidate sex determination. Although, some deviations in the sex ratios were reported (discussed later in this Chapter), the use of these techniques, has confirmed and extended our understanding of the sex determination systems in *O. niloticus*, *O. mossambicus* and *O. aureus* (Penman *et al.,* 1987b; Mair *et al.,* 1987, 1991a,b; Müller-Belecke and Hörstgen-Schwark, 1995; Myers *et al*., 1995; Sarder *et al.,* 1999).

To elucidate sex determination, the technique of sex reversal has also been applied in several tilapia species of the genus *Oreochromis* (Clemens and Inslee, 1968; Guerrero, 1975, 1979; Hopkins *et al.,* 1979; Mair *et al.,* 1987, 1991a,b; Lahav, 1993; Baroiller, 1996). The sex ratios of the offspring produced from crosses of normal females or males with sex-reversed males or females has been analysed to elucidate sex determination. All female progeny were reported in *O. mossambicus* and *O. niloticus* by crossing normal females to sex reversed neomales, suggesting the XX/XY sex determination in these

species (Clemens and Inslee, 1968; Mair *et al.,* 1991a.). Crosses between the sex reversed and normal individuals were also reported in *O. aureus*. Results indicated the WZ/ZZ sex determination in this species, as ZZ normal males produced nearly 100% males when crossed to ZZ sex reversed neofemales. However, slight to moderate (up to 40%) deviations from 100% male sex ratios have been reported in several sex reversal experiments in this species (Guerrero, 1975; Hopkins *et al.,* 1979; Mair *et al.,* 1987b, 1991b; Lahav, 1993; Desprez *et al.,* 1995).

1.4.2. Autosomal influence

To explain many unexpected sex ratios from different crosses discussed earlier, several ideas based on autosomal influences were put forward (Avtalion and Hammerman, 1978; Kallman, 1984; Majumdar, 1984; Mair *et al.*, 1990, 1991a,b; Wohlfarth and Wedekind, 1991).

An autosomally (single locus: AA or aa) influenced, three gonosomal (W, X, Y where Y=Z) model of the sex determining system was proposed on the basis of interspecific studies (Table 1.5) (Avtalion and Hammerman, 1978). Based on this model, *O. mossambicus* and *O. niloticus* were classified as AAXX females and AAXY males, and *O. aureus*, *O. macrochir*, and *O. urolepsis hornorum* were classified as aaWY females and aaYY males (Table 1.5). This is a gene balanced model assuming that if the sum effects of the four sex determining chromosomes and the autosomal locus exceeds a certain threshold, the individual develops into a male, if less, into a female (Avtalion and Hammerman, 1978).

Table 1.5. Influence of autosomal pairs on sex determination in interspecific crosses. m: male, f: female (from Avtalion and Hammerman, 1978).

Sex	Autosomal factors		
Chromosomes	AA	Aa	aa
YY	m	m	m
WY	m	m	f
XY	m	m	f
WW	m	f	f
WX	f	f	f
XX	f	f	f

The model hypothesised by Avtalion and Hammerman (1978) provided better predictions about the sex ratios from interspecific crosses, but not from intraspecific crosses. For intraspecific data, Mair *et al.* (1991b) suggested the possibility of an autosomal recessive sex modifying gene in *O. aureus*, epistatic to the major sex determining factors W and Z (Y).

Another hypothesis of autosomal influence of sex determination in *O. niloticus* was postulated by Hussain *et al.* (1994a) to explain the occurrence of varying proportions of males in heterozygous and homozygous meiotic and mitotic gynogenetic progeny. This hypothesis describes the existence of an autosomal sex-modifying locus (with alleles 'SR' and 'sr') epistatic to the gonosomal locus which induces female to male sex reversal when 'sr' is recessive homozygous. This hypothesis again failed to explain some of the aberrant sex ratios observed in crosses of hormonally sex reversed fish, for example, the occurrence of females in the progeny of YY males (Mair *et al.*, 1997). Mair *et al.* (1997) proposed the action of several autosomal sex modifying genes. Recently, Sarder *et al.* (1999) speculated on the partial penetrative nature of an autosomal sex modifying gene to explain the sex ratios in inbred clonal populations of *O. niloticus*.

Wohlfarth and Wedekind (1991) suggested that in intraspecific studies, sex ratio

behaves as a quantitative trait (i.e. polygenic and susceptible to environment). However, their suggestion has not been experimentally proved as yet. Moreover, Mair *et al.* (1991a) commented that sex inversion and gynogenesis experiments refute the polygenic model of sex determination in tilapias.

1.4.3. Recombination between sex determining gene(s) and their centromere

To explain the predominance of females in the F1 generation of gynogenetic *O. aureus*, Penman *et al.* (1987a) suggested recombination between the sex determining gene(s) and the centromere during the prophase of the first meiotic division. Single crossovers would produce only female progeny, and double crossovers would yield equal numbers of males and females. Recombinant females would occur depending on the rate of crossing over between the sex determining gene(s) and the centromere. Avtalion and Don (1990) further investigated this hypothesis in three generations of gynogenetic *O. aureus* and supported the same hypothesis as proposed by Penman *et al.* (1987a).

Hussain *et al.* (1994a) calculated a recombination rate of y = 0.85 between the centromere and an autosomal sex determining locus (*SDL*-2) in *O. niloticus,* based on the low proportion of males among meiotic gynogenetics derived from one female. A higher recombination rate of y = 1.00 between the centromere and the *SDL*-2 has been reported by Müller-Belecke and Hörstgen-Schwark (1995) on the basis of the absence of males among the 163 meiotic gynogenetic individuals produced by two females which had males among their mitotic gynogenetic progeny. However, this theory failed to explain a low proportion of males in the offspring of a mitotic gynogenetic male when

mated to normal brood stock females in their study. The authors suggested the occurrence of two or more autosomal minor sex determining factors.

1.4.4. ESD in tilapia

ESD, particularly temperature effects on sex determination, has been reported in several tilapia species, including *O. niloticus* (Baroiller *et al.*, 1995a,b, 1996; Abucay *et al.*, 1999), *O. mossambicus* (Mair *et al.*, 1990) and *O. aureus* (Mair *et al.*, 1990; Desprez and Mélard, 1998). The sensitivity to temperature varies from species to species. *O. niloticus* and *O. aureus* respond significantly to high temperature (34-37ºC), producing higher percentages of males at those temperatures; with the one exception is *O. mossambicus* which responded to low temperature (19°C) (Mair *et al*., 1990).

It has been reported that, among the tilapia species, *O. aureus* is the most sensitive to high temperature (Desprez and Mélard, 1998). High variability in the response to high temperature has also been reported within a given species (Abucay *et al.*, 1999) and even within a strain (reported to have originated from crossing two different strains) in *O. niloticus* (Baroiller *et al.*, 1995a,b, 1996). Baroiller *et al.* (1995a,b) showed that high temperature (36ºC) can induce female to male sex reversal in *O. niloticus*. However, Abucay *et al.* (1999) showed that the same high temperature can have more complex effects in *O. niloticus,* with high temperature causing both female to male and male to female sex reversal. These studies imply that a genotype-temperature interaction may exist in tilapia, as proposed in a different species of fish, the silverside *Menidia menidia* (Conover and Kynrad, 1981).

1.4.5. Chromosomal analysis in tilapia

All of the above studies have been based on the observation of the sex ratios produced from different crosses. Only a limited amount of work has been done so far on the cytological and molecular basis of sex determination in these species. Nijjhar (1983) described heteromorphic sex chromosomes in *O. niloticus* by analysing metaphase chromosomes. However, subsequently Majumdar and McAndrew (1986) failed to identify any heteromorphic sex chromosomes in this or other tilapia species from metaphase chromosomes. Foresti *et al.* (1993) and Carrasco *et al.* (1999) presented evidence for the largest pair of chromosomes being the sex chromosomes in *O. niloticus* by studying the synaptonemal complex (SC) in three genotypes, XX, XY and YY. This unpaired region observed during SC formation was observed in 25.7% of XY SC spreads. This unpaired region of the largest chromosome pair (chromosome number 1) has been suggested to be associated with the heterogamety and therefore to be the location of major sex determining loci in the *O. niloticus*. It was also hypothesized that this unpaired region is an indication of early stage of sex chromosome differentiation in *O. niloticus*, with a reduction or cessation of recombination between the largest chromosome pair (Carrasco *et al*., 1999).

Recently sex-chromosome specific degenerate oligonucleotide primed PCR (DOP-PCR) probes have been developed in *O. niloticus* by microdissection, and subsequent fluorescent *in situ* hybridization (FISH) of these probes back to the sex chromosomes has been reported (Harvey *et al*., in press). However, no sex-specific DNA markers have been isolated in *O. niloticus* by other molecular genetic techniques. Based on the evidence of GSD and evidence for sex chromosomes in this species, the project

described in this thesis was designed to find sex-specific molecular markers in tilapia by using a DNA fingerprinting technique, amplified fragment length polymorphisms (AFLPs) (Vos *et al*., 1995).

1.5. DNA fingerprinting and aquaculture research

The term 'DNA fingerprints' was first coined by Alec Jeffreys to describe the pattern of hyper-variable, individual-specific profiles on the Southern blots of human genomic DNA generated by the hybridization of tandemly-arrayed repetitive sequences (Jeffreys *et al.*, 1985a,b). Since then the term has been applied to both the Southern- and PCR-based applications on a wide variety of organisms ranging from prokaryotes to mammals in a wide variety of fields including forensic medicine (Bär and Hummel, 1991), parentage assignment (Westneat, 1990; Rico *et al.*, 1991), linkage analysis (Jeffreys *et al.*, 1986), genetic diversity studies (Reeve *et al.*, 1990; Schartl *et al.*, 1990; Turner *et al.*, 1998) and individual and family identification (Gilbert *et al.*, 1990; Wirgin *et al.*, 1991).

Ideally, a fingerprinting technique should require no prior investments in terms of sequence analysis, primer synthesis or characterization of DNA probes. A number of fingerprinting methods which meet these demands have been developed over the past few years, including random amplified polymorphic DNA (RAPD: Williams *et al.*, 1990), DNA amplification fingerprinting (DAF: Caetano-Anolles *et al.*, 1991), arbitrarily primed PCR (AP-PCR: Welsh and McClelland, 1990, 1991), and amplified fragment length polymorphisms (AFLPs: Vos *et al.*, 1995).

In principle, a multilocus DNA fingerprint can be generated either by the

simultaneous application of several probes, each one specific for a particular locus, or by applying a single DNA probe that simultaneously detects several loci (Krawczak and Schmidtke, 1994). However, in practice, length variation is surveyed at many VNTR (variable number of tandem repeats) loci simultaneously by multilocus fingerprinting. Due to the large number of loci examined and the extremely variable nature of this particular class of repeated DNA, each profile of bands (the so called 'fingerprint') is usually highly informative and individual-specific. Numerous probes are available that hybridize to VNTR loci possessing similar repeat unit sequences. A major practical advantage of multilocus fingerprinting, however, is the availability of probes that cross-hybridize in distant taxa. Although multilocus fingerprinting has been employed in several population level applications with varying degrees of success, it has some drawbacks. DNA profiles are often very complex, and it is usually not possible to identify both members of allelic pairs at individual loci. In addition, it often generates non-reproducible results, with the intensity of some bands in the mutilocus fingerprint and the presence of others varying between gels containing the same samples, even under carefully controlled conditions. Another disadvantage is the Southern blot and hybridization methodology. This method is much less sensitive, requires more DNA and is more time consuming than PCR-based approaches (O'Rielly and Wright, 1995).

In single locus profiling, allelic variation is surveyed at individual VNTR loci, using one of two approaches. One is Southern hybridization-based, and the other is PCR-based (O'Reilly and Wright, 1995). The first method involves restriction endonuclease digestion of genomic DNA, separation of fragments by electrophoresis through agarose gels, and Southern blotting on to DNA binding membranes (Jeffreys *et al.*, 1988;

Bentzen and Wright, 1993; Taylor *et al.*, 1994). Membranes are then probed with denatured, labelled DNA from a single VNTR locus, preferably the unique flanking region. The second method is to PCR amplifying the locus using primers flanking the array (Jeffreys *et al.*, 1988, 1994). PCR products are separated by gel electrophoresis and can be visualized by a variety of methods, including, under certain conditions, post-separation staining with ethidium bromide. Both the approaches require the isolation and cloning of single VNTR loci. In the second method, DNA sequence is needed from both sides of the array, which, in some instances, may require considerable additional effort. However, PCR-based VNTR systems are much less labour intensive and may be conducted in a standard molecular biology laboratory with a minimum of expensive equipment (O'Reilly and Wright, 1995).

DNA fingerprinting based on arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990, 1991) or random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) has also been applied in various contexts in fisheries research. AP-PCR or RAPD are PCR-based fingerprinting techniques where DNA polymorphisms can be examined by using arbitrary (usually 12-34 bases in AP-PCR and 8-10 bases in RAPD) oligonucleotide primers on genomic DNA. Generally, two cycles of PCR are performed under conditions of low stringency with a single random primer, followed by PCR at high stringency with specific primers (Williams *et al*., 1990; Welsh and McClelland, 1990, 1991). The number and size of amplified fragments depends on length and sequence of the primers. Polymorphisms generated by RAPDs or AP-PCR are dominant and are inherited in a Mendelian fashion. AP-PCR and RAPD techniques are easier to perform compared to other techniques and no prior sequence information is necessary.

However, these techniques are very sensitive to the PCR reaction conditions, template DNA concentration and purity, and PCR temperature profiles.

In recent years, DNA level polymorphisms or DNA fingerprints (both mitochondrial and nuclear) have been studied in a large number of wild and cultured fish species in a wide variety of fields, including individual and population identification, breeding, sex determination, linkage mapping and linkage analysis, parentage and pedigree analysis, and parasitology and pathology (reviewed by Dinesh *et al*., 1993; Wright, 1993; Zhang and Tang, 1993; Blears *et al*., 1998; Mueller and Wolfenbarger, 1999). Such studies involved many fish species including ayu (Han *et al*., 1992; Takagi *et al*., 1995), stripe and white bass and their F1 hybrid (Wirgin *et al*., 1991; Bosworth *et al*., 1994), *Poecilia latipinna* (Laughlin *et al*., 1994), several species of salmonids (Refseth and Jakobsen, 1994; Heath *et al*., 1994; Spruell, *et al*., 1994; Young *et al*., 1996), platyfish (Borowsky *et al*., 1995), bluegill sunfish (Colbourne *et al*., 1996), ginbuna (Dong *et al*., 1996; Tetsuya *et al*., 1997), channel catfish (Bosworth and Wolters, 1997), Indian major carps (Majumdar *et al*., 1997), medaka (Shimada and Shima, 1998), Javanese carp (Siraj *et al*., 1998), three-spined stickleback (Griffiths *et al*., 2000), seabass (Felip *et al*., 2000), African catfish (Kovács *et al*., 2001) and common carp (Ben-Dom *et al*., 2001). These studies utilized a wide variety of DNA markers, such as RAPDs, AP-PCR, microsatellites and AFLPs. DNA fingerprinting using AFLPs and microsatellites are discussed in Chapter 3 and Chapter 5 respectively.

1.5.1. Application of DNA fingerprinting in tilapia

DNA fingerprinting has wide-range applications to aquaculture and fisheries biology, including the study of quantitative genetics, parentage analysis, estimates of allele frequencies and population study (for review see Wright, 1993; McAndrew, 2000). Techniques of DNA fingerprinting have been successfully used in genome mapping in tilapia (Kocher *et al.*, 1998; Agresti *et al.*, 2000), in population studies and parentage analysis (Harris *et al.*, 1991; Bentzen *et al.*, 1991; Bardakci and Skibinski, 1994; Naish *et al.*, 1995; Majumdar *et al*., 1997).

DNA fingerprinting has also been applied to evaluate the success of genetic manipulations such as gynogenesis, androgenesis and clonal status in tilapia (Carter *et al.*, 1991; Jenneckens *et al.*, 1999; Sarder *et al.*, 1999). DNA fingerprinting using human-derived Jeffreys' minisatellite probes successfully verified the gynogenetic status in tilapia. However, these fail to distinguish meiotic from mitotic gynogenetics (Carter *et al.*, 1991; Sarder *et al.*, 1999) because of the dominant nature of the fingerprints. Biochemical markers, such as allozyme analysis has also been used to identify meiotic and mitotic gynogenetics in tilapia species and certain loci are very useful to distinguish meiotic and mitotic gynogenetic individuals (Hussain *et al.*, 1993; Sarder *et al.*, 1999).

1.6. Aims and structure of the present thesis

The primary goal of the experiments described in this thesis was to identify sex-linked molecular markers in Nile tilapia (*Oreochromis niloticus*) by using a suitable molecular genetic technique. AFLP was selected for this purpose, based on consideration of its efficiency and advantages. The thesis presents information about three main topics: genetic manipulation, sex determination and parentage analysis of genetically manipulated populations using two different molecular approaches. This thesis also reports for the first time evidence of spontaneous diploidization of maternal chromosome sets in this species. A schematic diagram of the structure of the thesis is shown in Fig.1.1.

In brief, the research focused on the following aims:

1. Production of XX and YY *Oreochromis niloticus* by mitotic gynogenesis.
2. Application of multilocus DNA fingerprinting for preliminary verification of success of gynogenesis using Jeffreys' 33.15 human-derived minisatellite probe.
3. Search for sex-linked markers in XX and YY homozygous individuals produced by mitotic gynogenesis using Amplified Fragment Length polymorphisms (AFLPs).
4. Isolation and characterization of sex-linked AFLP markers.
5. Linkage analysis of AFLP markers and the sex locus in *Oreochromis niloticus*.
6. Parentage analysis in normal and genetically manipulated populations using polymorphic microsatellite loci and AFLPs.
7. Heterozygosity analysis among a meiotic gynogenetic population using polymorphic microsatellite loci.

8. Analysis of linkage between a putative autosomal sex determining locus/i and red body colour in *Oreochromis niloticus*.

9. Assessment of spontaneous diploidization of maternal chromosome sets.

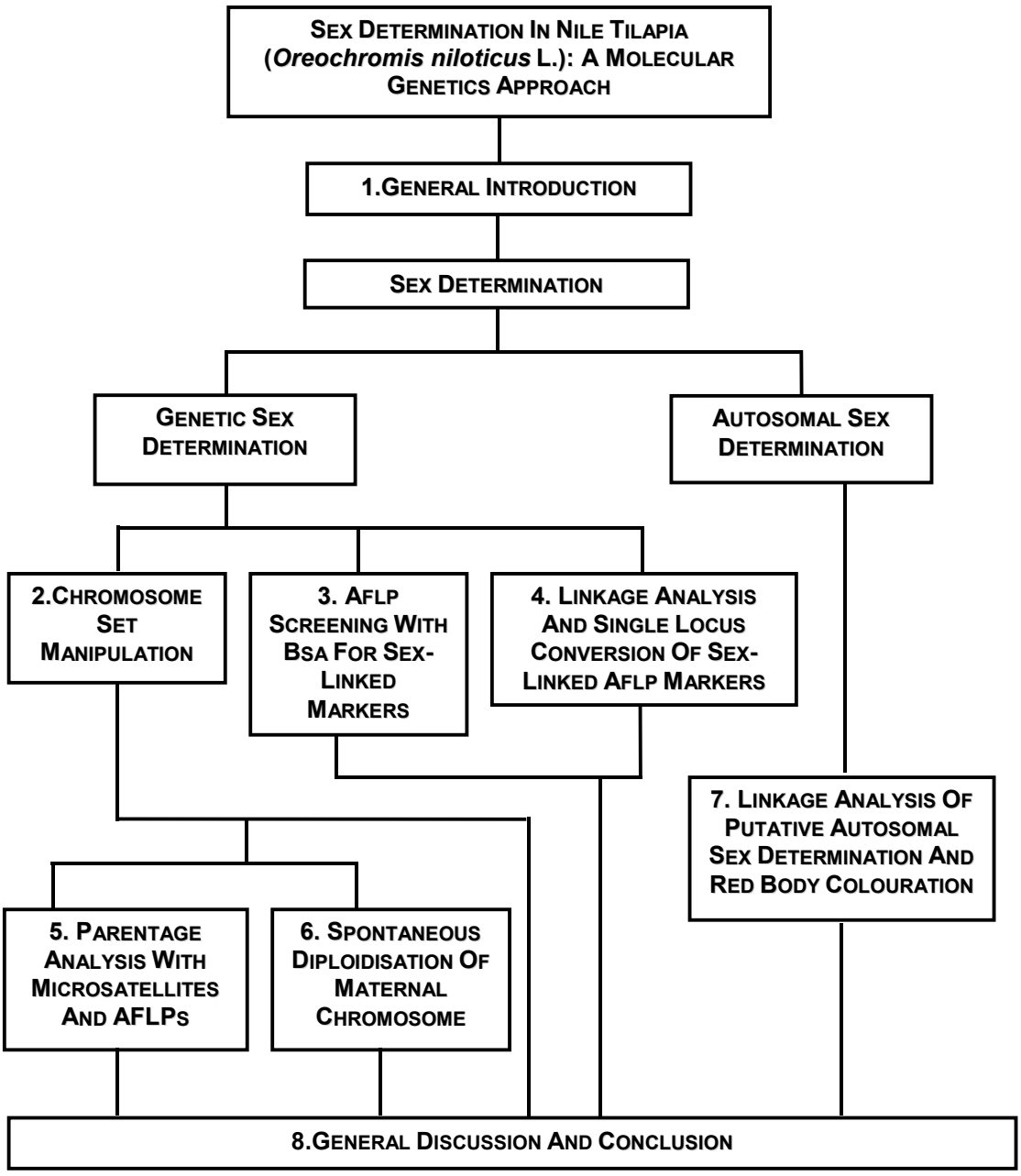


Fig. 1.1. Schematic diagram of the structure of this thesis.

Chapter 2 presents information about the production of homozygous XX and YY Nile tilapia *Oreochromis niloticus*, while Chapter 3 focuses on AFLP screening for the isolation of sex-linked markers. Chapter 4 presents the isolation of sex-linked AFLP markers and their sex-linkage. Chapter 5 discusses the application of two recently developed molecular techniques and their usefulness in parentage analysis among genetically manipulated populations of *O. niloticus*. Chapter 6 reports for the first time the occurrence of spontaneous diploidization in *O. niloticus*. Chapter 7 describes linkage analysis of a putative autosomal sex determining locus/i and the red body colouration in this species. Finally Chapter 8 presents a general discussion of the content of the thesis, and indicates further research possibilities.

Chapter 2

Production Of Homozygous XX And YY *Oreochromis niloticus*

2.1. Introduction

Highly inbred fish are of great importance in both aquaculture and basic research. They have the potential to be used in a variety of research areas including sex determination, immunology, toxicology and quantitative genetics. However, conventional breeding methods require the maintenance of several lines for up to 20 generations to produce fish homozygous at most loci. Such an intensive breeding programme would certainly be long term and require much labour. Additionally, inbred lines can be lost through inbreeding depression before the required degree of homozygosity has been achieved.

Instead of conventional sib mating, the rate of inbreeding can also be increased by parthenogenesis (Purdom and Lincoln, 1973). For this purpose, techniques of chromosome set manipulation such as induced gynogenesis or androgenesis are routinely used to produce homozygous inbred fish (reviewed by Hussain, 1996; Arai, 2001). These techniques of chromosome set manipulation greatly reduce the time required, as homozygous fish can be produced in a single generation and inbred clones can then be produced in the second generation. Induced gynogenesis is also important for aquaculture as crosses between inbred lines may generate heterosis for commercially important traits. It is also important for sex control of cultured species where sexual dimorphism is a determinant or to prevent unwanted reproduction.

Induced gynogenesis has proved to be a potent tool for the rapid production of homozygous strains in a number of fish species including zebrafish (Streisinger *et al.*, 1981), common carp (Komen *et al.*, 1991) and tilapia (Myers *et al.*, 1995; Hussain *et al.*, 1998; Jenneckens *et al.*, 1999; Sarder *et al.*, 1999; Müller-Belecke and Hörstgen-

Schwark, 2000). It has also proved to be an appropriate technology for other applied and basic research, such as mapping genes relative to their centromeres (Ihssen *et al.*, 1990), the study of single locus effects (Ihssen *et al.*, 1990; Hussain *et al*., 1994a; Markert *et al*., 1999), conducting immunological assays (Kaastrup *et al.*, 1989; Wiegertjes *et al*., 1994; Sarder, 1998), elucidating sex determination (Mair *et al.*, 1991a; Galbusera *et al*., 2000), studying endocrinology (Mair, 1993), production of transgenic fish by gene transfer in 'incomplete gynogenesis' (Thorgaard, 1992) and the development of genetic linkage maps using haploid gynogenesis (Kocher *et al.*, 1998). It is also useful to produce monosex populations (Penman *et al.*, 1987a, 1989; Scott *et al.*, 1989; Mair *et al.*, 1991a, b; Pongthana *et al.*, 1995) for aquaculture.

2.1.2. Gynogenesis

2.1.2.1. Natural gynogenesis

Natural gynogenesis is a special type of sexual reproduction in which insemination is obligatory but the sperm nucleus does not take part in development, and the ovum nucleus, with an unreduced number of chromosomes, becomes the zygote nucleus. Natural gynogenesis is not uncommon in fishes and is found in certain unisexual populations. Some populations of crucian carp (*Carassius auratus*) and several species of small viviparous fishes of the family Poeciliidae (*Poecilia* and *Poecilopsis*) reproduce naturally by gynogenesis (Golovinskaia, 1968; Cherfas, 1981; Thorgaard, 1983).

2.1.2.2. Induced gynogenesis

The second meiotic division in fish eggs occurs after fertilization, just before the fusion of the male and female pronuclei to form the zygote. The external fertilization in most fishes is another added advantage to manipulate chromosome sets. During the normal course of cell division after fertilization, either the second meiotic division or first mitosis can be manipulated or prevented in fish by applying suitable physical shocks (such as temperature or pressure) or treatment with some chemicals. Induced gynogenesis utilizes these advantages to produce individuals which will only carry the maternal genome. Induced diploid gynogenesis can be achieved in two steps: (1) the genetic inactivation of the sperm cell and (2) omission of the reduction of the female chromosomal complex (Cherfas, 1981; Nagy, 1987).

This technique involves fertilization of eggs with genetically inactivated sperms and subsequent diploidization of the haploid genome by an early (second meiotic division) or late (first mitosis) physical (such as heat, cold or pressure) or chemical shock which will disrupt the normal course of cell division. Several chemicals have been used to block polar body extrusion or mitotic division in fertilized eggs. Mosaic polyploid-diploid Atlantic salmon and brook trout were produced by exposing fertilized eggs to Cytochalasin B and Colchicine respectively (Refstie *et al.,* 1977; Smith and Lemonie, 1979). Anaesthetics such as nitrous oxide and freon have been used to block the first meiotic division (Johnstone *et al.*, 1989). In comparison to the success and ease of application of other methods, chemicals are not so suitable for mass production (Thorgaard, 1983).

The first successful example of artificial inactivation of sperm chromosomes without

the impairment of sperm's fertilizing capacity was described by Hertwig in 1911 in frogs (reviewed by Cherfas, 1981; Thorgaard, 1986; Ihssen *et al.*, 1990; Purdom, 1993). Hertwig irradiated frog sperm with radium gamma rays prior to fertilization. He observed massive, early embryonic mortality at low doses of irradiation and massive but delayed mortalities at higher doses. The embryos resulting from the latter were all abnormal. This paradoxical phenomenon is known as 'Hertwig effect'. The probable explanation of the 'Hertwig effect' lies in the partial inactivation of the sperm chromatin at low doses with the result that the embryos develop with an aneuploid chromosomal condition, which is detrimental to survival in very early in development. At higher doses, however, the sperm chromatin is completely inactivated or destroyed, and the maternal haploid chromosomes participate in further development. This gynogenetic haploid condition seems to be far less deleterious to development than aneuploidy (Ijiri and Egami, 1980).

Opermann (1913: cited by Ihssen *et al.*, 1990) became the first author to test the 'Hertwig effect' in fish. He used ionizing rays of radium and thorium on the sperm of brown trout (*Salmo trutta*) and observed a typical 'Hertwig effect' (Cherfas, 1981; Chourrout, 1987; Purdom, 1993; Thorgaard, 1983; Ihssen *et al.*, 1990). Since then, a variety of radiation types and chemicals have been used to inactivate fish sperm chromosomes, including ionizing radiation of γ-rays usually from ^{60}Co or ^{137}Cs sources (Purdom, 1969; Lincoln *et al.*, 1974; Nagy *et al.*, 1978; Chourrout *et al.*, 1980; Onozato, 1982; Refstie *et al.*, 1982) and X-rays (Romashov *et al.*, 1963: cited by Stanley and Sneed, 1974; Thorgaard, 1983).

A number of chemicals have also been used in induced gynogenesis studies in fish

and amphibians to inactivate the paternal genome. Chemicals which have been used successfully include toluidene blue (reviewed by Thorgaard, 1983; Chourrout, 1987), ethyleneurea (Jones and Whiting, 1975), and dimethylsulfate (Tsoi, 1969: cited and reviewed by Thorgaard, 1983). However, supernumerary chromosome fragments were detected by Thorgaard (1983) in dimethylsulfate treated spermatozoa, although their frequency was lower than in gamma (γ) rays-treated sperm. Residual paternal characteristics or chromosome fragments may sometimes be found in gynogenetic embryos even after high level γ-irradiation and X-irradiation of sperm (Chourrout and Quillet, 1982; Onozato, 1982). Sperm treated with radiation or chemicals usually show a lower viability and fertilizing capacity than those treated with UV radiation (Chourrout, 1987). Application of appropriate ultraviolet radiation (UV) to sperm before fertilization is routinely used to destroy the paternal genome. The appropriate dose for the sperm irradiation is very important for the successful production of gynogenetic individuals.

Haploid gynogenetic individuals are produced if such genetically inactivated sperms are used to fertilize eggs. Such haploid gynogenetic individuals normally survive until hatching but very few survive up to first feeding, with only one report of haploid gynogenetic *O. mossambicus* surviving up to equivalent age of maturation of normal diploid control individuals (Varadaraj, 1993). Haploid gynogenetic individuals can be used in linkage mapping. Indeed, the first genetic linkage map in *O. niloticus* was constructed using the haploid gynogenetic individuals (Kocher *et al*., 1998).

The combination of UV irradiation of sperm with an 'early' shock to prevent second polar body extrusion results in a form of diploid gynogenesis which is generally referred to as "meiotic" gynogenesis. However, the fertilization of eggs with UV treated sperm

and late shock treatment which restores diploidy by endomitosis also produces diploid gynogenetic individuals, which is referred to as "mitotic" gynogenesis. Individuals originating from mitotic gynogenesis are completely homozygous because of the duplication of a single chromosome set. However, within a single batch of eggs, individuals are not identical to each other because of the heterozygosity in the dam and the crossing over which occurred during the first meiotic division. In contrast, meiotic gynogenetics are partially heterozygous, again depending on the level of heterozygosity in the mother and the degree of recombination occurring during meiosis due to crossing over (Nace *et al.*, 1970, Hussain *et al.*, 1994a; reviewed by Penman and McAndrew, 2000; Arai, 2001).

Meiotic gynogenesis has been achieved in many fish species, including grass carp (Stanley and Sneed, 1974; Stanley, 1976), plaice and flounder (Purdom *et al.*, 1976; Thompson *et al.*, 1981), common carp (Nagy *et al.*, 1978; Nagy and Csanyi, 1982; Hollebecq *et al.*, 1986; Komen *et al.*, 1988), zebrafish (Streisinger *et al.*, 1981), rainbow trout (Chourrout and Quillet, 1982; Thorgaard *et al.*, 1983; Chourrout, 1984; Lou and Purdom, 1984a; Thompson and Scott, 1984; Kaastrup and Horlyck, 1987), Indian major carps (John *et al.*, 1984), cyprinid loach (Suzuki *et al.*, 1985), African catfish (Volckaert *et al*., 1994, 1997) and silver barb (Pongthana *et al.*, 1995).

Mitotic gynogenetic fish have also been produced in a number of species by suppressing the first mitotic division. Streisinger *et al.* (1981) was the first to report the successful mitotic gynogenesis in the zebrafish. Successful mitotic gynogenesis has also been reported in a number of other species, including common carp (Nagy, 1987; Komen *et al.*, 1991), medaka (Ijiri, 1987), ayu (Taniguchi *et al.*, 1988), Indian major

carp (Hussain *et al.*, 1994b) and African catfish (Galbusera *et al*., 2000).

2.1.2.3. Gynogenesis in Tilapia

Recent studies have attempted to induce gynogenesis in tilapia with the objective of elucidating sex-determining mechanism (Avtalion and Don, 1990; Pandian and Varadaraj, 1990; Mair *et al.*, 1991a,b). Induced gynogenesis has proven to be very useful in this respect. For instance, gynogenesis was used to confirm female homogamety in *O. niloticus and O. mossambicus*, to detect gene-centromere recombination for the sex determining loci in *O. aureus* and *O. niloticus* (Penman *et al*., 1987a; Avtalion and Don, 1990; Mair *et al*., 1991a,b; for review see Penman and McAndrew, 2000) and in the development of genetic linkage maps using haploid gynogenesis (Kocher *et al.*, 1998). This technique was also used to develop completely homozygous clones (Hussain *et al*., 1998; Jenenckens *et al*., 1999; Sarder *et al*., 1999) and heterozygous outbred clones in *O. niloticus* (Hussain *et al*., 1998; Sarder *et al*., 1999). Most of these studies were concentrated exclusively on the commercially important *Oreochromis* species*, O. niloticus, O. aureus* and *O. mossambicus.* There are no known reports of chromosome set manipulation studies in other genera of tilapia (Mair, 1993). Among the above mentioned three species most studies have been done in *O. niloticus.*

Both meiotic (Chourrout and Itskovich, 1983; Penman *et al.*, 1987b; Mair *et al*., 1987, 1991a,b; Varadaraj 1990a,b; Hussain *et al.* 1993; Sarder *et al.*, 1999; Müller-Belecke and Hörstgen-Schwark, 2000) and mitotic (Mair *et al*., 1987; Hussain *et al.*, 1993; Myers *et al.*, 1995; Müller-Belecke and Hörstgen-Schwark, 1995; Sarder *et al.*,

1999) gynogenetic individuals have been produced in the above mentioned three *Oreochromis* species using different shock treatments (cold, heat and pressure). However, most researchers have used UV irradiation to inactivate the sperm genome. Varadaraj (1990a) and Peruzzi *et al.* (1993) also reported the production of gynogenetics in *O. mossambicus* and *O. niloticus* by heterologous fertilization using common carp, crucian carp and koi carp sperm.

Haploid gynogenetic individuals have been produced in all the three species of *Oreochromis*, and Don and Avtalion (1988) and Penman (1989) have observed 'Hertwig effect'. Haploids are usually non-viable (with one exception reported by Varadaraj, 1993) in all three species, usually dying within 48 hours post hatching, and exhibiting a typical haploid syndrome such as shortening and deformation of the body, excessive hydration of the pericardium and impairment of pigmentation. The haploids pass through early embryonic development relatively well, but most die by hatching or first feeding or at the latest in the course of the next few days (Cherfas, 1981; Purdom, 1993; Mair, 1993). Spontaneous diploidization (Cherfas *et al*., 1991, 1995) of the maternal genome has not been reported in any of the three species.

2.1.2.4. Identification of gynogenetic diploids

It is important to correctly identify the gynogenetic diploids, i.e. those individuals where the sperm did not contribute genetically to the embryo. This confirmation can be obtained by several methods. Perhaps the simplest method for gynogenesis studies is the use of irradiated sperm from a related species to trigger development (Nace *et al.*, 1970). The use of foreign sperm in gynogenesis studies requires that the suitable sperm-donor

species be available. Within a species, colour, morphological, or biochemical markers can be conveniently used to provide proof of gynogenetic inheritance. Morphological, biochemical and DNA level markers have been applied to monitor the success of genetic manipulations by studying the inheritance of the parental genome in several fish species (Nagy *et al.*, 1978; Don and Avtalion, 1988; Young *et al*., 1996; Galbusera *et al.*, 2000). Sperm carrying dominant alleles have been used to provide proof of all-maternal inheritance in rainbow trout (Chourrout, 1980) and zebrafish (Streisinger *et al.*, 1981). In carp, Nagy *et al.* (1978) used irradiated sperm from normal (scaled) carp to fertilize eggs of homozygous recessive individuals with a scattered (ss) scale pattern, the observation of 100% scattered progeny supporting gynogenesis. Nagy *et al.* (1978) also used biochemical variation at the transferrin locus to provide proof of gynogenesis.

Genetic markers can also provide evidence of whether diploid gynogenesis results from a blockage of the first mitotic division or from a blockage of polar body extrusion. The progeny of a heterozygous female should all be homozygous at all loci, if diploidy results from suppression of first mitosis. Suppression of first polar body extrusion, which is unlikely because this event is normally completed before ovulation (Cherfas, 1981), would result in almost 100% heterozygous progeny for genes near the centromere, decreasing to 66% heterozygosity for genes segregating randomly in relation to their centromere (where the dam is heterorozygous at all loci). Nace *et al.*, (1970) suggested that suppression of second polar body extrusion would result in predominantly homozygous progeny for genes near the centromere, with 66% of progeny being heterozygous for more distally located genes. However, 100% heterozygosity for one allozyme locus (*ADA**) has also been reported in *Oreochromis*

niloticus suggesting strong interference during crossing over resulting in one chiasma per chromosome arm (Hussain *et al*., 1994a). In recent years, microsatellites have become the most efficient genetic markers for population and parentage determination. But several microsatellite loci are required for such studies to obtain an appropriate amount of genetic polymorphism (Terauchi and Konuma, 1994; Herbinger *et al*., 1995; Wenburg *et al*., 1996; Shikano *et al*., 1997, 2002; Takahashi *et al*., 1998; Fishback *et al*., 1999; Takagi *et al*., 1999). For example, using four or five microsatellite loci, Herbinger *et al*. (1995) were able to match 90% offspring of communally reared rainbow trout to one or two parental couples out of the 100 possible parental pairs. However, to identify mitotic gynogenetic individuals using microsatellites, typing of founder female and donor male with several microsatellite loci is necessaray to eliminate paternal transmission and failure of female diploidization. Preferentially those microsatellite loci should be selected which are heterozygous in both founder female and donor male with clearly different allele sizes.

Recent developments in molecular techniques enabled researchers to use molecular markers to study maternal genome in gynogenetic offspring. Biochemical markers, such as allozyme loci have been used to evaluate the success of gynogenesis in tilapia (Hussain *et al.*, 1993; Müller-Belecke and Hörstgen-Schwark, 1995; Sarder *et al*., 1999; Karayucel, 1999). DNA fingerprinting generated by Jeffreys' (Jeffreys *et al*., 1985a,b) human derived minisatellite probes 33.6 and 33.15 (Carter *et al.*, 1991; Sarder *et al.*, 1999) has been used in several fish species to evaluate the success of induced gynogenesis, including tilapia (Carter *et al.*, 1991) and African catfish (Volckaert *et al*., 1994). In recent years PCR-based fingerprinting techniques, such as AFLPs, have been

successfully used to evaluate the success of meiotic gynogenesis in seabass (Felip *et al.*, 2000). Other DNA fingerprinting techniques such as, micro- and mini-satellite typing, RFLP (restriction fragment length polymorphism), RAPD (randomly amplified polymorphic DNA) have also been applied to verify gynogenetic inheritance in several fish species (Van Eenennaam *et al.*, 1996; Jenneckens *et al.*, 1999; Galbusera *et al.*, 2000; Peruzzi and Chatain, 2000).

2.1.3. Objectives of the study

The objective of the present study was to produce homozygous XX and YY *O. niloticus* by mitotic gynogenesis in ova of XY sex-reversed neofemales. The success of this gynogenesis was then verified by multilocus DNA fingerprinting using Jeffreys' 33.15 human-derived minisatellite probe. The sex genotype of the gynogenetic fish (XX or YY) was assessed by progeny testing.

2.2. Materials and methods

2.2.1. Fish stock

The *Oreochromis niloticus* used in this experiment came from the Tilapia Reference Collection at the Institute of Aquaculture (IoA), University of Stirling, Scotland. They were originally collected from a wild population in Lake Manzala, Egypt in 1979 (McAndrew and Majumdar, 1983). The original fish stock has been managed to maintain their genetic variation over the generations, and their overall genetic variation has been studied by McAndrew and Majumdar (1983), Myers *et al.* (1995) and Sodsuk *et al.* (1995).

2.2.2. General management and maintenance facilities

The stocking, rearing, nursing and incubating activities were carried out in re-circulating tropical fresh water aquarium facilities at the IoA. The temperature of the aquaria was maintained at 27 ± 1°C with a controlled photoperiod of 12 hr of light and 12 hours of dark.

Brood fish were kept in glass aquaria for constant monitoring, particularly of the maturity states of females. Fertilized eggs were incubated in a separate unit fitted with round bottom plastic jars. The water prior to entering into the incubation units was treated with a 30 W UV sterilization unit (flow rate 20 l min^{-1}, UV dosage ca 62000 μW/sec/cm). The water flow rates of incubation jars were controlled by regulatory valves. During the incubation period, eggs were monitored regularly. Dead eggs were removed regularly and survival at different stages of embryonic development was scored.

After hatching and absorption of the yolk sac, the fry were transferred to recirculating fry rearing systems. These systems consisted of a series of 10 l perspex tanks with a water depth of 13 cm. Newly hatched 50-60 fry were stocked in each tank for a period of one month for nursing.

After one month of nursing in the fry rearing system, fry were transferred to re-circulating advanced fry rearing systems comprising a series of 30 l circular plastic tanks with a water depth of 18 cm. Again, 50-60 individuals were stocked in each tank. They were reared in the circular tanks for up to 3 months, and then transferred to large tanks (2 m diameter) to allow further growth. The basic water quality (such as pH, ammonia, nitrate and nitrite) parameters were checked every fortnight.

Fish of all sizes (from fry to brood) were fed with an appropriate size (designated as no. 3-5) of commercial trout feed (Trouw Aquaculture Nutrition, UK) at least twice a day. Early fry were fed at least 3 times a day with powdered food (0.25-1.0 mm in diameter) prepared by grinding no. 3 size feed in a coffee grinder. As the fish grew the feed size was increased. Advanced fry and fingerlings, weighing between 10 g and 40 g, received number 3-sized feed at a rate of 3-7.5% of body weight at least twice a day. Fish weighing 40 g - 80 g and 80 g and above were fed with number 4 and number 5-sized feed respectively, at a rate of 2-3% of their body weight per day.

To allow breeding to be monitored and to allow fish from different families to be kept in a same stocking tank, fish were tagged at 3-4 months old with Passive Integrated Transponder (PIT) tags (Trovan, Inc., USA; Avid Inc., USA). Each tag contains an individual code (Trovan tag -10 digit code; Avid tag - 9 digit code) that can be read by a specific tag reader. This small tag was placed into the body cavity of the fish through a

small lateral incision made just above the anus with a sharp sterile scalpel blade. After insertion of the tag orahesive protective powder (Squibb and Sons Ltd., UK.) was spread over the incision to assist adhesion and to prevent infection. A proprietary tagging syringe with a sharp specially designed needle was also used to tag bigger fish.

Fish were anaesthetised to avoid excessive handling stress prior to breeding, tagging and sampling (blood collection, fin clipping). For this purpose benzocaine (ethyl 4-aminobenzoate) solution at a final concentration of 1: 10,000 was used as an anaesthetic. A stock solution was first prepared by dissolving benzocaine powder at 10% (w/v) in ethanol.

All procedures of fish breeding, anaesthesia, tagging, sampling and genetic manipulation were performed under project (PPL 60/1967) and personal (PIL 60/7417) licenses issued by the U.K. Home Office.

2.2.3. Mitotic gynogenesis

2.2.3.1. Fish stock

The methodology for mitotic gynogenesis followed in this experiment was that of Sarder *et al.* (1999) with some minor modifications. Broodstock for this experiment was collected from the Stirling XY neofemale stock. Eleven mature XY neofemales and one mature XX neomale (Table 2.1.) were transferred from the stocking tanks into separate glass aquaria to facilitate proper monitoring. Several XX neomales were tested first and one was selected for the gynogenesis experiment as sperm donor on the basis of sperm quality and concentration. This XX neomale (005-117-817) was produced by hormonal sex reversal and also used for progeny testing of the XY neofemales and female mitotic

gynogenetic offspring. Another XX clonal (completely homozygous at all loci and identical to its full sib as well as mother) neomale (009-783-894) was also used for progeny testing of XY neofemales (Table 2.1). Clonal neomale used in this experiment were produced earlier in a separate experiment and the production and propagation of clonal *O. niloticus* were described by Sarder *et al*. (1999).

Table 2.1. List of XY neofemales, XX neomales and XX outbred clonal females (Obc) *Oreochromis niloticus* used for the production of XX and YY inbred lines by mitotic gynogenesis and progeny testing.

Family no.	PIT tag no.	Sex phenotype	Sex genotype	Other Phenotype	Other Genotype
1	00-013E-10FE	Female	XY	Red	RR
2	002-010-838	Female	XY	Blond	blbl
3	00-013E-3EE6	Female	XY	Red	RR
4	002-046-327	Female	XY	Red	Rr
5	000-366-365	Female	XY	Red	Rr
6	005-883-591	Female	XY	Blond	blbl
7	001-281-002	Female	XY	Blond	blbl
8	00-013C-B44D	Female	XY	Red	RR
9	00-013D-FA3C	Female	XY	Red	RR
10	00-01F7-76ED	Female	XY	Red	RR
11	009-822-311	Female	XY	Blond	blbl
Obc1	00-012C-13B7	Female	XX	Blond	Blbl
Obc2	00-012C-0FD5	Female	XX	Blond	Blbl
M1	005-117-817	Male	XX	Wildtype	rr
M2	009-783-894	Male	XX	Blond	Blbl

For progeny testing of male mitotic gynogenetic progeny, two XX outbred clonal (Obc) females were used. Obc females were produced by crossing females of one homozygous clonal line with sex reversed XX homozygous neomales from another clonal line in an earlier experiment. In the present experiment, the above mentioned (Table 2.1) two Obc females were collected from the existing stock of IoA.

2.2.3.2. Collection and ultraviolet (UV) irradiation of milt

Milt was collected from the male by hand stripping. Before stripping, faeces and mucus were cleaned off the urogenital papilla by using a piece of soft tissue. Hand

stripping involves application of gentle downward pressure with the thumb and index fingers from below the pectoral fin to the genital opening of the fish. The milt was collected in a glass capillary tube by placing it at the opening of the urethra. Milt contaminated with urine was discarded. Milt was kept at 4°C until use. Sperm used for gynogenesis were first checked for motility under a microscope and then the sperm concentration was determined using a haemacytometer. For this, 10 µl of milt was added to 490 µl of modified fish Ringers (MFR) solution, pH 8.0 (MFR; 112 mM NaCl; 68 mM KCl; 2.5 mM $NaHCO_3$; 15 mM $CaCl_2.6H_2O$), in a micro-centrifuge tube to make the total volume of 500 µl and mixed by gentle shaking. Then 10 µl of diluted milt from the first microcentrifuge tube was placed into another microcentrifuge tube containing 90 µl of MFR to give a total volume of 100 µl. The sperm was mixed again and about 12 µl of diluted sperm was placed carefully on each side of the haemacytometer under a cover slip. When the sperm settled down after a few mins, the number of sperm in five large squares was determined and the sperm concentration calculated as described by Sarder *et al.* (1999).

Diluted milt was transferred to a 30 mm diameter Petri dish for UV irradiation, where the depth of the diluted sperm solution was approximately 1.2 mm. The bottom of this Petri dish had previously been scoured with fine sand to remove its hydrophobicity and to allow the sperm suspension to spread evenly across the Petri dish. The UV irradiation was carried out at 4°C using a 254 nm wavelength UV lamp (Ultra-Violet Products, San Gabriel, California) at a dose of 250-260 µW cm^{-2} for 2 mins with gentle stirring using a mechanical shaker. Before irradiation, the intensity of the UV irradiation was measured by a radiometer (Ultra-Violet Products, San Gabriel, California).

2.2.3.3. Spawning females

Mature females of *O. niloticus* normally spawn at approximately 2-3 weeks' interval in aquarium conditions. Mature females can normally be identified by their swollen urogenital papilla and rounded abdomen and sometimes by pre-spawning behaviour (such as gulping at the tank bottom – indicative of nest building and cleaning). The eggs were collected from the mature females by hand stripping. Eggs were collected in a clean plastic Petri dish (100 mm in diameter) and were washed carefully with water from the recirculatory system several times to get rid of any blood and ovarian fluid.

2.2.3.4. Fertilization and heat shock

Eggs were divided into three batches. The first batch was fertilized with UV irradiated milt and the second batch with normal sperm. The first and second batches were transferred to the incubation unit as UV control and normal control respectively. The third batch was fertilized with UV irradiated sperm and used to produce mitotic gynogenetic individuals by applying a heat shock.

The heat shock method followed here was as described by Sarder (1998) with minor modifications. Two different temperature controlled water baths (Jencons Scientific Limited, England) were used for heat shocking. Eggs were fertilized *in vitro* with UV treated sperm in a clean Petri dish and then transferred directly to a netted plastic tea strainer placed into the water bath at 28°C. Fertilized eggs were kept at 28°C for 25-29 mins allowing them to proceed towards the first mitotic division. At 25-29 mins post fertilization, the strainer containing the fertilized eggs was transferred to another water bath set at 42-42.5°C for the heat shock, which was applied for 4 mins to induce

diploidization by prevention of the first cleavage. After the heat shock period was over, the strainer with the eggs was removed immediately back to the water bath set at 28°C for few mins to allow acclimatisation, and finally transferred to the incubator.

The schematic diagram of the production of XX and YY homozygous gynogenetics in the same generation and the generalized structure of an expected gynogenetic family are presented in Fig. 2.1a and Fig. 2.1b. The UV control group is not presented in the family structure as they normally died after hatching. One XX neomale (005-117-817) was used as sperm donor for all the families to produce XX and YY homozygous (mitotic) gynogenetic progeny, as well as for the production of related diploid control groups. The other XX neomale (009-783-894), which was a sex reversed clonal neomale, was used to produce normal diploid XX and XY progeny as unrelated control groups (Table 2.1 and Fig. 2.1).

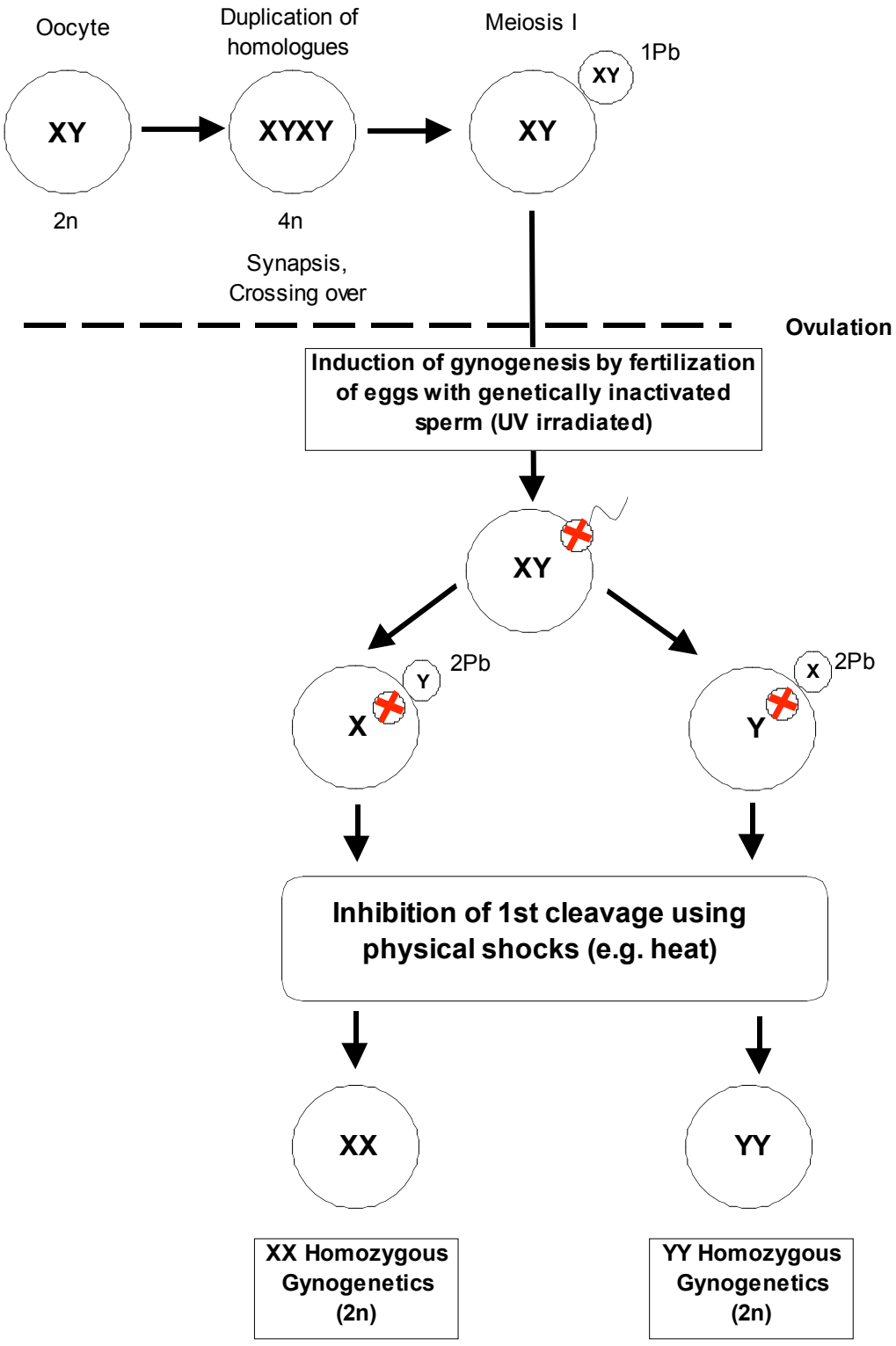


Fig. 2.1a. Schematic diagram of mitotic gynogenesis using XY neofemale for the production of homozygous XX and YY gynogenetic progeny in a single generation.

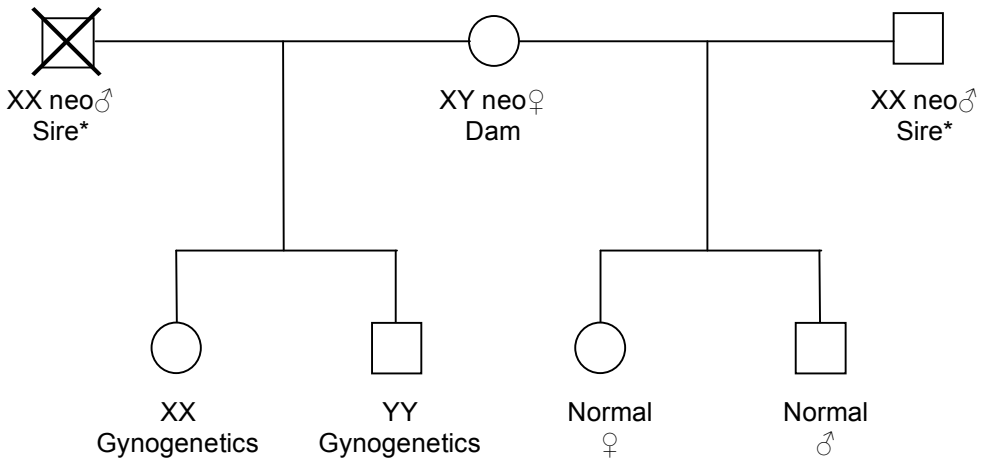


Fig. 2.1b. Schematic diagram of family tree showing the structure of an expected gynogenetic family from a XY neofemale. X: no genetic contribution; *: same sire was used with all XY neofemales in gynogenetic experiment.

2.2.4. Sexing tilapia

Two sexing methods were used. For large, mature fish, external examination of the urogenital openings could easily reveal the sex. The male has a single urinary and genital opening, whereas the female has separate openings for the urinary tract and oviducal canal (Chervinski, 1983). The second method was more accurate but involved dissection and examination of gonads, which required the fish to be sacrificed (Guerrero and Shelton, 1974). In this method, each fish was killed by an overdose of benzocaine and subsequent destruction of the brain. The aceto-carmine method is only used for small fish (i.e. too small for sexing on the basis of the papilla). The gonads were then dissected out on to clean glass slides and squashed with a drop of aceto-carmine stain by a cover slip. The sex of fish could then be easily revealed by examining the squashed gonads under 500 to 1000 magnification using a compound microscope. Although this method required the fish to be killed, it is quick, accurate and can be used to sex fish as small as 25 mm in standard length.

2.2.5. DNA extraction

2.2.5.1. Sample collection

Both fin and blood samples were collected for DNA extraction. Blood samples were collected from the caudal vein of fish using 1 ml syringes with 25GX5⁄8″ to 23GX1″ needles. Modified Cortland's saline (124 mM NaCl; 1.1 mM $CaCl_2.2H_2O$; 2.6 mM NaH_2PO_4; 12 mM $NaHCO_3$; 2 mM $MgSO_4$ and 5.6 mM Glucose) with 10 mM EDTA was used as an anticoagulant. Approximately one volume of Cortland's saline and two volumes of blood were taken in the syringe. The collected blood was transferred into a 1.5 ml sterile microcentrifuge tube and centrifuged at 1500g for 4 mins on a benchtop centrifuge. After centrifugation, the supernatant was removed and the precipitated red blood cells were immediately immersed in a flask containing liquid nitrogen. The frozen blood cells were stored at -20°C until required. Fin samples were collected by clipping around 3 mm of tissue from the tip of the caudal fin using sterile scissors. Samples were stored in absolute ethanol at 4°C.

2.2.5.2. Extraction of total genomic DNA

Total genomic DNA was extracted from both blood and fin tissues. Ten µl of blood or approximately 50 mg of fin tissue were used. Fin was minced using small, sharp scissors. Minced fin, fresh or thawed blood was transferred into sterile 2 ml screw cap tubes containing 435 µl of TEN buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 250 mM NaCl), 10 µl of 20 mg ml^{-1} ProteinaseK (Kramel Biotech, UK) and 50 µl of 10% (w/v) SDS (sodium lauryl sulphate, Sigma, Dorset, UK) solution. In the case of blood,

the SDS was added after dispersion of the sample in buffer. The mixture was mixed gently and incubated at 55°C in a rotating oven overnight (6-18 hrs).

After incubation, 10 μl of 20 mg ml^{-1} DNAse free RNAse (RNAse A; Sigma, UK) was added to the mixture, which was incubated at 37°C for 1 hr. After 1 hr of incubation, 500 μl of buffered phenol was added to the mixture, which was mixed at room temperature for 15-20 mins. The mixture was then centrifuged at 16,000g for 12 mins and the supernatant transferred to another sterile tube. This step was repeated once and 500 μl of 24:1 (v/v) chloroform:isoamyl alcohol was then added to the solution and mixed at room temperature for 15-20 mins. This step was repeated once. The mixture was then centrifuged again as previously described and the supernatant transferred to another sterile tube. Then 0.6-0.8 volume of chilled isopropanol was added to the solution, which was shaken vigorously for approximately one minute to precipitate the DNA.

The DNA pellet was then transferred to another sterile tube containing 500-700 μl of 70% ethanol, using a sterile pipette tip. The pellet was then washed twice in 70% ethanol. Finally, the ethanol was discarded and the pellet was dried and re-suspended in 100 μl of $TE_{0.1}$ (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) at 45-50°C for 30 mins, with total resuspension taking several days or weeks. The re-suspended DNA solution was kept at 4°C for immediate use or frozen at -20°C for long-term storage.

2.2.5.3. Quality and quantity of DNA

The quality of DNA was checked by electrophoresis on a 0.7% agarose gel, using 0.5X TAE (1 l of 50X solution contain 242 g Tris base, 57.1 ml glacial acetic acid and

100 ml 0.5M EDTA, pH 8.0) running buffer, for 3-4 hrs at a constant 5V cm^{-1}. The gel was stained with ethidium bromide (10 mg ml^{-1}) at a rate of 5 mg/100 ml and visualized using a UV transilluminator.

The quantity of DNA was determined by comparing the ratio of absorbance at 260 and 280 nm in a spectrophotometer. The quantity of DNA was also assessed by agarose gel electrophoresis of high molecular weight DNA with a standard DNA size marker.

2.2.6. Multilocus DNA fingerprinting

Multilocus DNA fingerprints were generated by restriction digestion of high molecular weight total genomic DNA, Southern transfer of restricted DNA and subsequent hybridization with Jeffreys' 33.15 (Jeffreys *et al.*, 1985a) human-derived minisatellite probe.

2.2.6.1. Restriction of total genomic DNA

High molecular weight total genomic DNA was digested with the restriction endonuclease *Hinf*I (5′…G/ANTC…3′). Then 100 µl of restriction mixture was prepared in a 1.5 ml sterile tube for each sample. A master mix was prepared by adding 10 µl of 10X reaction buffer, 10 µl of spermidine hydrochloride (40 mM), 1 µl of acetylated bovine serum albumen (BSA; 10 mg ml^{-1}), *Hinf* I (New England Biolab, UK) restriction enzyme (4 units μg^{-1}) and 20-25 µg of high molecular weight genomic DNA. Finally sterile distilled water was added to make a final volume of 100 µl. Restriction digestion reactions were then maintained at 37°C for 12-16 hrs.

After digestion, the reaction mix was centrifuged briefly and 200 µl of $TE_{0.1}$ and 300 µl of buffered phenol was added to the restricted DNA solution, centrifuged briefly and supernatant was collected. The restricted DNA was then extracted using an equal volume of 24:1 chloroform : isoamyl alcohol and precipitated with chilled isopropanol as described previously. The restricted DNA pellet was resuspended in $TE_{0.1}$ buffer and stored at 4°C until use. The success of restriction digestion was checked by electrophoresis of the restricted DNA on a 0.7% agarose gel with 0.5X TAE running buffer.

2.2.6.2. Southern blotting

For Southern blotting, 5 µg of each restricted DNA sample was electrophoresed on a 0.7% agarose gel with 0.5X TPE (1 l of 10X stock solution contain 108 g Tris base; 15.5 ml 85% phosphoric acid and 40 ml 0.5M EDTA, pH 8.0) running buffer at 1.5V cm^{-1} for 24 hrs. 50% of the buffer was changed and mixed well after 12 hrs.

At the end of gel electrophoresis, the DNA from the gel was transferred on to a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, UK) by vacuum blotting (Vacu Gene™, Pharmacia, UK) using the standard procedure. After blotting, membranes were rinsed in 2X SSC (1 l of 20X stock solution containing 175.3 g sodium chloride, 88.2 g sodium citrate, pH 7.0, sterilise by autoclaving) for 10 mins. The membrane was then air dried and baked at 80°C for 2 hrs and stored at room temperature in a dry place until further use.

2.2.6.3. Hybridization

The membrane was hybridized with Jeffreys' 33.15 probe. The probe was labelled with alkaline phosphatase and chemiluminescent detection was performed by Lumi-Phos® 530 (Cellmark Diagnostics, Zeneca, UK). The hybridization and washing of the membrane and the autoradigraphy were performed according to the manufacturer's instructions (Cellmark Diagnostics, Zeneca, UK) except that an additional pre-washing step was carried out for 1 hr at 65°C with 0.1X SSC and 0.5% SDS.

2.2.7. Statistical analysis

For analysis of survival rates Kruskal-Wallis tests were performed to compare the survival at different stages of embryonic development between the control and mitotic gynogenetics using Minitab (V. 13) statistical analysis software. To interpret the sex ratios, heterogeneity chi-square analysis was performed for individual family. Chi-square analysis was performed for expected proportions on pooled data. To assess the correlation between percent survival and maleness in normal cross progeny Pearson's correlation analysis was performed using SPSS (V. 10 for Windows) statistical analysis software.

2.3. Results

2.3.1. Survival of mitotic gynogenetic *O. niloticus*

The survival rate of mitotic gynogenetic *O. niloticus* and their corresponding control groups at three different stages of development are presented in Table 2.2. Seven out of the 11 neofemales produced mitotic gynogenetic individuals in varying proportions. Survival of the mitotic gynogenetics at all stages was significantly ($P < 0.05$) lower than that of the control groups (Table 2.2, Fig. 2.2).

The results also showed that the average survival rate in all three stages of development was relatively low in control groups. Among the control groups, the survival at pigmentation, hatching and yolk sac absorption ranged from 63-87%, 54-81% and 44-71% respectively (Table 2.2.).

Table 2.2. Survival (%) of control and mitotic gynogenetic *Oreochromis niloticus* at different stages of embryonic development (P: pigmentation; H: hatching; YSA: yolk sac absorption).

Founder	Family	Control group			Mitotic gynogenetic group			
female tag no.	no.	P	H	YSA	P	H	YSA	YSA*
00-013E-10FE	1	85.45	73.15	63.36	44.2	20.64	9.34	14.74
002-010-838	2	63.13	54.06	44.18	42.75	15.4	0.96	2.17
00-013E-3EE6	3	63.32	54.12	31.52	21.6	2.71	1.76	5.58
002-046-327	4	87.2	81.8	71.12	52.33	16.45	4.8	6.75
000-366-365	5	84.0	74.68	65.87	55.93	33.9	16.4	24.9
005-883-591	6	79.84	70.82	64.8	43.69	27.9	0.52	0.8
001-281-002	7	70.5	64.15	54.5	45.1	5.6	0.25	0.46

* relative to control

Among the mitotic gynogenetic groups, the survival at pigmentation, hatching and yolk sac absorption stage ranged from 21-56%, 2-34% and 0.25-16% respectively (Table 2.2).

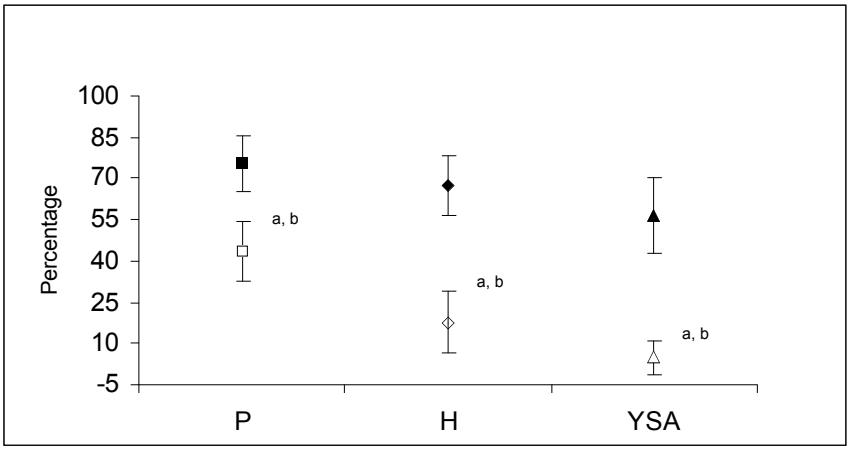


Fig.2.2. Mean survival (±SD) in control and mitotic gynogenetic *Oreochromis niloticus* in three different stages of embryonic development. Data are presented at pigmentation, hatching and yolk sac absorption stages; P: pigmentation; H: hatching; YSA: yolk sac absorption; black: control; open: gynogenetics, [a]: significantly different ($P < 0.05$) from control groups; [b]: significantly different ($P < 0.05$) from each developmental stages in gynogenetic groups.

The results also revealed that the survival among the mitotic gynogenetic groups at all three developmental stages was significantly lower ($P < 0.05$; Kruskal-Wallis test) than the control groups. Among the mitotic gynogenetic groups, a significant ($P < 0.05$; Kruskal-Wallis test) fall in the survival rate was observed between each developmental stages (Fig. 2.2).

2.3.2. Multilocus DNA fingerprinting

The success of gynogenesis in seven families of *O. niloticus* was verified by multilocus DNA fingerprinting. The multilocus DNA fingerprints generated by Jeffreys' human-derived 33.15 hybridization probe successfully identified gynogenetic individuals (Fig. 2.3).

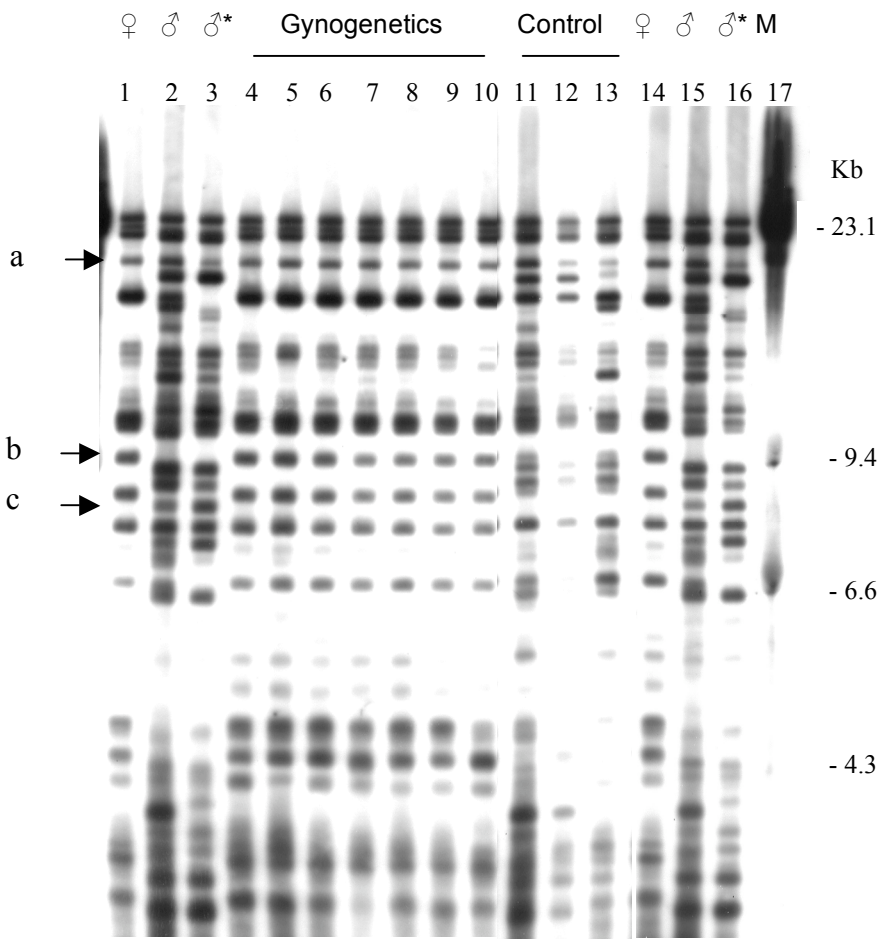


Fig. 2.3. DNA fingerprint generated by Jeffreys' 33.15 human-derived minisatellite probe in *Oreochromis niloticus*. Lane legends: 1-Founder female, 2-donor male, 3-unrelated male, 4 to 10- mitotic gynogenetics, 11 to 13- control, 14-founder female, 15-donor male, 16-unrelated male, 17(M)- DNA size marker (λ-DNA-*Hin*d III digest). a-monomorphic band; b-female specific band; c- male specific band; *: unrelated male.

Fig. 2.3 shows an example of the fingerprint pattern generated by the Jeffreys' 33.15 probe in one of the families of mitotic gynogenetics. The fingerprint pattern typically revealed three different types of bands, monomorphic bands (i.e. present in all individuals), maternal bands and paternal bands. All the mitotic gynogenetic individuals inherited maternal bands and none of the paternal bands, indicating the success of

gynogenesis in this experiment. The inheritance pattern among the control fish was characterized by the appearance of both maternal and paternal bands as would be expected from normal sexual reproduction. Multilocus fingerprinting was performed for all the gynogenetic families produced in this experiment. In all families the fingerprints generated by this probe verified the success of this procedure. No paternal bands were observed in gynogenetic groups.

2.3.3. Sex ratios of mitotic gynogenetic and control groups

The sex ratios of the mitotic gynogenetics and the control groups are presented in Table 2.3 and Fig. 2.4. The sex ratios among the gynogenetic *O. niloticus* were skewed to males in all gynogenetic families except family 4, with families 2, 3 and 7 producing all male progeny.

Table 2.3. Sex ratios of mitotic gynogenetics and control group (with male 1 and 2). Sex ratios are presented as female:male (F:M) with percentage of males in parentheses. (male 1- 005-117-817: XX sex reversed male used as sperm donor; male 2- 009-783-894: XX sex reversed clonal neomale; NT-not tested).

Family	Sex ratios					
	Mitotic gynogenetics		Control			
			Male1		Male 2	
	F:M	chi-square	F:M	chi-square	F:M	chi-square
1	13:51 (80)*	22.56	38:96 (72)*	25.10	48:56 (54)	0.60
2	0:9 (100)*	9.0	31:69 (69)*	14.44	61:39 (39)*	4.84
3	0:20 (100)*	20.0	10:44 (81)*	24.08	40:49 (55)	0.90
4	28:22 (44)	0.72	54:77 (59)	3.96	35:31(47)	0.24
5	18:31 (62)	3.44	70:59 (56)	0.92	NT	NT
6	5:14 (74)*	4.26	48:96 (67)*	16.0	16:30 (65)*	4.26
7	0:5 (100)*	5.0	22:57 (71)*	15.5	23:27 (54)	0.32

* significantly different from 1:1 sex ratio at $P<0.05$

Two XX neomales were used to test the sex ratios among the control crosses. When male 1 (sperm donor) was used, the progeny sex ratios were skewed to males in all of

the families except family 4 and family 5. However, when male 2 was used, significant deviations from 1:1 sex ratios were observed in only two families (family 2 and family 6).

When male 1 was used, the overall sex ratios among the mitotic gynogenetic and the control groups showed significant ($P < 0.05$) deviation from 1:1 sex ratios; however, with male 2 the overall sex ratios of the control groups did not differ from the expected 1:1 sex ratio (Fig. 2.4).

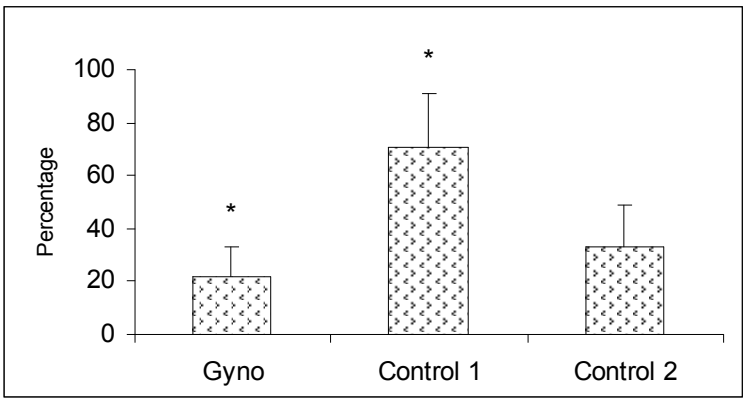


Fig. 2.4. The percentage of males (Mean ± SD) in gynogenetic *Oreochromis niloticus* and their control crosses, control 1 was crossed with male 1 and control 2 was crossed with male 2, *: significantly ($P < 0.05$) different from 1:1 sex ratio.

Comparison of the sex ratios of these control males (male 1 and male 2) with the percentage survival revealed a significant correlation between sex ratios and survival in male 1 ($P < 0.05$; Pearson's Correlation). This suggests that the male-biased sex ratio in the progeny of male 1 could be a consequence of sex-specific mortality. However, the correlation between the survival and the sex ratio among the gynogenetic groups could not be assessed, because the gynogenetic individuals for each neofemale came from several small groups.

2.3.4. Progeny sex ratios of mitotic gynogenetics

Several randomly selected YY and XX gynogenetic progeny from families 1, 4, 5 and 6 were progeny tested with XX outbred clonal females (Obc) and XX sex reversed neomales respectively. Progenies from families 2, 3 and 7 were not tested because of the small sample size and predominantly all male progeny. Table 2.4 presents the sex ratio data from each YY gynogenetic male tested.

YY males from family 4 produced 100% males when crossed with XX Obc females. Of the four YY males tested from family 1, two gave 100% male progeny, and two gave a very small proportion of female progeny. Seven YY males were tested from family 5, three of them producing 100% male progeny and four producing up to 31.7% female progeny. The only YY male tested from family 6 gave 100% male progeny (Table 2.4).

Table 2.4. Sex ratios of YY gynogenetic males from four different families of *Oreochromis niloticus*. These were progeny tested by crossing to outbred clonal females (Obc).

Family	Founder female tag number	YY gynogenetic male tag number	F1 Obc XX Female tag no.	Total number of fish sexed	Number of males	Number of females	% males
1	00-013E-10FE	00-0137-1BBD	00-012C-13B7	50	50	0	100
		00-0136-E502	00-012C-13B7	60	60	0	100
		00-013B-BED9	00-012C-13B7	76	75	1	98.7
		00-013E-01D0	00-012C-13B7	50	49	1	98
		00-0137-74DF	00-012C-13B7	50	50	0	100
		00-0137-2A6E	00-012C-13B7	50	50	0	100
		00-0135-15FO	00-012C-13B7	47	47	0	100
4	002-046-327	00-013F-8556	00-012D-D47B	50	50	0	100
		00-0121-4BFE	00-012D-D47B	26	26	0	100
		00-012E-A48A	00-012C-13B7	50	50	0	100
		00-013E-322F	00-012D-D47B	25	25	0	100
5	000-366-365	00-0137-7F49	00-012C-13B7	50	50	0	100
		00-01E0-A99D	00-012C-13B7	48	48	0	100
		00-01DE-E203	00-012C-13B7	50	50	0	100
		00-012D-DEFC	00-012C-13B7	8	7	1	87.5
		00-013E-4871	00-012C-0FD5	60	51	9	85
		00-013E-30FD	00-012D-D47B	28	21	7	75
		00-012C-13CF	00-012C-0FD5	41	28	13	68.3
6	005-883-591	00-0134-2CFE	00-012C-13B7	50	50	0	100

All of the tested YY mitotic gynogenetic males from the four different families produced sex ratios which were significantly different from 1:1 ($P < 0.05$). However, YY males tested from family 5 produced around 11% female progeny when crossed with an XX Obc female (Table 2.4 and Table 2.6).

Similarly several randomly selected XX gynogenetic females from family 1, 4, 5 and 6 were progeny tested with XX neomale respectively. These results are presented in Table 2.5.

All the XX gynogenetic females tested from family 1, 4 and 6 produced 100% females when crossed to XX neomale, however, two females out of four in family 5 produced a small proportion of males (up to 6%) (Table 2.5).

Table 2.5. Sex ratios of XX gynogenetic females from four different families of *Oreochromis niloticus* which were progeny tested by crossing to an XX neomale.

Family	Founder female tag number	XX gynogenetic female tag number	XX neomale used	Total number of fish sexed	Number of females	Number of males	% females
1	00-013E-10FE	00-0121-15ED	005-117-817	31	31	0	100
		00-01E0-3C7D	005-117-817	33	33	0	100
		00-0135-BE59	005-117-817	37	37	0	100
		00-01E0-A552	005-117-817	24	24	0	100
		00-0134-34FC	005-117-817	31	31	0	100
		00-013C-A861	005-117-817	40	40	0	100
4	002-046-327	00-013E-097C	005-117-817	27	27	0	100
		00-01E0-3E42	005-117-817	32	32	0	100
		00-01E0-3ECC	005-117-817	25	25	0	100
5	000-366-365	00-0136-F3F8	005-117-817	49	46	3	94
		00-01DF-59E1	005-117-817	39	39	0	100
		00-01E0-AC24	005-117-817	36	36	0	100
		00-013E-0308	005-117-817	59	57	2	96
6	005-883-591	00-01E0-3DDF	005-117-817	25	25	0	100

The overall sex ratios of all of the YY and XX gynogenetic males and females tested from four families were significantly different from 1:1 sex ratios ($P < 0.05$) and also close to 100% male and female respectively. However, around 11% females were

observed to be produced by the YY gynogenetic males from family 5 when crossed to Obc females. Additionally, around 3% male progeny were observed when XX gynogenetic females from the same family (family 5) were crossed to an XX neomale (Table 2.6).

Table 2.6. Overall progeny sex ratios from YY male and XX female mitotic gynogenetics. In progeny testing of YY mitotic gynogenetic males, two XX outbred clone (Obc) females were used, sex ratios are presented as male:female and percent of males are presented in parentheses. For XX mitotic gynogenetic females, one XX sex reversed neomale was used for progeny testing. Sex ratios are presented as female:male and percent of females are presented in parentheses.

Gynogenetic families	Sex ratios	
	YY mitotic gynogenetics ♂ : ♀ (% ♂)	XX mitotic gynogenetics ♀ : ♂ (%♀)
1	379:2 (99.47)	196:0 (100)
4	151:0 (100)	84:0 (100)
5	255:30 (89.47)	178:5 (97.26)
6	50:0 (100)	25:0 (100)

2.4. Discussion

The present study aimed to produce homozygous XX and YY *Oreochromis niloticus* by gynogenesis. Therefore, emphasis was given to the production of homozygous fish rather than to further optimize the gynogenesis technique. Gynogenesis is now a routine chromosome manipulation technique and has been applied to a large number of different fish species with variable degrees of success. Therefore, the discussion in this Chapter will mainly focus on the fish produced by gynogenesis in *O. niloticus*.

Multilocus DNA fingerprinting revealed the gynogenetic status of all the UV / late heat shock treated fish produced in this study. The fingerprints showed the inheritance of maternal bands in the mitotic gynogenetic offspring and the absence of paternal bands. The use of Jeffreys' human-derived minisatellite probes (both 33.6 and 33.15) to determine the success of gynogenesis has also been described in African catfish (Volckaert *et al.*, 1994). Although in the present experiment no paternal chromosomal transmission was observed in any of the gynogenetic offspring across the families, Carter *et al.* (1991) observed some paternal transmission by using this technique. The level of polymorphisms observed by the multilocus fingerprinting between each of the XY neofemales and the XX donor male, were sufficient to prove the maternal inheritance among the gynogenetic progeny, supported by complete mortality (and haploidy) in UV control group.

In the present study, gynogenesis was carried out according to the procedures described by Sarder *et al.* (1999). The UV irradiation of sperm for 2 minutes at 250-265μW cm^{-2} successfully destroyed the sperm chromosomes as shown by the lack of survival in UV control group. Higher UV dose, of 300-310 μW, has also been used for

tilapia sperm irradiation (Hussain *et al.*, 1993). The effect of UV intensity and duration were not optimized in this study, therefore its implication on the survival rate cannot be discussed. However, in an earlier study, Sarder (1998) observed a higher survival rate in mitotic gynogenetics when using a lower UV dose and suggested that higher UV dose caused more damage to the sperm, resulting in poorer fertilization rates and consequently in a lower survival rate of mitotic gynogenetics.

XY neofemales were used in the present experiments to produce both XX and YY homozygous fish within families. For this purpose, 11 XY *O. niloticus* neofemales were used. Seven females produced mitotic gynogenetic progeny with a variable degree of survival and the other four did not produce any mitotic gynogenetics. In these cases all embryos died before the pigmentation stage.

A low survival rate was observed in all three stages of embryonic development, not only in the mitotic gynogenetics, but also in the control groups. Poor survival in both the control and mitotic gynogenetics in the present experiment might be due to the poor egg quality of the XY neofemales as well as a low resistance to heat shock treatment. A significant drop in the survival in the different stages of embryonic development was also observed in the present experiment among the mitotic gynogenetics. This could have happened due to the eggs of those females not being resistant to heat shock and/or the XY neofemales may have had recessive lethal alleles at some loci, resulting in lethal effects killing homozygous individuals. Individual variation in susceptibility to heat shocks and a high rate of expression of recessive deleterious and lethal genes among homozygous mitotic gynogenetics resulting in poor survival has been suggested by

Onozato (1984), Lou and Purdom (1984b), Purdom *et al.* (1985), Mair *et al.* (1987), Hussain *et al.* (1993) and Sarder *et al.* (1999).

Sex ratios among the mitotic gynogenetic progeny in the present experiment were skewed to males in all the six families except family 4 and 5. The small sample sizes in family 2, 3, 6 and 7 could have some influence on these skewed sex ratios. Regarding the sex ratios of both meiotic and mitotic gynogenetic progeny in *O. niloticus*, two unexpected conditions have been reported: (i) unexpected males among XX gynogenetic progeny and (ii) an excess of males among meiotic gynogenetic groups produced from XY neofemales. However, the present experiment reports a third situation: (iii) an excess of males among mitotic gynogenetic groups produced from XY neofemales.

In the situation (i) the occurrence of unexpected males among meiotic and mitotic XX gynogenetic progeny (Table 2.5) has been explained by postulating the influence of an epistatic sex reversing autosomal locus (*S*ex *D*etermining *L*ocus-2, *SDL*-2) (Mair *et al*., 1991a; Hussain *et al*., 1994a; Sarder *et al.*, 1999). The occurrence of unexpected males even in one family of an homozygous clone produced from XX females has also been reported in this species (Sarder *et al*., 1999), which further supports the concept of the partial penetrative nature of a putative autosomal recessive sex reversal locus. Similar type of sex reversal in gynogenetic XX females has also been reported in common carp (*Cyprinus carpio*) (Komen *et al*., 1992). Authors suggested an autosomal recessive sex determining gene, termed masculinization (*mas-1*), which produces males in the homozygous state (XX *mas-1*/ *mas-1*) and females and intersexes in the heterozygous state (XX *mas-1*/ *mas+1*).

In situation (ii) the male-skewed sex ratios among the gynogenetic progeny produced from XY neofemales has been suggested to be the possible consequence of crossing over between the sex determining locus and the centromere (Mair *et al*., 1991a). In contrast, the same authors found the expected 1:1 sex ratios among the mitotic gynogenetic progeny produced from the same fish.

In situation (iii) i.e. the present experiment, there is a significant deviation from 1:1 sex ratios among mitotic gynogenetic progeny produced from XY neofemales in five out of seven families.

Previous experiments on the production of YY males in this species showed that YY males are slightly less viable and grow more slowly than XY males (Mair *et al*., 1994, 1997). In contrast, in the present experiment, it could be that sex-biased mortality occurred predominately among the XX gynogenetic offspring resulting in male-skewed sex ratios. Therefore, it can be stated that, progeny sex ratios resulting from mitotic gynogenesis of XY neofemales should follow 1:1 sex ratios, unless female-biased mortality of any sorts, either genetic or non-genetic operates in the experimental population.

To evaluate the sex ratios of mitotic gynogenetic founder XY neofemales, two XX neomales were used to produce control fish: M1 was an XX methyl testosterone (MT)-treated neomale while M2 was an XX MT-treated homozygous clonal neomale. Sex ratios produced from both of these neomales in most cases were male-skewed but sufficient to prove the XY genotype of the founder females. However, from the overall average sex ratios, M1 showed a significantly skewed male sex ratio while M2 did not significantly differ from 1:1 sex ratio (Fig. 2.3). A significant correlation between the

survival and sex ratios among the progeny of M1 was also observed, which suggested this skewed male sex ratio was also produced by sex-specific mortality.

Several randomly selected YY and XX mitotic gynogenetic individuals from families 1, 4, 5 and 6 were also progeny tested. YY males from family 1, 4 and 6 produced 100% males while YY males from family 5 produced a mean of 88% males, ranging from 68-100% males. Mair *et al.* (1991a) reported 100% males produced from two YY meiotic gynogenetics and 92.5-100% males from four YY males when crossed with XX normal and XY sex reversed females. They suggested the occurrence of multiple autosomal sex modifying genes.

Progeny testing of XX mitotic gynogenetic females from families 1, 4 and 6 produced 100% females while XX progenies from family 5 produced 98% females when crossed with M1. A possible explanation of the small proportion of males produced by two out of four females of family 5 tested in the present experiment, is that, the XY founder neofemale of these XX mitotic gynogenetic individuals was homozygous at a recessive autosomal sex determining locus. Therefore, considering the free recombinations, 50% of the XX gynogenetic progeny were homozygous recessive at the same locus. Moreover, presumably XX neomale (M1) was recessive heterozygous. Thus, crosses of such XX females homozygous at a recessive sex-modifying locus with XX neomale that is heterozygous at that locus might produces a small proportion of males in the progeny.

Such unexpected males in the gynogenetic and clonal lines in tilapia species have been reported and discussed by several authors and several hypotheses have been put forwarded to explain this phenomenon. These included single or numerous sex-modifying factors (Mair *et al.*, 1991a, 1997), an autosomal sex reversal locus (*SDL*2)

(Hussain *et al.,* 1994a), environmental factors (Abucay *et al.*, 1997) and partial paternal inheritance due to incomplete sperm inactivation (Sarder, 1998). This phenomenon of autosomal sex reversal will also be discussed in Chapter 7 of this thesis.

Male sterility was observed in two YY gynogenetic males from family 6 and blocked genital openings were also observed in several XX mitotic gynogenetic females across the families in this experiment. Abnormal and deformed genital openings were also observed in several XX female mitotic gynogenetics.

From the above discussion, it can be stated that the gynogenesis technique was successful in producing homozygous XX and YY fish in *Oreochromis niloticus.* In summary, despite skewed sex ratios in mitotic gynogenetics, which in some cases gave 100% males, all progeny-tested mitotic gynogenetics proved to be XX or YY as expected from the phenotypic sex. Therefore, these homozygous XX and YY individuals were suitable for subsequent work involving application of different molecular techniques to try to isolate sex-linked molecular markers or for parentage studies using molecular markers.

Chapter 3

Screening For Sex-Linked Markers Using Amplified Fragment Length Polymorphisms (AFLPs)

3.1. Introduction

Evidence of genetic sex determination (GSD) is primarily based on the results of cytological studies, with heteromorphic chromosome pairs (termed sex chromosomes and designated X, Y, W and Z) indicating either male or female hetero- or homogamety. Such monofactorial chromosomal models satisfactorily explain the sex determining systems in most mammals, but fail to explain sex determination in many other groups of vertebrates, where more than one pair of sex chromosomes as well as autosomes may act to determine sex. Moreover, in lower vertebrates such as fish, the occurrence of morphologically distinct sex chromosomes is rare in most groups. This suggests that many teleosts exhibit a relatively primitive condition of sex chromosome evolution where the accumulation of gross chromosomal rearrangements is insufficient to be visible at the cytological level (Devlin *et al.*, 1991).

Sex determination systems (e.g. XX/XY or WZ/ZZ) can be identified by other techniques, such as intraspecific crosses, hybridization and sex ratios among genetically manipulated populations (from gynogenesis or androgenesis). In recent years, improved technology has revealed sex chromosomes in studies of meiotic chromosome pairing. Recent developments in molecular genetics also open the door to better understanding and identification of sex chromosomes by allowing the direct analysis of DNA sequence.

Understanding sex determination in fish is of particular importance due to their economic importance. Because of variable growth rates and other beneficial attributes of economic interest (such as colour and shape), fish farmers often prefer to culture only a single sex. For example, male cichlids grow faster than females and female salmonids mature later than males. Monosex culture also prevents unwanted reproduction in

culture ponds and thereby can increase product quality as well as price. Studying sex determination in fish may also improve understanding of the evolution of sex-chromosome differentiation and sex determination in higher vertebrates (Trombka and Avtalion, 1993).

The Nile tilapia, *Oreochromis niloticus*, is widely used in tropical aquaculture and is also becoming a popular species for culture in temperate countries (Mair *et al.*, 1997). The males in this species grow faster than the females (Marr *et al.*, 1966; Fryer and Iles, 1972; Lowe-McConnell, 1987; McAndrew and Majumdar, 1989; Marengoni *et al.*, 1998). Monosex (all male) culture of this species is also an important technique to avoid precocious maturation and unwanted reproduction in culture ponds.

Primarily, *O. niloticus* displays GSD of an XX female/XY male type (Jalabert *et al.*, 1974; Penman *et al.*, 1987a; Mair *et al.*, 1991a; Carrasco *et al.*, 1999). However, sex determination in this species is also influenced by environmental factors (Baroiller *et al.*, 1995a,b; 1996; Abucay *et al.*, 1999) and by autosomal genes (Majumdar and McAndrew, 1983; Mair *et al.*, 1991a; Hussain *et al.*, 1994a; Müller-Belecke and Hörstgen-Schwark, 1995; Baroiller *et al.*, 1996; Sarder *et al.*, 1999). The phenotypic sex of *O. niloticus* can also easily be inverted by hormonal sex reversal (reviewed by McAndrew, 1993; Macintosh and Little, 1995; Green *et al.*, 1997).

Due to the importance of all male culture for this species, identification of sex in the earlier stages is very important. Phenotypic sex can be identified by the histological examination of gonads in the fry 10-12 weeks after hatching or by examining the genital papilla in adult fish. However, due to the influence of temperature and autosomal genes, the sexual phenotype is not always a reliable indicator of the X and Y chromosome

complement.

The tilapia genome has been studied extensively, with the karyotypes of most of the various tilapia species shown to be highly similar, consisting of 22 pairs with no morphologically distinct sex chromosomes. In fact, only two chromosome pairs are recognizable, the remaining 20 being similar in size and morphology (Majumdar and McAndrew, 1986). A recent study of the meiotic chromosomes of *O. niloticus* by analysis of the synaptonemal complex revealed an unpaired region in the largest chromosome pair that is found only in XY animals (Carrasco *et al.*, 1999). Additionally, it has been shown by Harvey *et al.* (in press) that sequence differences exist between the X and Y chromosomes within this region. Chromosome microdissection and subsequent amplification by degenerate oligonucleotide primed PCR (DOP-PCR) was used to produce *in situ* hybridization probes specific to *O. niloticus* chromosomes. Analysis of the comparative hybridization of X and Y chromosome derived probes to metaphases from different genotypes revealed sequence differences within the putative sex determining region.

At the molecular level, the genome size of several tilapia species has been measured at around 1 pg (1000 Mb), about one third of that of many mammalian genomes. Genome size varies by up to 44% among tilapia species, from 0.84 to 1.21 pg (Majumdar and McAndrew, 1986), probably because of the evolution of repetitive element families (Kocher *et al.*, 1998). The overall genome size is relatively small, only about twice as large as the smallest teleost genome (Hinegardner and Rosen, 1972).

The use of molecular markers for the identification of sex relies on the presence of GSD. DNA level markers have been found to be efficient in identifying sex in

vertebrates (Griffiths, 1991; Griffiths and Tiwari, 1993). As discussed in Chapter 1, a number of molecular techniques to generate sex-specific or sex-linked markers have been investigated in both plants and animals (Devlin *et al.*, 1991, 1994, 1998; Reamon-Büttner *et al.*, 1998; Griffiths and Orr, 1999; Griffiths *et al.*, 2000; Reamon-Büttner and Jung, 2000; Kovács *et al*., 2001).

One of these techniques is amplified fragment length polymorphisms (AFLPs), a polymerase chain reaction (PCR)-based method for the rapid screening of genetic diversity (Vos *et al.* 1995; Müeller and Wolfenbarger, 1999). As discussed in Chapter 1, AFLP methods rapidly generate hundreds of highly replicable markers from the DNA of any organism; thus allowing high-resolution genotyping. The time and cost efficiency, replicability and resolution of AFLPs are superior or equal to those of other markers [allozymes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellites], except that AFLP methods primarily generate dominant rather than co-dominant markers.

3.1.2. Amplified fragment length polymorphisms (AFLPs)

Amplified fragment length polymorphisms (AFLPs) are a means of fingerprinting DNAs of any origin and complexity without any prior knowledge of sequence. The AFLP technique can be applied to any DNA sample, giving it the potential to become a universal DNA fingerprinting system. Since its description in 1995 (Vos *et al.*, 1995), the AFLP technique has been applied in various ways, such as monitoring the inheritance of agronomic traits in plant and animal breeding, the diagnosis of genetically inherited diseases, pedigree and parentage analysis, forensic typing, screening of DNA

markers linked to genetic traits, microbial typing and high density genetic mapping. AFLP has also been used to generate RNA fingerprints using cDNA as template. Although the AFLP technique primarily generates dominant rather than co-dominant markers, it has several advantages over other DNA fingerprinting techniques. The most important of these is the capacity to inspect an entire genome for polymorphisms by rapidly generating hundreds of highly replicable markers (Vos *et al.*, 1995; reviewed by Blears *et al.*, 1998; Mueller and Wolfenbarger, 1999).

3.1.2.1. Principle of AFLP technique

The main principle of the AFLP technique is the selective amplification of restricted DNA fragments from a digest of total genomic DNA using PCR. The technique was first described by Vos *et al.* (1995) and subsequently reviewed by Blears *et al.* (1998) and Mueller and Wolfenbarger (1999).

The AFLP technique involves four steps: digestion of total genomic DNA, ligation of adapters, PCR amplification of selected fragments and gel analysis. First, total genomic DNA is digested with two restriction enzymes (usually a rare and frequent cutter). Double stranded oligonucleotide adapters are ligated to the restricted DNA fragments. The selective PCR amplification can then be performed in one of two ways, direct PCR amplification of restricted ligated products using primers complimentary to the adapter and restriction site sequence with additional selective nucleotides at their 3′- end, or a two step PCR amplification. Firstly, PCR amplification (pre-selective amplification) with one additional selective nucleotide at the 3′- end of the selective primers and secondly, two more nucleotides are added to the 3′- ends of the selective primers and the

product of the first PCR amplification is used as template for the final selective amplification. The use of selective primers reduces the complexity of the mixture, with only those fragments with complementary nucleotides extending beyond the restriction site being amplified by the selective primers under stringent annealing conditions. Polymorphisms are revealed by electrophoresis of the fragments on a denaturing polyacrylamide gel.

3.1.2.2. Sensitivity of the AFLP technique

High purity genomic DNA is required for AFLP studies to ensure complete digestion by the restriction endonucleases. Incomplete restriction of DNA will generate partial fragments, predominantly of high molecular weight that might produce an altered banding pattern and which in turn can be misinterpreted as false polymorphisms. However, the AFLP procedure is not sensitive to the template DNA concentration. The procedure is optimized in such a way that the amplification reaction stops when the labelled primer is consumed (Vos *et al.*, 1995). This ensures approximately equal intensity bands despite variations in template concentration (Blears *et al.*, 1998). The nucleotide sequences flanking the restriction site will no longer be random for a small pool of restriction fragments and variations in the banding patterns may be observed at very high template dilution (Blears *et al.*, 1998).

3.1.2.3. Advantages of AFLP

Usually small quantities of genomic DNA are required for the AFLP procedure (0.05μg for small genomes ranging from 50-500 megabases and 0.5 μg for genomes of

500-5000 megabases). The AFLP technique commonly generates 100-200 products that can be distinguished in a gel lane after a single amplification, thus allowing a much greater number of loci to be analysed than any other currently available PCR based fingerprinting technique. AFLP analysis is also superior in terms of its reproducibility. Additionally, a virtually unlimited number of markers can be generated by simply varying the restriction enzymes, and the nature and number of selective nucleotides (Blears *et al.*, 1998).

AFLP analysis also has considerable advantages over other molecular-based techniques including RFLP (restriction fragment length polymorphism) and RAPD. In the AFLP technique, the addition of selective nucleotides can generate polymorphisms beyond the restriction sites, while in the RFLP, only the restriction site is scanned for the differences in sequences. AFLP also has the capacity to detect more point mutations than RFLP. The frequency of detecting insertions and deletions are also the same in AFLP (Becker *et al.*, 1995). More importantly, no prior knowledge of sequence is required for AFLP analysis. Additionally, polymorphic AFLP bands can be cloned and sequences can be obtained. Cloned AFLP fragments can be converted into PCR-based markers such as sequence–tagged sites (STS) or cleaved amplified polymorphic sequence (CAPS) markers (de Jong *et al.*, 1997; Qu *et al.*, 1998).

3.1.2.4. AFLPs and aquaculture/fisheries research

AFLP analysis has gained popularity among fisheries researchers because of its advantages over other molecular systems. In recent years, a number of studies have been conducted in several species of fish mainly for the genetic analysis of populations, the

construction of high density linkage maps, analysis of quantitative trait loci and in marker assisted breeding programmes.

AFLP analysis has been used to construct genetic linkage maps in several fish species, including zebrafish, *Danio rerio* (Johnson *et al.*, 1996), Nile tilapia, *Oreochromis niloticus* (Kocher *et al.*, 1998; Agresti *et al.*, 2000), rainbow trout, *Oncorhynchus mykiss* (Young *et al.*, 1998) and channel catfish, *Ictalurus punctatus* (Liu *et al.*, 1999). This technique has also been used to study TGF beta (Transforming growth factor beta) gene expression in zebrafish (Rubinstein *et al.,* 2000) and population genetics in ayu, *Plecoglossus altivelis* (Seki *et al.*, 1999), channel catfish *Ictalurus punctatus*, blue catfish *I. furcatus* (Liu *et al.*, 1998) and Malayasian river catfish, *Mystus nemurus* (Chong *et al.*, 2000). It has also been used to verify gynogenesis in the seabass, *Dicentrarchus labrax* (Felip *et al.*, 2000), and to study hybridization and introgression between anadromous rainbow trout (*Oncorhynchus mykiss*) and coastal cutthroat trout, *O. clarki clarki* (Young *et al.*, 2001).

3.1.2.5. Sex identification using AFLPs

The prerequisite to generate sex-linked markers by any molecular technique is males and females of known genotype. Polymorphisms generated by AFLPs have been successfully used to identify sex and sex linkage in a number of plants and animal species. AFLP markers have been used successfully to identify sex in asparagus (*Asparagus officinalis*) (Reamon-Büttner, *et al.* 1998). Sex-specific AFLP markers have also been isolated and successfully used to identify sex in two species of birds, the ostrich (*Struthio camelus*) and the shag (*Phalacrocorax aristotelis*) (Griffiths and Orr,

1999), in one species of fish, three-spine stickleback, (*Gasterosteus aculeatus*) (Griffiths *et al.*, 2000), in the silk worm (Tan *et al.*, 2001) and in the Asian rice gall midge (Behura *et al.*, 2000). In several species, sex-linked AFLP markers have also been converted into single locus markers to be used in male/female PCR assays or in Southern analysis (Griffiths and Orr, 1999; Griffiths *et al.*, 2000; Reamon-Büttner and Jung, 2000).

The present Chapter describes a search for sex-specific or sex-linked DNA markers in *O. niloticus* using genetically manipulated homozygous XX females and YY males and the AFLP technique.

3.2 Materials and methods

3.2.1. Experimental fish

XX and YY homozygous *Oreochromis niloticus* and the corresponding control fish used in this experiment were produced from XY *O. niloticus* neofemales by mitotic gynogenesis. Mitotic gynogenesis was performed by fertilizing the eggs with UV irradiated sperm followed by the suppression of first mitotic division using heat shock (Sarder *et al*., 1999).

The XX and YY homozygous progeny used in this experiment came from two gynogenetic families (Dam 1 and Dam 4) as described in Chapter 2. The generalized family structure for each family is presented in Fig. 3.1.

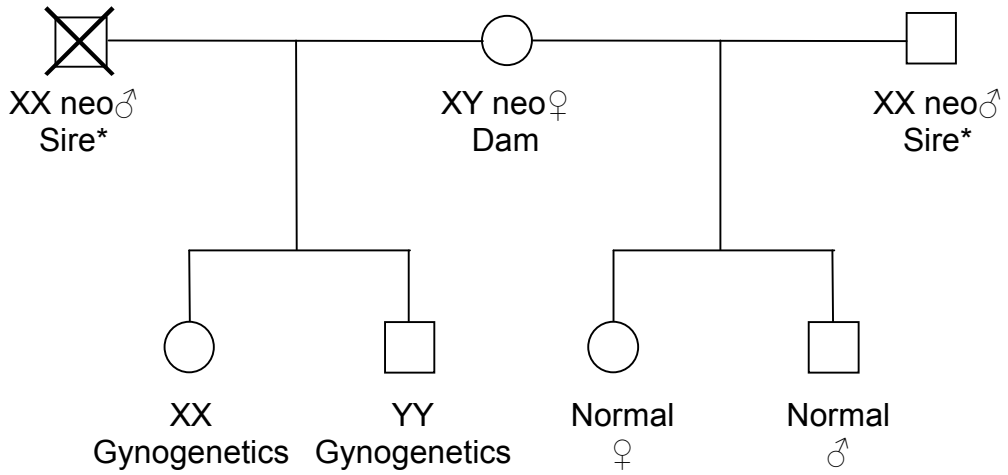


Fig. 3.1. Family structure of experimental fish used in the present experiment. **X**: represents the non-genetic contribution from sire; * same sire was used with both dams.

The gynogenetic and homozygotic status were verified by multilocus DNA fingerprinting using Jeffreys' 33.15 (Jeffreys *et al.*, 1985a) human-derived probe (described in Chapter 2) and several microsatellite loci (described in Chapter 5). Homozygous XX and YY fish were progeny tested before screening for sex-linked

AFLP markers. General procedures for mitotic gynogenesis, fish management and maintenance and progeny testing were as described in Chapter 2.

3.2.2 DNA Extraction

DNA was extracted from blood collected from the caudal vein as described in Chapter 2.

3.2.3 AFLP procedure

The AFLP procedure described in this section is as described by Vos *et al.* (1995) with minor modifications. This technique involved four different steps (Fig. 3.2).

3.2.3.1. Restriction digestion of genomic DNA and adaptor ligation

The digestion of genomic DNA and the ligation of restricted fragment specific adaptors were done in a one step reaction in the same tube. Because of a base change incorporated in the adaptor sequence, the ligation of adaptors does not restore the original restriction sites. This change in the recognition sites prevents restriction from taking place after ligation has occurred.

Restriction and ligation reactions can be performed in the same tube because of this change in the recognition sites. Adaptor to adaptor ligation is prevented by using non-phosphorylated adaptors. Because of those two features adaptors are ligated to almost all restriction fragments.

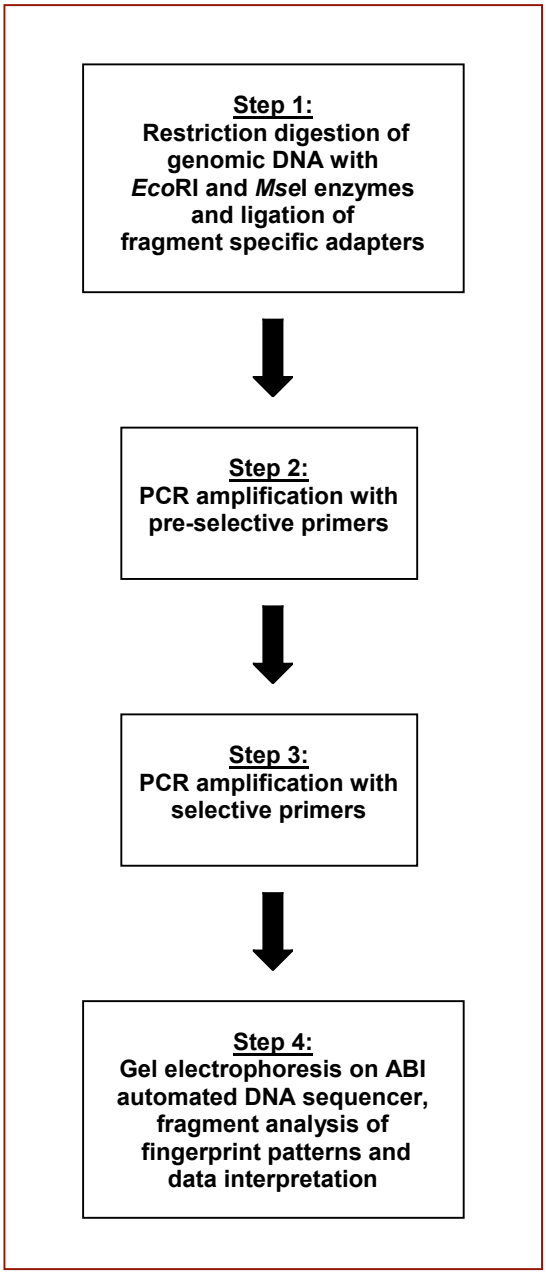


Fig.3.2. Schematic diagram of AFLP procedures followed in this experiment.

AFLP adaptors consist of a core sequence and an enzyme-specific sequence (Vos *et al.* (1995; Table 3.1). Adaptor oligos were synthesized at 0.01 μmol scale by MWG-

Biotech (UK Ltd.). First, dried oligos were dissolved in 1X STE (50 mM NaCl; 10 mM Tris pH 8.0; 1 mM EDTA) at a concentration of 650 μM.

Table 3.1. Single strand AFLP adaptor sequences.

Adapter name	Sequence- 5' - 3'
*Eco*RI-A1	5' – CTCGTAGACTGCGTACC
*Eco*RI-A2	CTGACGCATGGTTAA – 5'
*Mse*I-A1	5' – GACGATGAGTCCTGAG
*Mse*I-A2	TACTCAGGACTCAT – 5'

Equimolar amounts of each of the *Eco*RI, and of each of the *Mse*I adaptor oligos were then mixed into different 0.5 ml sterile tubes for annealing. The contents of the tubes were mixed gently by flicking the bottom of the tubes and then heated at 95°C in a heat block for 3 mins. After 3 mins, the entire block with the tubes was removed from the heat block and kept on the bench to cool slowly to room temperature. The 325 μM concentrated stocks were then diluted to working concentration with $TE_{0.1}$ (10 mM Tris, pH 8.0; 0.1 mM EDTA), and both the diluted and concentrated stocks were aliquoted into several small sterile tubes and frozen at –20°C to avoid frequent freeze-thaw cycles.

Two different restriction enzymes, a six base cutter, *Eco*RI (5′-G/AATTC-3′), and a four base cutter, *Mse*I (5′-T/TAA-3′), were used to digest the genomic DNA. These two enzymes generated three different types of restriction fragments: i) fragments with *Eco*RI sites at both ends; ii) fragments with *Mse*I sites at both ends and iii) fragments with an *Eco*RI site on one end and an *Mse*I site on the other end. More than 90% of the fragments are expected to have four base cutter sites at both ends (type ii).

To ligate the double stranded adaptors (Table 3.1) complimentary to the sticky ends of the corresponding restriction sites, an enzyme master mix was prepared by mixing 1X

T4 DNA ligase buffer with ATP, 0.5 M NaCl, 1 mg ml^{-1} BSA, 50 units of *Eco*RI, 10 units of *Mse*I and 10 Weiss units of T4 DNA ligase (New England Biolab, UK). Sterile distilled water was added to make the final volume 10 µl. Concentrated stocks of restriction enzymes were used to avoid exceeding 5% glycerol in the reactions. Keeping the enzyme master mix on ice, a ligation reaction mix was prepared. For each sample, 5.5 µl of ligation reaction mix was prepared in a sterile 1.5 ml tube by mixing 1X T4 DNA ligase buffer with ATP, 0.5 M NaCl, 1mg ml^{-1} BSA, 5 µM *Eco*RI adaptor, 50 µM *Mse*I adaptor and 1 µl of enzyme master mix. The ligation mixtures were mixed thoroughly, centrifuged briefly and were kept on ice until use. 500 ng of genomic DNA was added to each ligation reaction mix to give a total volume of 11 µl. The restriction ligation reaction was performed by incubating the tubes overnight at room temperature or for two hrs at 37°C in a thermal cycler. After incubation, a 20 fold dilution of the restriction ligation mix was prepared with $TE_{0.1}$, mixed thoroughly and stored at 4 to 6°C for immediate use or at –20°C for long term storage.

3.2.3.2. PCR Amplification with pre-selective AFLP primers

AFLP primers for pre-selective and selective amplification contain three types of DNA sequence: the 5′ region complementary to the adaptor (core), the restriction site sequence (enzyme) and the 3′ selective nucleotide(s) (extension). Two types of pre-selective and selective AFLP primers were used (Vos *et al.*, 1995), each complementary to each restriction enzyme and adaptor with one to three additional selective nucleotides at the 3′ ends (Table 3.2 and Table 3.3).

Table 3.2. AFLP pre-selective primers.

Primer name	Sequence- 5' - 3'		
	Core	Enzyme	Extension
*Eco*RI-A	GACTGCGTACC	AATTC	A
*Mse*I-C	GATGAGTCCTGAG	TAA	C

AFLP pre-selective primers were also synthesized at 0.01 μmol scale by MWG-Biotech (UK Ltd.). Pre-selective primers were resuspended in $TE_{0.1}$ to 100 μM concentration and aliquoted into several sterile tubes as 6 μM working solution. Both stock and working primer solutions were stored at -20°C and avoided frequent freezing and thawing cycles.

AFLP pre-selective PCR amplification was carried out in a 20 μl reaction volume containing 10 ng of restricted and ligated products, 1X PCR buffer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.3 μM of each pre-selective primers and 0.05 units of Taq polymerase (Advanced Biotechnologies, UK) and re-distilled water. PCR cycling conditions were: 2 mins at 72°C, 20 cycles of 20 secs at 94°C, 30 secs at 56°C and 2 mins at 72°C, 30 mins at 60°C. Reactions were then held at 4°C. All PCRs were carried out in a programmable thermal cycler (PTC-100™, MJ Research, Inc.)

After pre-selective PCR amplification, 10 μl of pre-selective products were electrophoresed on 0.8% agarose with 1X TAE running buffer to confirm successful pre-selective amplification (Fig. 3.3). The pre-selective products were then diluted with 19 volumes of $TE_{0.1}$ and kept at 4 to 6°C for immediate use or at -20°C for long term storage.

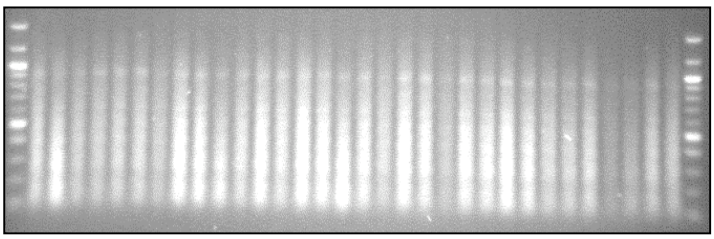


Fig. 3.3. An example of the products of a pre-selective amplification electrophoresed on a 0.8% agarose gel.

Only those genomic fragments that have an adaptor on each end amplify exponentially during this pre-selective amplification. This step effectively purifies the target away from sequences that amplify only linearly (those with one modified end).

3.2.3.3. AFLP selective PCR amplification

Eight different selective primers were designed for each enzyme. The sequences of the selective primers were the same as the pre-selective primers except two additional nucleotides were added at the 3′ ends. Like the AFLP adaptors and the pre-selective primers, selective primers were also synthesized at 0.01 μmol scale by MWG-Biotech (UK Ltd.). The *Eco*RI primers were labeled at their 5′ ends with one of three different ABI fluorescent dyes (FAM, NED and JOE) for fragment analysis using an ABI PRISM™ 377 DNA sequencer (PE Applied Biosystems). The selective primers were resuspended in $TE_{0.1}$ to produce 100 μM stock solutions, with working solutions of 5 μM and 1 μM made for the *Mse*I and *Eco*RI primers respectively. ABI dye labeled *Eco*RI primers were kept in dark boxes and shielded from excessive light to prevent photoactive degradation.

Table 3.3. AFLP selective amplification primers with 3' selective nucleotides; NL-not labelled.

Primer name	3' Pre-selective extension	3' Selective extension	ABI dye labelling	Primer name	3' Pre-selective extension	3' Selective extension	ABI dye labelling
*Eco*RI-(1)	A	CT	FAM	*Mse*I-(A)	C	AA	NL
*Eco*RI-(2)	A	CA	FAM	*Mse*I-(B)	C	AC	NL
*Eco*RI-(3)	A	AC	NED	*Mse*I-(C)	C	AG	NL
*Eco*RI-(4)	A	CC	NED	*Mse*I-(D)	C	AT	NL
*Eco*RI-(5)	A	GC	NED	*Mse*I-(E)	C	TA	NL
*Eco*RI-(6)	A	AG	JOE	*Mse*I-(F)	C	TC	NL
*Eco*RI-(7)	A	GG	JOE	*Mse*I-(G)	C	TG	NL
*Eco*RI-(8)	A	CG	JOE	*Mse*I-(H)	C	TT	NL

For AFLP analysis, 64 selective primer combinations (eight *Mse*I and *Eco*RI primers) were used. An abbreviated name was given to each primer combination for later description in this thesis. A list of these is presented in Table 3.4.

Table 3.4. Nomenclature of AFLP selective primer combinations (with selective nucleotide extension only). Each *Mse*I primer was given a letter (A-H) and each *Eco*RI was given a number (1-8). Thus, for example C3 primer combination represents AFLP selective amplification using primer combination *Mse*I-CAG/*Eco*RI-AAC.

Selective primers	*E*-ACT (1)	*E*-ACA (2)	*E*-AAC (3)	*E*-ACC (4)	*E*-AGC (5)	*E*-AAG (6)	*E*-AGG (7)	*E*-ACG (8)
M-CAA (A)	A1	A2	A3	A4	A5	A6	A7	A8
M-CAC (B)	B1	B2	B3	B4	B5	B6	B7	B8
M-CAG (C)	C1	C2	C3	C4	C5	C6	C7	C8
M-CAT (D)	D1	D2	D3	D4	D5	D6	D7	D8
M-CTA (E)	E1	E2	E3	E4	E5	E6	E7	E8
M-CTC (F)	F1	F2	F3	F4	F5	F6	F7	F8
M-CTG (G)	G1	G2	G3	G4	G5	G6	G7	G8
M-CTT (H)	H1	H2	H3	H4	H5	H6	H7	H8

AFLP selective PCR reactions were carried out in a 20 µl reaction volume containing 1X PCR buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.25 µM of selective *Eco*RI primer, 0.05 µM of selective *Mse*I primer, 0.1 unit of Taq DNA polymerase (Advanced Biotechnologies, UK) and 3 µl of diluted pre-selective products (0.5 ng) and made up to 20 µl by adding re-distilled water. The PCR conditions were: an initial denaturation step of 2 mins at 94°C, 10 cycles of 20 secs at 94°C, 30 secs at 66°C and 2 mins at 72°C with

a 1°C decrease in annealing temperature at each step; 20 cycles of 20 secs at 94°C, 30 secs at 56°C, 2 mins at 72°C; 30 mins at 60°C and a hold at 4°C. Selectively amplified PCR products were stored at 2-6°C. After selective amplification, 5 μl of product were electrophoresed on a 1.5% agarose gel with 0.5X TAE running buffer to check the successful selective amplification.

Selective amplification with *Eco*RI and *Mse*I selective primers preferentially amplifies labelled *Eco*RI-*Mse*I ended fragments, as *Eco*RI-*Eco*RI fragments require high annealing temperature. *Mse*I-*Mse*I fragments will be amplified but will not be visualised as the *Mse*I primers are not labelled.

3.2.3.4. Electrophoresis on ABI Prism™ 377 DNA sequencer and data analysis

Electrophoresis of AFLP selectively amplified fragments was performed on an ABI Prism™ 377 DNA sequencer according to the manufacturer's instructions (GeneScan™ Analysis software Users Manual, The PE Corporation). A 0.2 mm thick 5% denaturing LongRanger® (Biowhittaker Molecular Applications, ME, USA) gel was prepared with 1X TBE running buffer. First, the glass plates (36 cm well to read distance) were washed in warm water and rinsed with distilled water. Plates were then air dried completely before pouring the gel solution. A 50 ml gel mix was prepared by mixing 18 g urea, 26 ml double distilled water, 5 ml of 50% LongRanger® solution and 0.5 g mixed bed resin. The gel mixture was stirred for half an hour (to bring it to the room temperature) and filtered through a 0.20 μm filter. 5 ml of 10X TBE buffer was also added during filtration. The filtered gel mixture was degassed by exposure to vacuum for 4 mins. After de-gasing, 250 μl of freshly prepared 10% ammonium persulfate (APS) and 25 μl

of N,N,N′,N′-Tetramethylethylenediamine (TEMED, Sigma, UK) was added to the gel solution, which was mixed and poured carefully using a 50 ml syringe. The gel was then left for 2-2.5 hrs to allow polymerization.

Selectively amplified AFLP products were mixed with a loading buffer mix containing deionized formamide, 50 mg ml^{-1} blue dextran with 25 mM EDTA (pH 8.0) and GeneScan™-500 [ROX] size standard (PE Applied Biosystems) in a ratio of 5:1:2. For each sample 0.4 µl of selectively amplified PCR product was mixed with 1.2 µL of loading buffer mix, mixed well and centrifuged briefly. Each sample was heated at 95°C for 3 minutes, quickly chilled on ice and then loaded onto the gel. Samples were electrophoresed in ABI Prism™ 377 sequencer with filter set F at 2500 V for 4 hrs.

After electrophoresis, the gel image was tracked, the appropriate matrix standard was installed and the lanes were extracted according to the instruction manual. After extraction, analysis parameters were set and data were analysed using GeneScan™ (V. 2.1) and Genotyper™ (V. 2.1) analysis software (PE Corporation).

3.2.4. Experimental design for the screening of Sex-Linked AFLP Markers (SLAMs)

64 AFLP primer combinations (Table 3.4) were used for this screening, with screening started using grand pools of XX and YY homozygous individuals. Subsequently, primer pairs showing good amplification were screened against individual family pools and individual families.

The XX grand pool contained eight, progeny tested XX mitotic gynogenetic individuals from two families (Dam 1 and Dam 4) while the YY grand pool contained

eight, progeny tested YY mitotic gynogenetic individuals from two families (Dam 1 and Dam 4) (Fig. 3.4). In pool level screening, each step was repeated at least once.

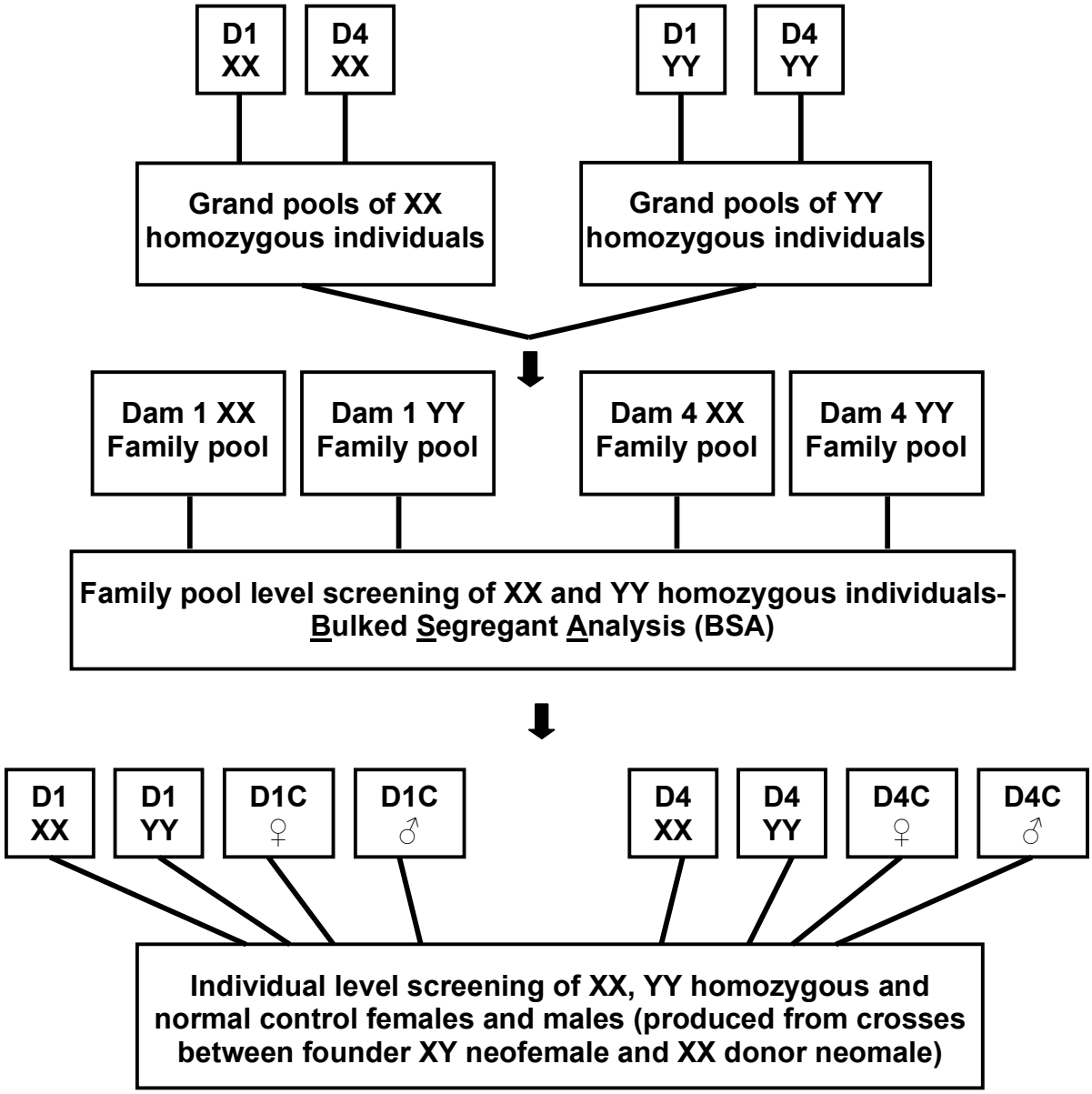


Fig. 3.4. Schematic diagram of AFLP screening for sex-linked markers in *Oreochromis niloticus*; (D1= Dam 1; D4= Dam 4; C: normal control).

Later, BSA (Bulked Segregant Analysis; Michelmore *et al.*, 1991) was performed separately in two family pools to screen Sex-Linked AFLP Markers (SLAMs). The Dam

1 pool comprised 26 XX and YY homozygous individuals (13 XX and 13 YY) and the Dam 4 pool comprised 32 XX and YY homozygous individuals (16 XX and 16 YY individuals) (Fig. 3.1).

After family pool level screening, individual family sets were designed and screened accordingly. Family set Dam 1 contained the XY founder neofemale (mother), XX donor neomale (father), 13 XX female and 13 YY male gynogenetics and 15 normal control females and 15 normal control males (produced by crossing the XY founder neofemale and XX donor neomale). Family set Dam 4 contained the XY founder neofemale (mother), XX donor neomale (father), 16 XX female and 16 YY male gynogenetics and 10 normal control females and 14 normal control males (produced by crossing the XY founder neofemale and XX donor neomale).

The following criteria were used to select primer combinations suitable for further analysis:

1. Primer pair did not show identical pattern of bands between XX and YY - primer pair selected for further evaluation
2. Too many or too few bands per primer combination - primer pair discarded
3. Consistent high back ground - primer pair discarded

3.2.5. Identification of true sex-linked markers

Any AFLP primers that produced bands in only one sex initially during family pool level screening, were re-screened with more individuals to decrease the probability of false identification of a true sex-linked marker by following this formula: $p = q^m . (1 - q)^f$ where, q = m/(m+f); m and f are males and females (e.g. 5♂♂ + 5♀♀; p = 0.00098 which is < 0.001) (Lessells and Mateman, 1998; Griffiths *et al.*, 2000).

3.2.6. Statistical analysis

Chi-square analysis with Fisher's exact test was performed to test the significance of sex-linked AFLP markers using SPSS (10.0 for Windows) statistical software.

3.3. Results

3.3.1. AFLP screening in grand pools

The search for sex-linked markers in *O. niloticus* was started using 64 AFLP primer combinations (Table 3.4) on grand pools of XX and YY homozygous *O. niloticus* from two families (Dam 1 and Dam 4) of mitotic gynogenetics (Fig. 3.5). The grand pools contained eight, progeny tested XX and eight, progeny tested YY mitotic gynogenetic individuals respectively. A schematic diagram of this screening is presented in Fig. 3.5 and an example of an AFLP gel image is presented in Fig. 3.6. Results of the grand pool screening are presented in Table 3.5.

AFLP analysis using grand pools of XX and YY individuals resulted in a total of 3,138 AFLP bands. The number of bands produced by different primer combinations in grand pool screening ranged from 10-91 bands per combination, with an average of 49 bands produced by each primer combination. Grand pool screening was carried out twice and the bands on first screening were reproducible on second screening. After grand pool screening 33 primer combinations were discarded on the basis of the criteria described in Section 3.2.4. Although bands produced by the other 31 primer combinations did not show absolute sex-linkage, some of the bands generated by these 31 primer pairs showed indications of putative sex-linkage (such as, differences observed in intensity or height of peaks of AFLP bands between XX and YY pools using GeneScan™ fragment analysis software). Therefore these primer combinations were selected for further analysis in family pool level screening.

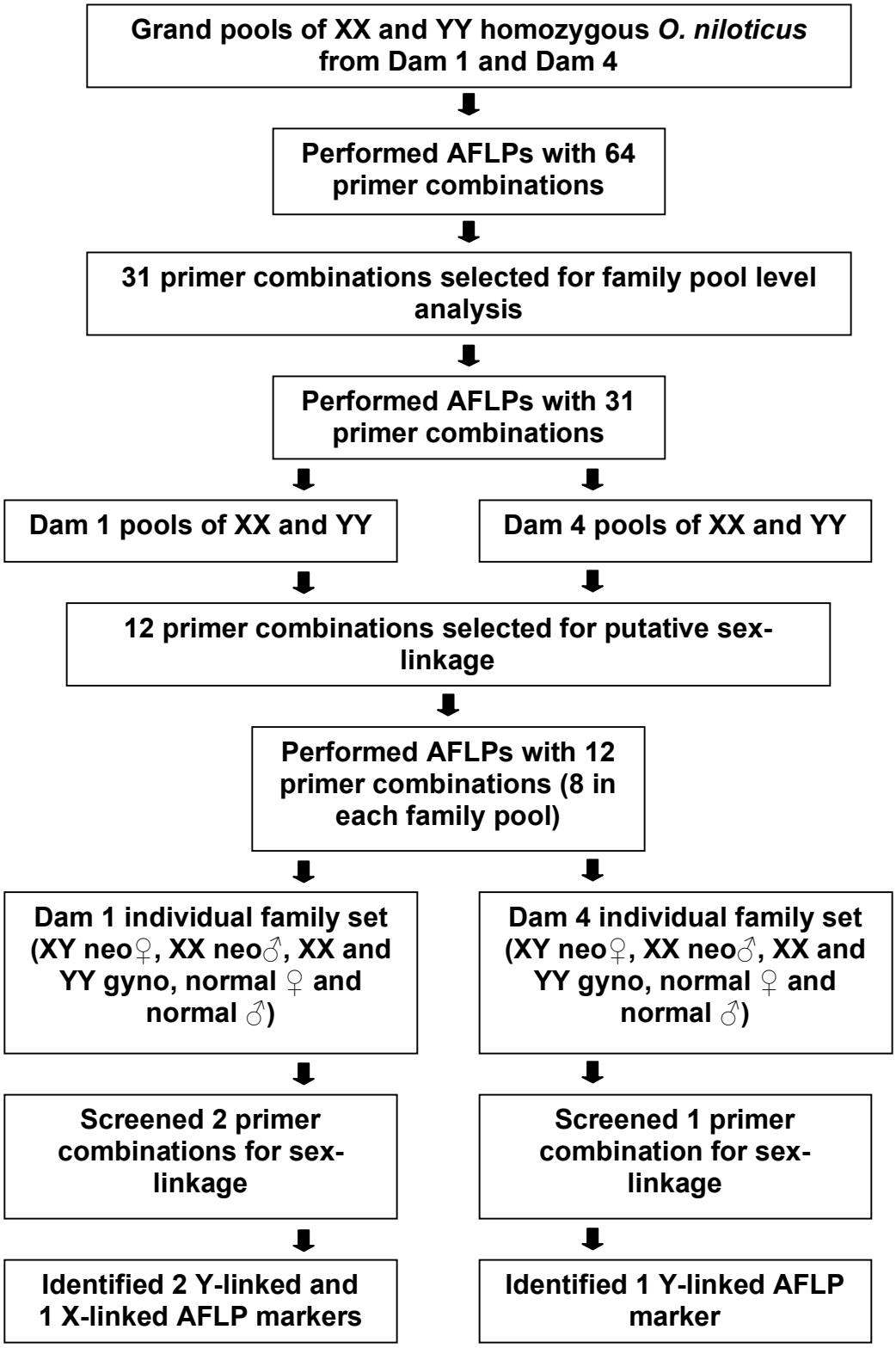


Fig.3.5. Schematic diagram of the screening used to identify sex-linked AFLP markers (SLAMs).

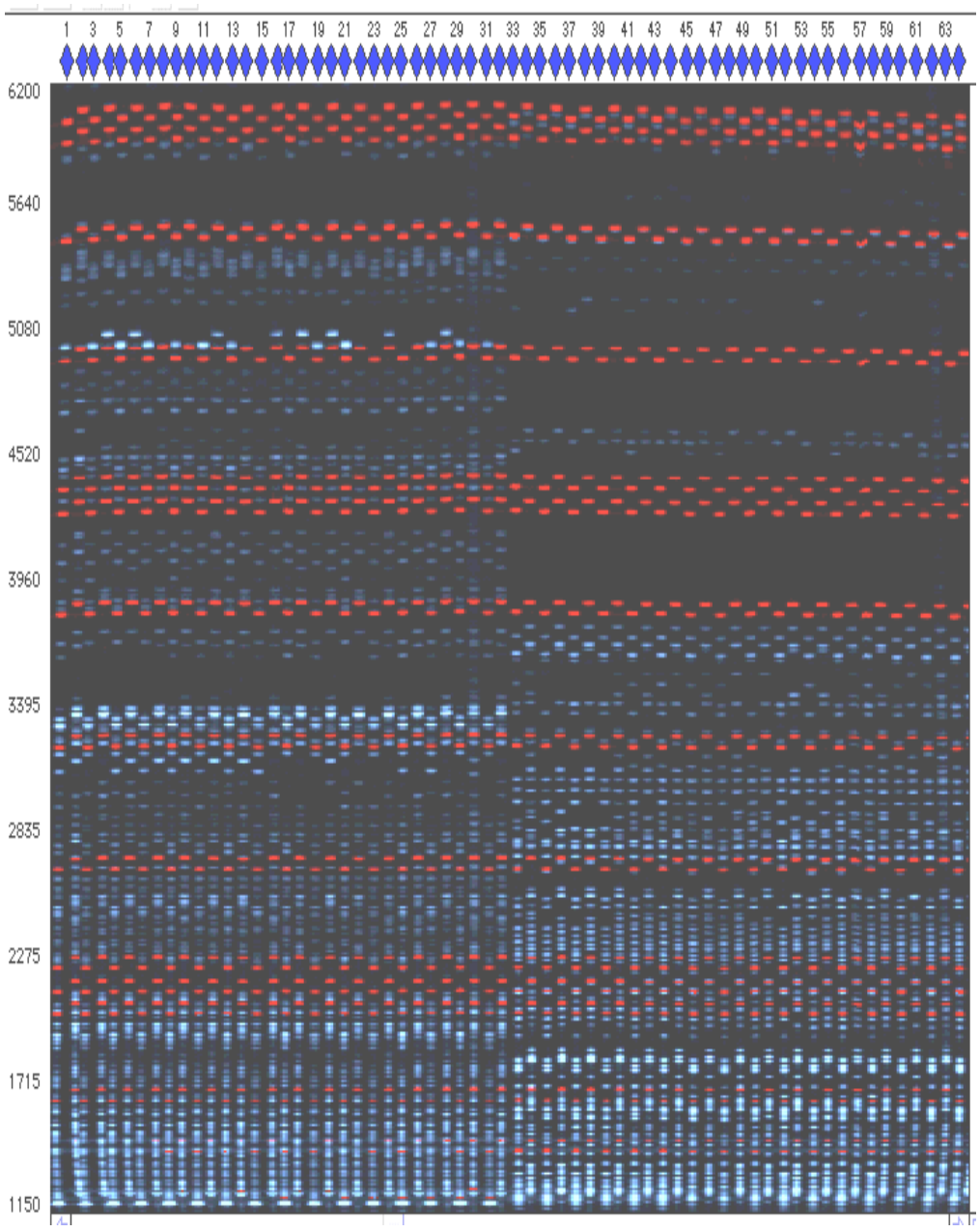


Fig. 3.6. An example of an AFLP gel generated by primer combination A2 (*Mse*I-CAA + *EcoR*I-ACA) in Dam 1 and Dam 4 individual family sets. Lane 1-32: Dam 1 and 33-64: Dam 4.

Table 3.5. AFLP screening with 64 primer combinations in grand pools of XX and YY homozygous *Oreochromis niloticus*.

AFLP Primer Combination	No. of Bands	AFLP Primer Combination	No. of Bands	AFLP Primer Combination	No. of Bands	AFLP Primer Combination	No. of Bands
A1	73	C1	59	E1	62	G1	74
A2	61	C2	68	E2	66	G2	73
A3	44	C3	40	E3	40	G3	37
A4	10	C4	19	E4	27	G4	31
A5	16	C5	21	E5	40	G5	22
A6	52	C6	84	E6	73	G6	68
A7	52	C7	71	E7	72	G7	64
A8	26	C8	25	E8	28	G8	32
B1	83	D1	56	F1	49	H1	84
B2	91	D2	70	F2	71	H2	84
B3	40	D3	53	F3	40	H3	36
B4	40	D4	49	F4	28	H4	33
B5	15	D5	10	F5	22	H5	31
B6	68	D6	50	F6	50	H6	30
B7	56	D7	65	F7	80	H7	53
B8	51	D8	18	F8	20	H8	82
		Total no. of bands screened in grand pool =					3138

3.3.2. AFLP screening in family pools

In the following step, bulked segregant analysis (Michelmore *et al.*, 1991) was performed in two (XX and YY) pools of two families (Dam 1 and Dam 4) comprising XX and YY homozygous individuals. Thirty one primer combinations selected on the basis of the results of the grand pool screening were used.

Two rounds of family pool screening were performed. In the first round screening, the family pools comprised small numbers (depending on the number of progeny tested XX and YY individuals available during the experiment) of XX and YY homozygous individuals. The Dam 1 pools comprised three XX and three YY homozygous individuals, while the Dam 4 pools comprised four XX and four YY homozygous individuals. AFLP analysis with 31 primer combinations was performed and 17 primer combinations were selected for a second round of family pool level screening on the

basis of the putative sex-linked bands produced by these primer combinations. Eight XX and eight YY homozygous individuals were added to the Dam 1 pools and eleven XX and eleven YY homozygous individuals to the Dam 4 pools to perform a second round of AFLP analysis with the 17 selected primer combinations.

The results of the two rounds of family pool level AFLP screening are presented in Table 3.6. The 31 AFLP primer combinations used in the two rounds of family pool level screening produced 1,698-1,704 markers in the Dam 1 pools and 1,749-1,757 markers in the Dam 4 pools. Comparisons of the markers between the four pools (i.e. among Dam 1 XX, Dam 1 YY, Dam 4 XX and Dam 4 YY) showed that 1,660 bands appeared to be common among the pools (Table 3.6).

From the family pool level analysis, 12 primer combinations covering two families were selected for further analysis, as they appeared to produce putatively sex-linked bands. Selections of the primer combinations for further analysis were based on the criteria 1 (described in section 3.2.4). These included those primer combinations that produced bands (both X and Y-linked) present on one pool and absent on the other within each family pools, guided by the electropherograms generated by GeneScan™ (V.2.1) and Genotyper™ (V.2.1) fragment analysis software. In this way 12 primer combinations were selected for further analysis. Among those 12 primer combinations, eight primer combinations appeared to produce putatively sex-linked bands in a single family with four combinations producing such bands in both families (Table 3.6). These 12 primer combinations produced 30 putatively sex-linked AFLP bands among the XX and YY pools in two families. Of the 30 putatively sex-linked markers, 8 were identified as X-linked and 22 as Y-linked in the two family pools (Table 3.6).

Table 3.6. AFLP screening results for the 31 primer combinations used on the two family pools of XX and YY homozygous *Oreochromis niloticus*.

AFLP primer combination	No. of bands				No. of putative sex-linked markers				No. of shared bands
	Dam 1 pools		Dam 4 pools		Dam 1 pools		Dam 4 pools		
	XX pool	YY pool	XX pool	YY pool	X-linked	Y-linked	X-linked	Y-linked	
A1	67	67	68	68	0	0	0	0	67
A2	81	82	81	81	0	1	0	0	81
A4	29	29	27	27	0	0	0	0	27
A5	13	13	13	13	0	0	0	0	13
A7	76	78	76	78	0	2	0	2	76
B1	85	83	85	85	0	0	2	0	85
B2	86	84	86	85	0	0	3	1	86
B3	39	39	38	38	0	0	0	0	38
B4	50	50	50	50	0	0	0	0	50
B7	54	54	50	50	0	0	0	0	50
B8	39	39	40	40	0	0	0	0	39
C2	79	79	79	79	0	0	0	0	79
C4	28	28	29	29	0	0	0	0	28
C5	27	27	27	27	0	0	0	0	27
C6	76	75	81	85	0	1	0	3	76
D2	76	76	83	83	0	0	0	0	76
D3	39	39	38	38	0	0	0	0	38
D4	36	36	53	53	0	0	1	1	36
D7	56	57	65	65	0	1	0	0	57
E1	65	65	71	71	0	0	0	0	65
E2	61	65	58	58	1	3	0	0	58
E6	49	49	59	60	1	1	0	1	49
F2	55	55	66	66	0	0	0	0	55
F6	34	34	48	48	0	0	0	0	34
G2	80	80	80	80	0	0	0	0	80
G3	37	37	35	35	0	0	0	0	35
H1	65	65	67	68	0	0	0	1	65
H5	26	26	33	33	0	0	0	0	26
H6	61	61	53	53	0	0	0	0	53
H7	52	53	39	39	0	1	0	0	39
H8	77	79	71	72	0	2	0	1	72
	1698	1704	1749	1757	2	12	6	10	1660

3.3.3. AFLP screening in individual family sets

At this stage, individual family sets were designed for the two families to test the 12 primer combinations selected from the family pool level AFLP analysis, with individual fish being tested. Each family set contained XY founder neofemale (mother), XX donor neomale (father), five XX and five YY homozygous females and males respectively, and ten normal females and ten normal males (produced by crossing the XY founder neofemale and XX neomale and only phenotypically sexed). Normal females and males were selected randomly within sex in Dam 1 family set, while in Dam 4 normal females were the only survivors whereas normal males were selected randomly within sex. The results of the AFLP analysis of the 12 primer combinations are presented in Table 3.7 and the frequency distribution of all 30 putatively sex-linked AFLP markers are presented in Fig. 3.7.

Table 3.7. Results of the first round of the AFLP screening using 12 primer combinations in two individual family sets of *Oreochromis niloticus*. The presence or absence of putatively sex-linked AFLP markers are shown in XY founder neofemale and donor XX neomale as well as their frequency in gynogenetic XX females and YY males and in normal females and normal males progeny (produced from crossing XY neofemale and XX neomale). P: present; A: absent.

AFLP Primer Combination	Family screened	Putative sex-linked AFLP markers number	size (bp) X-linked	size (bp) Y-linked	XY founder ♀	XX donor neo ♂	Frequency of markers' presence XX-Gyno (n=5)	YY-Gyno (n=5)	Normal ♀ (n=10)	Normal ♂ (n=10)
A2	Dam 1	1		285	P	P	0	2	10	10
A7	Dam 1	2		137	P	P	0	2	10	10
				151	P	P	0	1	10	10
	Dam 4	2		155	P	A	0	5	6	1
				307	P	P	0	1	10	10
B1	Dam 4	2	182		P	P	5	0	10	10
			212		P	A	3	0	9	4
B2	Dam 4	4	185		P	A	1	0	4	3
			230		P	P	5	0	6	7
			295		P	A	1	0	4	3
				325	P	P	0	5	8	5
C6	Dam 1	1		317	P	A	0	3	7	3
	Dam 4	3		65	P	A	0	1	0	2
				256	P	A	0	2	1	5
				257*	P	A	0	2	1	6
D4	Dam 4	2		179	P	A	0	5	6	2
			354		P	P	2	0	4	9
D7	Dam 1	1		310	P	A	0	2	9	5
E2	Dam 1	4		101	P	P	0	2	10	8
				273	P	P	0	2	9	8
				486	P	P	0	2	6	7
			420*		P	A	2	0	9	2
E6	Dam 1	2		425*	P	A	0	4	1	6
			420		P	P	5	0	10	10
	Dam 4	1		227*	P	A	0	5	0	8
H1	Dam 4	1		149	P	P	0	2	7	6
H7	Dam 1	1		303	P	P	0	4	6	9
H8	Dam 1	2		190	P	P	0	2	10	10
				382*	P	A	0	4	1	6
	Dam 4	1		233	P	P	0	2	3	6
12		30	8	22						

* Significant by chi-square contingency with Fisher's exact test

Data from the first round of family set AFLP screening were first checked for the presence of putatively sex-linked AFLP markers(s) in the XY neofemale (mother) and then absence in XX donor neomale (father) to identify Y-linked markers. These data were also analysed by chi-square contingency with one-sided Fisher's exact test, and

only those putatively sex-linked markers which showed significant ($P < 0.05$) differences in segregation with sex were selected for another round of screening with family sets containing new individuals (Table 3.7). For X-linked markers, absence of bands in YY gynogenetic groups were first considered and then their segregation were also analysed in normal cross individuals.

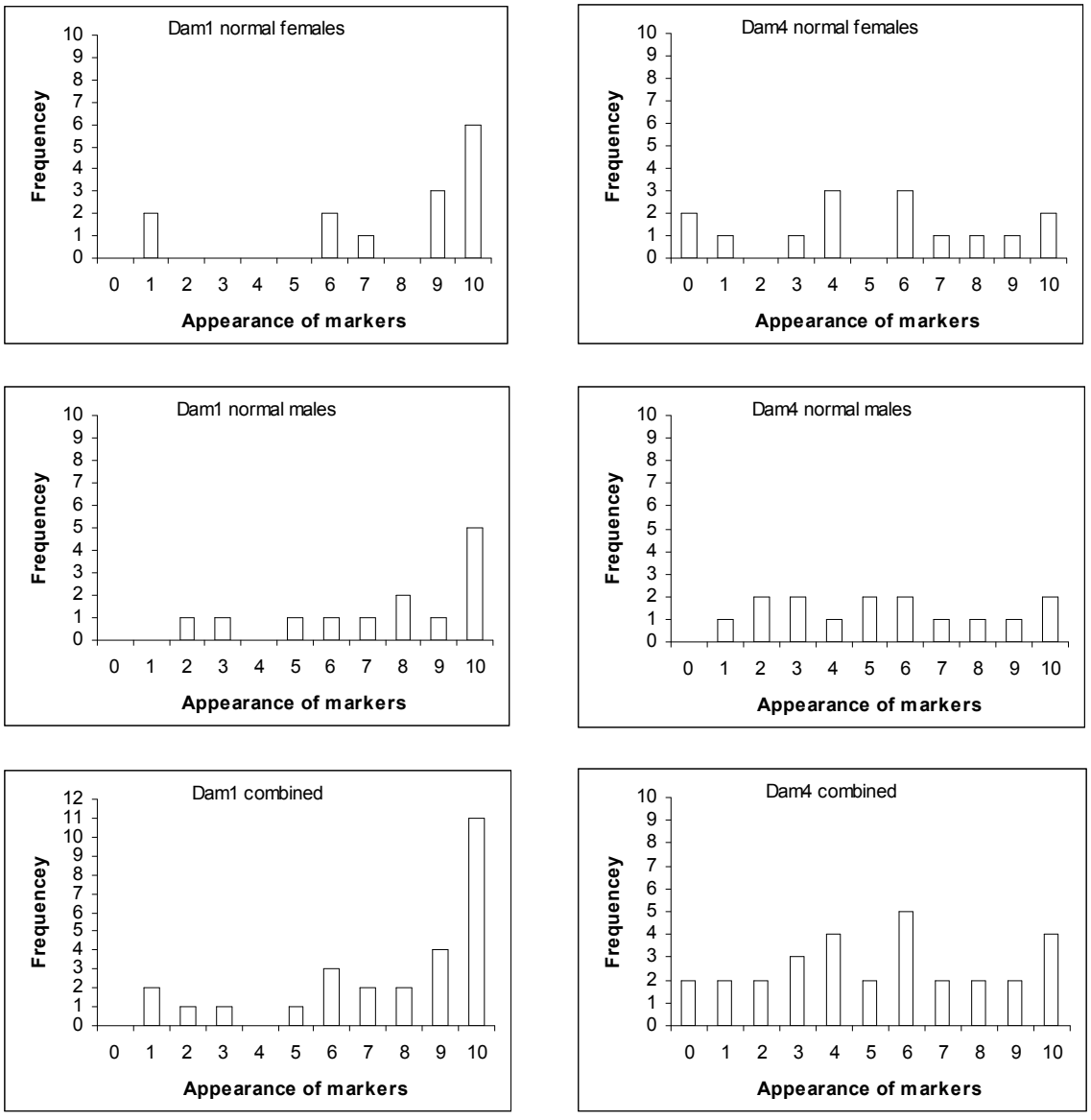


Fig. 3.7. Frequency distribution of presence of each band in normal control males and females after individual family set screening showing the zygotic conditions of 30 putative AFLP sex-linked markers.

Five putatively sex-linked bands which showed statistically significant differences in segregation with sex were identified in the first screen from four primer combinations. In the Dam 1 set, primer combination E6 (*Mse*I-CTA + *Eco*RI-AAG) and H8 (*Mse*I-CTT + *Eco*RI-ACG) each produced a Y-linked band, 425 base pairs (bp) in E6 and 382 bp in H8. In the Dam 4 set, three putatively sex-linked bands were identified, a 257 bp Y-linked band with the C6 (*Mse*I-CAG + *Eco*RI-AAG) primer pair, a 420 bp X-linked band with the E2 (*Mse*I-CTA + *Eco*RI-ACA) primer pair and a 227 bp Y-linked band with the E6 (*Mse*I-CTA + *Eco*RI-AAG) primer pair. None of the X-linked bands showed significant segregation with sex. Additionally, although, not significant, a further 420 bp putative X-linked band generated by the E6 primer combination in the Dam 1 set was included for further analysis (Table 3.7).

Among these four primer combinations, the E6 combination was common in both families (although it produced different size DNA fragments) and the same primer combination generated both male and female linked AFLP markers in Dam 1. Among the other three, each family showed sex-linked differences for two different primer combinations (Table 3.7).

In the final round of individual family set screening, more new individuals were added to both family sets and AFLP screening was performed with the four previously identified primer combinations to determine the association of the six putatively sex-linked markers in new individuals. Eight XX and eight YY homozygous and five normal control females and five normal control males were added to the Dam 1 family set, while in the Dam 4 family set, 11 XX and 11 YY homozygous individuals and four normal control males were added.

The results of this final round of AFLP screening in the individual family sets are presented in Table 3.8 and an example of the electropherogram generated by GeneScan™ (V. 2.1) is shown in Fig. 3.8.

Table 3.8. Results of the analysis of the four SLAMs by AFLP screening in two families of *Oreochromis niloticus*. Numbers of individuals are in parentheses. gyno: homozygous individuals produced by mitotic gynogenesis; normal: control individuals produced by normal cross between Dam 1 or Dam 4 with sire. The same sire was used for crosses with both dams; +: only one allele is known; AFLP primer combinations for corresponding SLAMs are in parentheses.

Types of individuals screened	SLAMs			
	*Oni*Y425+ (E6)	*Oni*Y227+ (E6)	*Oni*Y382+ (H8)	*Oni*X420+ (E6)
XX gyno - Dam 1 (13)	0	13	0	13
YY gyno - Dam 1 (13)	11	13	11	2
♀ Normal - Dam 1 X Sire (15)	0	15	0	15
♂ Normal - Dam 1 X Sire (15)	9	15	11	15
XX gyno - Dam 4 (16)	0	0	0	16
YY gyno - Dam 4 (16)	0	16	0	16
♀ Normal - Dam 4 X Sire (10)	0	0	0	10
♂ Normal - Dam 4 X Sire (14)	0	10	0	14

With this larger sample size, the sex-linked bands generated using primer combination E6 and H8 appeared to be consistent with the first round analysis in both families. However, sex-linked bands generated using primer combinations C6 and E2 appeared to be inconsistent and statistically insignificant. Thus, four family specific sex-linked AFLP markers (SLAMs) in two families of *O. niloticus* were identified. Three markers were male-linked (Y-linked) and one was female-linked (X-linked). These four SLAMs were given specific names according to the species (*Oni*), association with sex (X or Y) and size of markers. Thus, the markers amplified by primer combination E6 were named *Oni*Y425, *Oni*X420, *Oni*Y227 and that amplified by H8 was named *Oni*Y382.

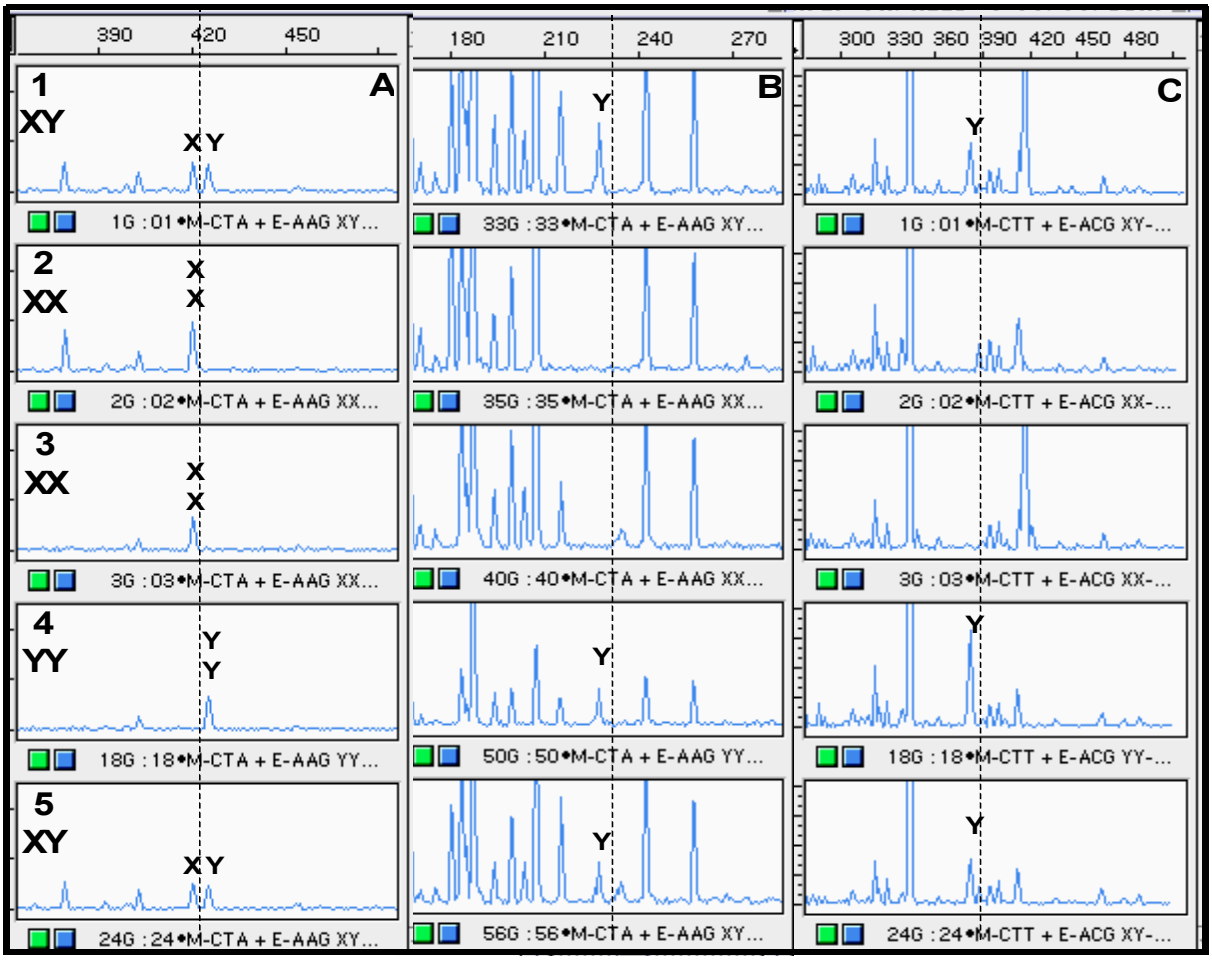


Fig. 3.8. An example of electropherogram generated by GeneScan™ (V. 2.1) software showing four SLAMs in five different individuals in *Oreochromis niloticus*; a: *Oni*X420 and *Oni*Y425 in Dam 1, b: *Oni*Y227 in Dam 4 and c: *Oni*Y382 in Dam 1.

The occurrence of family specific SLAMs was also analysed in the other family. For example, marker(s) sex-linked in Dam 1 individuals were also analysed in Dam 4 individuals. The results showed that the Dam 1-specific Y-linked markers *Oni*Y425 and *Oni*Y382 were absent in all Dam 4 individuals, while Dam 1-specific X-linked marker *Oni*X420 was present in all Dam 4 individuals. On the other hand, the Dam 4-specific Y-linked marker *Oni*Y227 was present in all Dam 1 individuals (Table 3.8).

The heterozygosity of the four SLAMs can also be assumed by analyzing their segregation patterns among the normal control individuals. The segregation patterns of *Oni*Y425 and *Oni*Y382 among the normal control individuals suggested their heterozygous status in Dam 1 and homozygous status in Dam 4. *Oni*Y227 appeared to be heterozygous in Dam 4 and homozygous in Dam 1. In contrast, *Oni*X420 appeared to be heterozygous in Dam 1 while homozygous in Dam 4 (Table 3.7 and 3.8).

Table 3.9. Summary results of SLAMs. Numbers of individuals are in parentheses. gyno: homozygous individuals produced by mitotic gynogenesis; normal: individuals produced by normal cross between Dam 1 or Dam 4 with XX sire (same sire was used for crosses with both dams), +: one allele is known.

Types of individuals	SLAMs			
	*Oni*Y425+	*Oni*Y227+	*Oni*Y382+	*Oni*X420+
♀ - XX gyno (29)	0	13	0	29
♀ - normal (25)	0	15	0	25
♀ - total (54)	0	28	0	54
♂ - YY gyno (29)	11	29	11	18
♂ - normal (29)	9	25	11	29
♂ - total (58)	20	54	22	47

The summarized results and percent frequency of occurrence of all four SLAMs are also presented in Table 3.9 and Fig. 3.9.

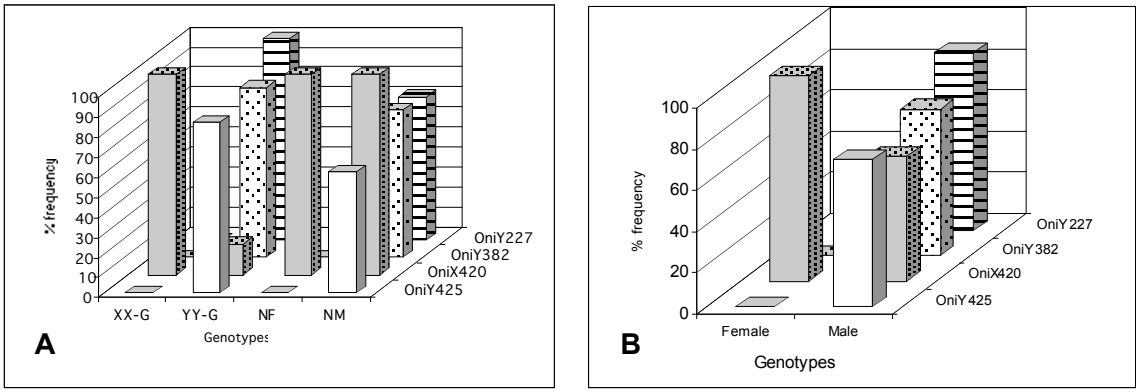


Fig. 3.9. Frequency distribution of the four SLAMs. A: in individual families; B: combined. G: gynogenetic individuals; NF: normal cross female; NF: normal cross male.

The probability (p) of false identification of true sex-linked markers in the two families of mitotic gynogenetic *O. niloticus* during AFLP screening are presented in Table 3.10.

Table 3.10. Probability of false identification of true sex-linked AFLP markers in two gynogenetic families of *Oreochromis niloticus*; number of females and males are in parentheses.

Families	Mitotic gynogenetics (XX, YY)	Control (XX, XY)
Dam 1	$p = 1.49 \times 10^{-8}$; n = (13, 13)	$p = 9.31 \times 10^{-10}$; n = (15, 15)
Dam 4	$p = 2.3 \times 10^{-10}$; n = (16, 16)	$p = 8.30 \times 10^{-8}$; n = (10, 14)

According to the data presented in Table 3.10, the present experiment used sufficient numbers of individuals to statistically identify true sex-linked markers and therefore it can be stated that the family-specific SLAMs of *O. niloticus* isolated in this experiment were not biased due to an insufficient number of individuals.

3.4. Discussion

Since its initial description, the AFLP fingerprinting technique has been applied in a wide variety of organisms to generate markers tightly linked to a particular locus. In the present experiment, AFLP analysis was used to identify sex-linked markers in the Nile tilapia, *Oreochromis niloticus*.

As is the case in many teleost fish, sex determination in *O. niloticus* is influenced by both autosomal and environmental factors in addition to the primary genetic signal. At the chromosomal level, the analysis of metaphase chromosomes does not show the presence of heteromorphic sex chromosomes (Majumdar and McAndrew, 1986), such as found in humans and other mammals. Only recently, synaptonemal analysis of meiotic chromosomes has revealed a region of un-pairing in the largest bivalent, which is only seen in XY individuals (Foresti *et al*., 1993; Carrasco *et al*., 1999). This suggests that sex-specific sequence differences do exist in this species.

The present experiment was designed to apply suitable molecular techniques to reveal these differences. However, as the available evidence suggested that there are only limited genetic differences between the X and Y chromosomes, genetically manipulated fish were used to maximize the chances of revealing any differences. For this purpose, several families of homozygous fish were produced which only differed from each other in their sex chromosome complement, with the rest of the genome randomly distributed and homozygous in all the individuals generated from a particular female (described in details in Chapter 2).

AFLP screening was performed initially using grand pools of XX and YY individuals. This screening revealed no sex-linked markers, but 33 primer combinations

were discarded on the basis of the selection criteria mention in section 3.2.4. However, subsequent family pools and individual-level screening identified one X-linked and three Y-linked markers within source family. Therefore, it is possible that if AFLP screening had begun with family pool-level screening instead of grand pool-level screening, more sex-linked markers would have been found. The appearance of these sex-linked markers in the family pool and individual level study indicates that sex-specific differences do exist in this species. The possibility of recombination between these markers and the sex locus can also be a reason for not finding any sex-linked markers during grand pool-level screening. It is also noticeable that the sex-linked markers *Oni*X420, *Oni*Y425 and *Oni*Y227 were generated from the same primer combinations in two different families, but they were not sex-linked in the other family (such as Dam 1 specific sex-linked markers *Oni*X420 and *Oni*Y425 showed no sex-linkage in Dam 4, and the same situation was also observed in the case of Dam 4 specific *Oni*Y227 in Dam 1), which is also an indication of not finding universal sex-linked markers in this species from the present study.

However, due to the nature of the restriction endonucleases used in the AFLP screening, certain sequence types may have been underrepresented in the pool of amplified markers. As the recognition sites for both *Eco*RI and *Mse*I are AT rich, GC regions of *O. niloticus* genome may be under represented within the marker pool. As the sex determining region in higher vertebrates is often confined to heterochromatin regions of the sex chromosome, which are commonly GC rich in sequence, this effect may have limited the chances of detecting sex-linked markers in this study. Further, it is possible that differences between the sex chromosomes in either the dosage or

distribution of repetitive elements occur, as such differences would be difficult to detect using this methodology. For better distribution of AFLP markers along the sex chromosomes as well as the genome, AFLP screening with different enzyme combinations would give wide coverage of chromosomes for AFLP loci linked to sex as well as other loci of interest.

Although the present experiment failed to detect markers which are 100% sex-specific in *O. niloticus*, this appears to be the first time in this species that sex chromosome-specific DNA markers of any kind have been identified. Once isolated and characterized these markers will allow physical mapping by subsequent suitable genomic library screening and fluorescence *in situ* hybridization. These markers would also enable us to find sex determining genes in this species using suitable molecular techniques, for example genomic library screening and subsequent hybridization with positive clones and chromosome walking. Therefore, these markers could be of potential use in answering many questions relating to sex determination in this species.

The following Chapter describes the isolation and further analysis of these markers and provides further discussion.

Chapter 4

Isolation, Sequencing, Single Locus Conversion And Linkage Analysis Of Slams

4.1. Introduction

Despite the reported use of the AFLP technique in various genetic analyses, little information is available regarding the cloning of AFLP fragments and conversion to other marker types. The conversion of AFLP bands into other marker types, such as restriction fragment length polymorphisms (RFLPs) or sequence-specific PCR markers (both sequence tag sites and internal primers) is necessary for high-throughput genotype scoring. The value of such a conversion is that other types of marker analysis are less expensive and can be more easily employed in large populations. In the few cases in which AFLP marker conversion has been attempted (Meksem *et al*., 1995, 2001; Cho *et al*., 1996; Qu *et al*., 1998; Griffiths and Orr, 1999; Griffiths *et al*., 2000), only a few of the corresponding RFLP or sequence-specific PCR markers retained the specificity indicated by the original AFLP markers.

Conversion of different types of markers, such as RFLPs, RAPDs and microsatellite markers, to sequence-specific PCR markers has been reported by several authors (Bradshaw *et al*., 1994; Salentijn *et al*., 1995; Brady *et al*., 1996; Talbert *et al*., 1996; Shan *et al.*, 1999). However, the efficiency and difficulties associated with conversion of AFLPs are largely unknown. Meksem *et al*. (2001) reported that the efficiency of AFLP band conversion can be improved by using BAC (bacterial artificial chromosomes) libraries and physical maps. Designing and use of sequence tag sites primers (STS) have also been reported as a way of conversion of AFLP markers into single locus markers. The loss of the original polymorphism during generation of STS primers from AFLP markers and the loss of the locus specificity of STS have also been reported in several

experiments involving conversion of AFLP bands into locus specific markers (Shan *et al*., 1999; Wei *et al*., 1999).

The polymorphisms normally identified by AFLP analysis include SNPs (single nucleotide polymorphisms), indels (insertions and deletions) and microsatellites (Bradeen and Simon, 1998; Wei *et al*., 1999; Meksem *et al*., 2001; Nakamura *et al*., 2001). However, detailed information on the nature of AFLP markers, especially on their physical locations and distribution along chromosomes, is limited. Southern analysis using cloned AFLP fragments as probes has revealed both single copy and repetitive sequences in several experiments on plants (Meksem *et al*., 1995, Cho *et al*., 1996).

Linkage of AFLP markers with various traits has been reported in a number of plant and animal species (Bradeen and Simon, 1998; Nakamura *et al*., 2001). However, linkage analysis of AFLP markers with sex has only been reported in few species of plants (Reamon-Büttner *et al*., 1998; Terauchi and Kahl, 1998) and animals (Gadau *et al*., 2001).

This chapter will focus on the isolation, sequencing and single locus conversion of sex-linked AFLP markers and will also describe the analysis of the linkage of these markers with the sex locus in *Oreochromis niloticus*.

4.2. Materials and methods

4.2.1. Radioisotope labelling of AFLP selective amplification

To clone and sequence sex-linked AFLP markers, a radioactive AFLP was performed. α^{32}P-dATP was directly incorporated into the PCR reaction mix for selective amplification. For each sample, 0.075 Mbq of α^{32}P-dATP was added to a 20 µl PCR reaction mix. The PCR reaction conditions were as described in section 3.2.3.3 for selective amplification.

4.2.2. Polyacrylamide gel electrophoresis

The PCR products of radioactive AFLPs were analysed on 6% denaturing polyacrylamide gels. Then 200 ml of 6% denaturing acrylamide solution was made by mixing 12 g of total acrylamide in a ratio of 24:1 acrylamide : bis acrylamide (BioRad Laboratories Ltd, Hemel Hempstead, UK), 20 ml of 10X TBE and 72 g urea and finally distilled water was added to make a final volume of 200 ml. For each gel, 75 ml of previously prepared 6% acrylamide solution was placed in a glass beaker and 750 µl of 10% freshly prepared APS (ammonium persulfate, Sigma, Dorset, UK) and 15 µL of N,N,N′,N′-Tetramethylethylenediamine (TEMED, Sigma, Dorset, UK) were added to the solution, stirred briefly and poured immediately. The non-toothed edge of a 96 well shark tooth comb was placed in between the plates to a depth of 2 to 3 mm and was tightly clamped with clips. The gel was then left for at least 3 hrs to allow polymerization. After polymerization, the gel was placed inside the gel apparatus (Gibco, BRL, Paisley UK). The toothed edge of the comb was inserted and the wells were flushed with 1X TBE buffer. The upper and lower buffer chambers of the gel rig

were filled with 1X TBE buffer. The gel was pre-run for between 45 mins and one hr at a constant 75 W power to heat the gel to approximately 50°C.

Loading buffer mix was prepared by mixing 98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol FF (Sigma, Dorset, UK) and 0.025% bromophenol blue. Equal volumes of labelled PCR product and loading buffer mix were mixed and 4 μL of that solution loaded onto the gel. Samples were electrophoresed for between two and two and half hours at a constant 75 W power.

After the gel was cool, the plates and combs were removed. 3 MM Whatman filter paper was then cut to the size of the large plate. The small plate was removed carefully and one piece of 3 MM Whatman paper was placed over the gel, pressed gently all over and used to remove the gel. Gels were dried in a commercial gel dryer (Hoefer®, Drygel Sr., SE 1160, Pharmacia Biotech) for 1 hr at 80°C and when dry, were placed in a cassette with an intensifying screen and a sheet of film (Kodak Biomax™ MS film, Eastman Kodak company, USA) overnight at -70°C. Film was developed using standard techniques.

4.2.2.1. DNA purification from dried polyacrylamide gels

The developed autoradiography film was superimposed on the dried gel and the sex-linked AFLP bands were marked with sterile needles and excised with sterile scalpel blades. Corresponding regions on those lanes on the gel containing samples that were negative for the band of interest were also excised from the dried gel as controls. Excised gel bands were placed in a sterile tube containing 50-100 μl $TE_{0.1}$ (0.1 mM Tris-EDTA) buffer. Tubes were incubated for 30-45 mins at 40°C, the buffer was then

discarded and the gel fragment rinsed with another 100 μL of $TE_{0.1}$. Finally, 25 μl of $TE_{0.1}$ was added and the sample was incubated overnight at 55°C. The solution containing DNA was purified using a GFX spin column (Pharmacia, UK). Extracted DNA was frozen at -20°C. This solution was directly used for PCR re-amplification with the same primer combination, using the same conditions as in the selective amplification.

4.2.3. Cloning PCR products and plasmid preparation

PCR products were cloned into vectors using the AdvanTage™ PCR cloning kit (Clontech Laboratories, Inc.) according to the manufacturer's instruction. Briefly this involved the ligation of amplified PCR products into the pT-Adv vector and subsequent transformation of this vector into TOP10F' *E. coli* competent cells. Cells were then selected on LB agar plates containing ampicillin (50 μg/ml), X-Gal (5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside; 40 mg/ml), and IPTG (Isopropyl beta-D-thiogalactopyranoside; 100 mM). White colonies were selected and grown overnight in 2-5 ml LB broth containing 50 μg/ml of either ampicillin or kanamycin for plasmid DNA preparation and further analysis. Plasmid DNA was extracted by using GFX *Micro* Plasmid Prep kit (Pharmacia Biotech) according to the manufacturer's instructions.

4.2.4. Sequencing and data analysis

PCR products and extracted plasmid DNA containing inserts were sequenced using Big Dye (version 2; PE corporation), with sequencing reactions electrophoresed on an ABI automated sequencer (ABI PRISM™ 377, PE Corporation). Sequencing gels were

prepared as described in section 3.2.3.4. Sequence data were analysed using BioEdit software and NCBI-BLAST (Altschul1 *et al*., 1990) search. A multiple sequence alignment programme (ClustalW: Thompson *et al*., 1994) was also use to compare the sequences.

4.2.5. Primer design for SLAMs

Two sets of PCR primers were designed for each of the four SLAMs: internal primers and STS primers (sequence tagged sites). Internal primers were designed inside the derived sequences, while STS primers were designed according to the following structure: 5'-*Eco*RI or *Mse*I complete restriction sequence + three selective bases + short sequence from sex-linked AFLP marker-3'.

PCR was optimized for all four pairs of internal primers on a randomly selected small number of progeny tested XX, XY and YY individuals (n = 7-10) from within the relevant family. After PCR optimization the internal primers were tested on all within source family individuals used in the original AFLP screening (XY neofemale, XX donor neomale, XX and YY gynogenetic individuals and normal control females and males in two families). STS primers were tested for only those markers that failed to show specific amplification with internal primers.

These primers were used in PCR reactions containing 25 to 50 ng of genomic DNA in a 10 µl PCR volume containing 1X PCR buffer, 200 µm of each dNTPs, 1.5 mM of $MgCl_2$, 130 µM of each primer and 0.8 units of *Taq* DNA polymerase (ABgene, UK). The generalized PCR conditions were: initial denaturation of 3 mins at 96°C, then 30

cycles of 94°C for 30 secs, 55-60°C for 30 secs, 72°C for 30 secs; then 72°C for 7 mins and 4°C for holding.

4.2.6. Southern analysis and probe labelling

Sex-linked AFLP markers (SLAMs) were used as probes for Southern analysis. To determine the amount of DNA required to allow the detection of a single copy of each marker, experiments were performed using salmon DNA spiked with differing amounts of plasmid DNA containing sex-linked AFLP inserts. For this purpose, high molecular weight salmon DNA was digested with *Hae*III restriction enzymes and plasmid DNA containing the SLAMs was extracted (as described in section 4.2.3). Plasmid DNA was linearised by digestion with the *Not* I restriction endonuclease. Plasmid DNA, equivalent to 1, 10, 100 and 200 copies of each SLAM, was added to salmon DNA based on the size of plasmid and the size of the tilapia genome (tilapia genome 1 pg = 1000 Mb; Majumdar and McAndrew, 1986; Kocher *et al.*, 1998). One, 10, 100 or 200 copies of plasmid DNA with inserts were mixed with 10 μg of restricted salmon DNA and samples were run on 1% agarose gel with 0.5X TAE running buffer for 24 hrs at a constant 1.2 Vcm^{-1}. Half of the buffer was changed and mixed after 12 hrs. DNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) using a capillary transfer protocol, according to the manufacturer's instructions.

Sex-linked AFLP probes were labelled by random priming (Dalgleish, 1987) incorporating α^{32}P-dATP. A labelling reaction mixture was prepared by mixing 3.0 μl OLB (solution A: 625 μl 2M Tris-HCl, pH 8.0 + 25 μl 5 M $mgCl_2$ + 350 μl H_2O + 18 μl

2-mercaptoethanol + 5 µl dCTP + 5 µl dGTP + 5 µl dTTP, each triphosphate dissolved in 3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 at a concentration of 0.1 M; solution B: 2 M HEPES, pH 6.6; solution C: hexadeoxyribonucleotides evenly suspended in 3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 at 90 OD units/ml; all solutions were stored at -20°C. OLB was prepared by mixing solutions A, B and C in the ratio of 2:5:3; stored at -20°C for up to 3 months), 0.6 µl of BSA (10 mg/ml), 10-15 ng of DNA, 1 µl DNA polymerase Klenow fragment (diluted 1 unit/ml according to manufacturer's recommended buffer; New England Biolab, UK), 0.075 Mbq of α^{32}P-dATP, dH_2O to a total volume of 15 µl. Labelling reaction mixtures were incubated for 1-2 hours at 37°C.

Southern hybridization was performed according to standard methods (Sambrook *et al.* 1989) at 60ºC. Membranes were pre-hybridized at 60ºC for 3-4 hrs in a hybridizing oven (Techne Hybridiser, HB-1, Techne Inc., USA) using pre-hybridization buffer (6X SSC; 5X Denhardt's reagent: 500 ml 50X filter sterilized stock Denhardt's reagent contain 5 g type 400 Ficoll- Amersham Pharmacia, 5 g polyvinylpyrrolidone- Sigma, 5 g Fraction V bovine serum albumin- Sigma; and 0.5% SDS). After pre-hybridization, α^{32}P-dATP labelled probe was added to the pre-hybridization buffer and hybridization was performed overnight at 60ºC. After hybridization, blots were washed once in 2X SSC/0.2% SDS for 5 mins and twice in 1X SSC/0.2% SDS for 15 mins at 60°C. Membranes were checked for radioactivity at this stage. If the signal was high, membranes were washed a further three times in 0.2X SSC /0.2% SDS for 20 mins at 60ºC. For higher stringency, the final wash was sometimes performed at 65ºC. Finally, the membranes were rinsed in 2X SSC at room temperature for 10 mins. After washing, membranes were wrapped in Saran wrap, and put inside an autoradiography cassette

with a sheet of film (Kodak Biomax™ MS film, Eastman Kodak Company, USA). The cassette was maintained at -70ºC for at least one day, with film being developed by the standard procedure. The autoradiography of this experiment was analysed and the required amount of the genomic DNA needed to be electrophoresed on agarose gel for single locus analysis was optimized.

O. niloticus genomic DNA was digested in three different ways: single digestion with *Mse*I or *Eco*RI and double digestion with both *Mse*I and *Eco*RI. For Southern analysis, 10 copies equivalent of restricted genomic DNA for all three different digests and 10 copies equivalent of SLAMs were electrophoresed on 1% agarose gel as described earlier in this section. For each probe, one positive and one negative sample were also analysed.

4.2.7. PCR-based tests with AFLP pre-selective and internal primers (derived from SLAMs) using AFLP pre-selectively amplified PCR products as template

A PCR-based test was performed using the AFLP pre-selective PCR product as template. In this experiment, AFLP pre-selective primers were used in combination with either of the forward or reverse internal primers derived from SLAMs. This experiment was designed to determine the reason for the polymorphism of the sex-linked AFLP markers. A 20 μl PCR reactions were performed using the same PCR conditions as the AFLP pre-selective amplification. The products of PCR amplification were analysed by 2% agarose gel electrophoresis with 0.5X TAE running buffer.

4.2.8. Linkage analysis of AFLP markers with the sex locus

Linkage analysis was performed following the results of AFLP screening and sequencing analysis of the markers in the normal cross groups, gynogenetics and pooled normal cross and gynogenetics in two families. Linkage analysis was performed between three AFLP markers (*Oni*X420/*Oni*Y425, *Oni*Y382 and *Oni*Y227) and the sex locus in *O. niloticus*. In each group the number of recombinants for each sex-linked AFLP marker with the sex locus of *O. niloticus* was scored and the information was used for calculating logarithm of maximum likelihood ratio and for interpreting linkage map distance. Linkage was also analysed between *Oni*X420/*Oni*Y425 and *Oni*Y382, because, these two markers were generated by two AFLP selective primer combinations (E6 and H8) in the same family (Dam1).

The logarithm of likelihood ratio (LOD score) was used as the measure of support for linkage versus absence of linkage. The following formula was used to calculate the LOD score:

$$Z(\theta) = \{ n \log(2) + k \log(\theta) + (n-k) \log(1-\theta)$$

Where the domain is usually $0 \leq \theta \leq {}^{1}/_{2}$;

θ : recombination fraction;

k : number of recombinants

n : number of individuals

The linkage analysis was performed with a LOD cut-off of 2.0 for sex-linked markers as proposed by Morton (1955: cited by Ott, 1991).

Complete interference was assumed i.e. the occurrence of multiple crossovers was excluded while map distance was measured between two loci as follows:

$x = \theta$; where x: map function (Morton, 1955: cited by Ott, 1991).

Therefore, the recombination fraction should be equal to the map function. The distance between the two loci (map distance) was expressed as centiMorgan units (cM).

To test the distribution of recombinants in male and female individuals from pooled data of normal crosses in two families, 2 X 2 chi-square contingency analysis was performed. 2 X 2 chi-square contingency analysis was also performed to test the distribution of recombinants in normal cross group and gynogenetic group for all three markers in two families.

4.2.9. AFLP screening in unrelated samples

At the end of family set level screening, a group of randomly selected individuals of three sexual genotypes (XX, XY and YY), unrelated to those individuals used earlier, were also screened for three SLAMs generated from the E6 (*Mse*I-CTA + *Eco*RI-AAG) AFLP primer combination. A list of these individuals is presented in Table 4.1.

Table 4.1. Showing the sex genotypes, origin, status and total number of unrelated individuals used for SLAMs screening; - indicates the non-progeny tested status.

Phenotypes	Genotypes	Origin	Number of fish tested
♀	XX	Normal	9
♀	-	Gynogenetic	4
♂	XY	Normal	10
♂	YY	Normal	5
♂	-	Gynogenetic	12

4.2.10. Statistical analysis

Chi-square contingency (2X2) analyses were performed in several occasions to test against relevant hypotheses.

4.3. Results

4.3.1. Separation of sex-linked AFLP bands from polyacrylamide gel

Radioactive AFLP reactions performed to isolate sex linked AFLP markers (SLAMs) successfully amplified the four previously identified sex-linked markers (Fig. 4.1).

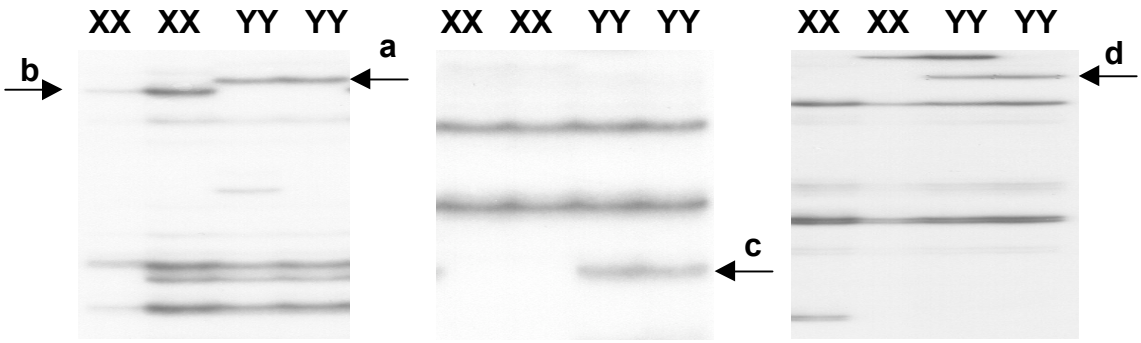


Fig. 4.1. Autoradiography of sex-linked AFLP markers derived from 6% denaturing polyacrylamide gel. a: *Oni*Y425; b: *Oni*X420; c: *Oni*Y227 and d: *Oni*Y382.

The SLAMs were isolated from the dried polyacrylamide gels, with DNA being extracted and subsequently re-amplified using fragment specific AFLP selective primers (Fig. 4.2). These re-amplified PCR products were subsequently sequenced and cloned.

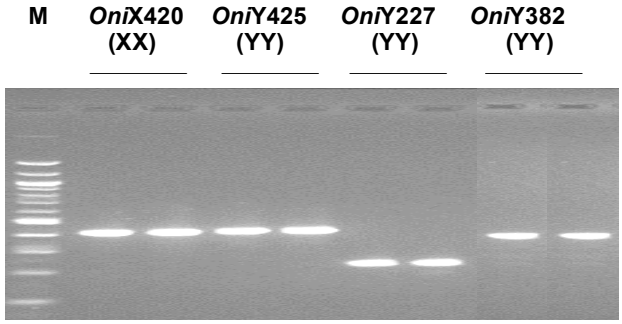


Fig.4.2. Re-amplified sex-linked AFLP markers on 1.5% agarose gel; M: 100 bp ladder DNA size marker.

4.3.2. Sequencing of SLAMs

Initially the re-amplified PCR products for all four sex-linked AFLP markers were directly sequenced without cloning into a vector. As mentioned in Chapter 3, each sex-linked AFLP band was given a specific name according to species, association with sex and the size of the band.

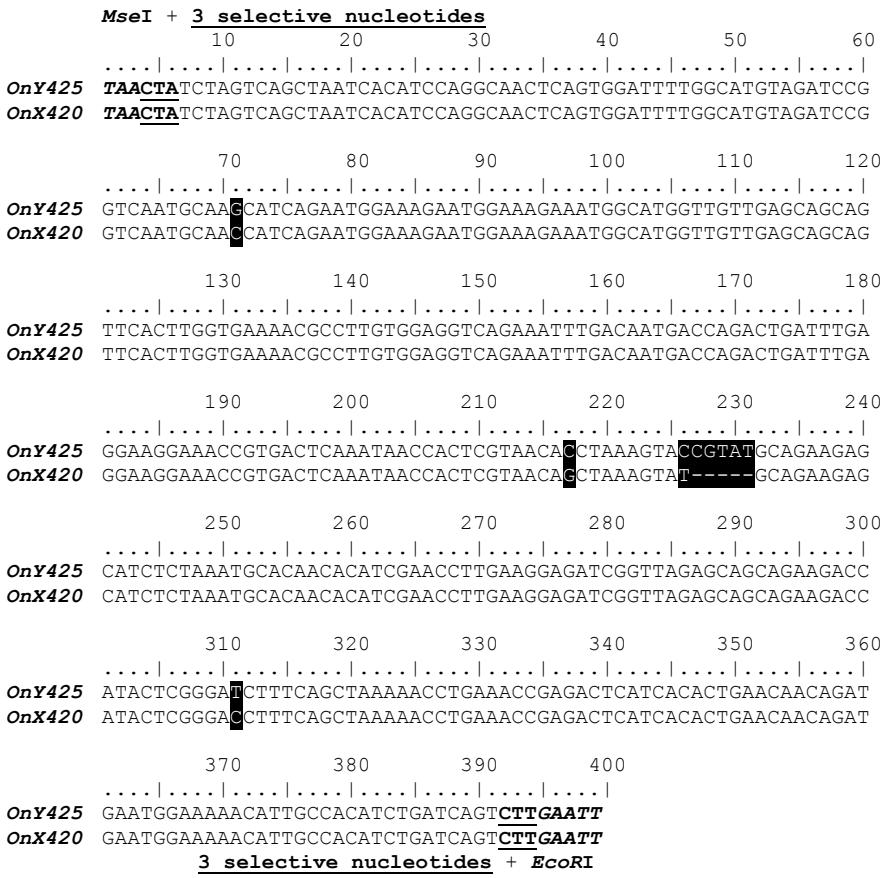


Fig. 4.3. Complete aligned sequences of *Oni*Y425 and *Oni*X420. Blocked areas represent the polymorphic regions.

Complete sequences for *Oni*Y425 and *Oni*X420 were obtained by direct sequencing of re-amplified PCR products. Both sequences were aligned and very high sequence alignment was observed. The sequence alignment between *Oni*Y425 and *Oni*X420 also

revealed a five base pair indel (insertion/deletion) and four SNPs (single nucleotide polymorphisms). This suggested that *Oni*Y425 and *Oni*X420 are allelic forms of a sex-linked locus in family 1 (Dam 1; Fig. 4.3).

Direct sequencing from re-amplified PCR product did not reveal complete sequence information for *Oni*Y382 and *Oni*Y227. To obtain the complete sequence for *Oni*Y382 and *Oni*Y227 and to compare the cloned sequence of *Oni*Y425 and *Oni*X420 with that obtained from the PCR products, freshly re-amplified products for all four SLAMs were cloned and sequenced using plasmid-specific forward and reverse sequencing primers. In each case, 4-6 positive clones were sequenced and the derived sequences for all of the clones from a particular SLAM were compared to identify any sequence variation. In the case of *Oni*Y425 and *Oni*X420, no sequence differences were observed between the sequences derived from PCR products and the sequences derived from the cloned AFLP markers.

Complete sequences were obtained for all four SLAMs, characterised by flanking with *Eco*RI and *Mse*I enzyme restriction sites + three selective AFLP primer specific nucleotides (Fig. 4.3 and Fig. 4.4). Interestingly, very short stretches of microsatellites (tetra-nucleotide and di-nucleotide repeats) were also identified in *Oni*Y227 (Fig. 4.4; shaded). Although *Oni*Y425, *Oni*X420 and *Oni*Y227 were generated by the same AFLP primer combination, no sequence similarity was observed when *Oni*Y227 was compared with *Oni*Y425 and *Oni*X420.

*Oni*Y227

***Mse*I + 3 selective nucleotides**
***TAA*CTA**TAGAAATCGGAGGCACAGGTATTCATCAGAGCCTGATAAACACATAATGCATGGAGCTTCACGAC
ATGGGTTTCAATGACCAAGGTTTCCCAGTGCTTTTGTCTCCAAAGTGTATACCAACATCTGATATGTTCTT
TTTGGGGGGGGGGGTTATTTGTTTGTTTGTTTTGTGTGAAGTTTGTTATTTTT***CTTGAATT***
3 selective nucleotides + *Eco*RI (in reversed order)

***Oni*Y382**

***Eco*RI + 3 selective nucleotides**

***AATTC*ACG**TGAAAACAAAAATATTCCAAAATAACTCAAAACTGGTCTCGTGGTGAATTTCAAAATTTCCTT
TGAGGTCAACTCAACAATAACCGAAACTCTAAACAGCGAAAACGATGGAACACGCACAGACTCGCACACAT
GGGCCTTCAGGTTGGGGGGGGGGGGGTCCAGTGTAAACAACAATGACATGATTTTATTTTCCTCCTTCCCA
CCGCTCAGTAACACAGCTTTCTCGCTTGCACTTTCTTTCCATTTCTTCTCACGAGGAAGAGGAGCACACAT
TGAGGCTCATTATGCTCGTTTCACTGTGAAATGTTCTAAGAAGACAAACGGCTATAACAGTTTATATAT***AA***
***G*TTA**

3 selective nucleotides + *Mse*I (in reversed order)

Fig. 4.4. Complete sequence of *Oni*Y227 and *Oni*Y382.

Comparison of the nucleotide and conceptual amino acid sequences of the four markers, by BLASTn and BLASTx , with previously published sequences did not reveal any significant similarity.

4.3.3. Single locus conversion of SLAMs

The internal and STS primers for the SLAMs are shown in Table 4.2. For *Oni*Y425 and *Oni*X420 a common forward primer (*Oni*XY420+425FC) and two marker specific reverse primers (*Oni*Y425R and *Oni*X420R) were designed, with the specific primers including the region of the five base pairs indels.

Table 4.2. Sex-linked AFLP markers (SLAMs) derived primer sequences.

SLAM derived primer name	Forward primer (5'-3')	Reverse primer (5'-3')
*Oni*Y425	TTGGCATGTAGATCCGGT	CTTCTGCATACGGTACTTTAGG
*Oni*X420	TTGGCATGTAGATCCGGT	TGCTCTTCTGCATACTTTAGC
*Oni*Y227	ACAAACTTCACACAAAACAAA	CACAGGTATTCATCAGAGCC
*Oni*Y382	ACATTTCACAGTGAAACGAGC	ACTCAAAACTGGTCTCGTGG
*Oni*Y425 STS	AATTCAAGACTGATCAGATGTGGCAATG	TAACTATCTAGTCAGCTAATCACATCCAGG
*Oni*X420 STS	AATTCAAGACTGATCAGATGTGGCAATG	TAACTATCTAGTCAGCTAATCACATCCAGG
*Oni*Y227 STS	AATTCAAGAAAAATAACAAACTTCACAC	TAACTATAGAAATCGGAGGCACAGG
*Oni*Y382 STS	AATTCACGTGAAAACAAAAATATTCCAAAATA	TAACTTATATATAAACTGTTATAGCCGTTTGTCTT

The internal primers designed for *Oni*Y425 successfully amplified male-specific bands (Y-linked) when a group of XX, XY and YY *O. niloticus* were tested within source family 1 (Fig. 4.5A). The internal primers designed for *Oni*X420, the female specific allele of *Oni*Y425, amplified in all XX and XY individuals and did not amplify in any of the YY individuals inside the family (Fig.4.5B). Similarly, internal primers designed for *Oni*Y227 in family 4 also correctly determined sex within the family samples (Fig.4.5C). In contrast, non-specific PCR amplification was observed when the internal primers for *Oni*Y382 were tested inside the family 1 samples (Fig. 4.5D).

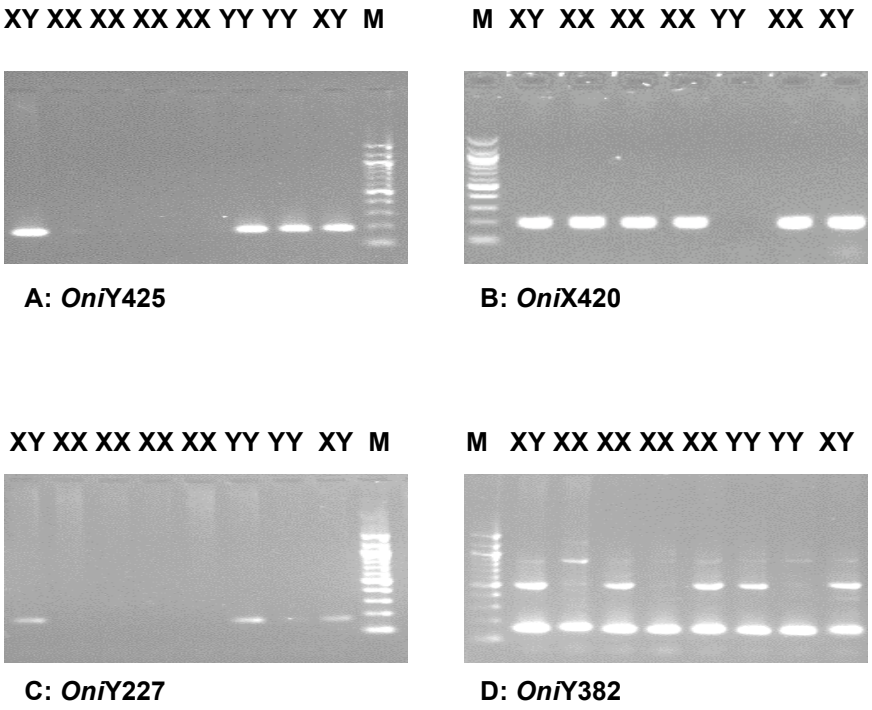


Fig.4.5. SLAMs derived internal primer amplification in within family samples. M: 100 bp ladder DNA size marker. A- *Oni*Y425; B- *Oni*X420; C- *Oni*Y227, second YY male was a recombinant with *Oni*X420/*Oni*X420 genotype; D- *Oni*Y382.

The single locus internal PCR primers for *Oni*Y425, *Oni*X420 and *Oni*Y227 successfully identified the genetic sex in samples within the source family, therefore

STS primers for these markers were not tested. In contrast, for *Oni*Y382 STS primers were tested, as non-specific PCR amplification (Fig. 4.5D) was observed with the internal primers. These primers, *Oni*Y382STSF and *Oni*Y382STSR, were designed to be specific to the 5'-*Eco*RI or *Mse*I restriction sequence + the three selective bases + a short sequence from *Oni*Y328. PCR amplification using the STS primers designed for *Oni*Y382 successfully determined the sex of individuals within the family samples (Fig. 4.6).

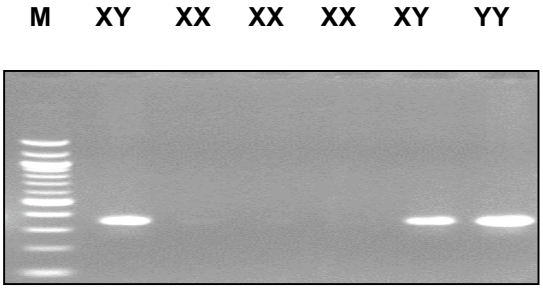


Fig. 4.6. Specific sex-linked PCR amplification of *Oni*Y382 STS primers in *Oreochromis niloticus*; M: 100 bp ladder DNA size standard.

Once all four SLAM-derived primers had been tested on a small number of family samples, they were tested on the same set of family samples which had been used in the AFLP screening (Chapter 3). In all cases, the PCR primers were successful in determining the sex inside these family samples.

4.3.4. Southern analysis

Two sex-linked AFLP markers, *Oni*Y227 and *Oni*Y382 were used as probes in Southern analysis. *Oni*Y425 and *Oni*X420 were not used for Southern analysis, as sequence analysis indicated that they were allelic forms of the same locus.

The quantity of digested genomic DNA required for single locus (copy) Southern analysis was successfully optimized using *Hae*III digested salmon DNA (Fig. 4.7).

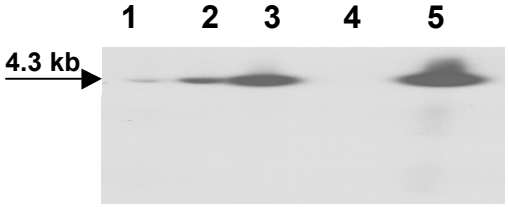


Fig. 4.7. Autoradiography with linearised pT-Adv vector (3.9 kb + *Oni*Y382). 1, 2, 3 and 5 are single copy, 10, 100 and 200 copies respectively used for the optimization of required quantity needed for single copy Southern hybridization; lane 4 is blank.

The results of the Southern hybridization of *Oni*Y227 and *Oni*Y382 AFLP sex-linked marker derived probes are presented in Fig. 4.8. In both cases, no sex-specific hybridization pattern was observed.

When the *Oni*Y382 probe was used, hybridization was observed to all three of the digests in both male and female samples. In *Mse*I digested male and female DNA, the *Oni*Y382 probe hybridized to produce three bands, whereas in the *Eco*RI digested samples, the probe produced significantly larger bands (around 3 kb). In double digested female and male DNA, the probe produced bands similar in size to the three bands observed in the *Mse*I digestion. However, a 382 bp band was also visible (Fig. 4.8).

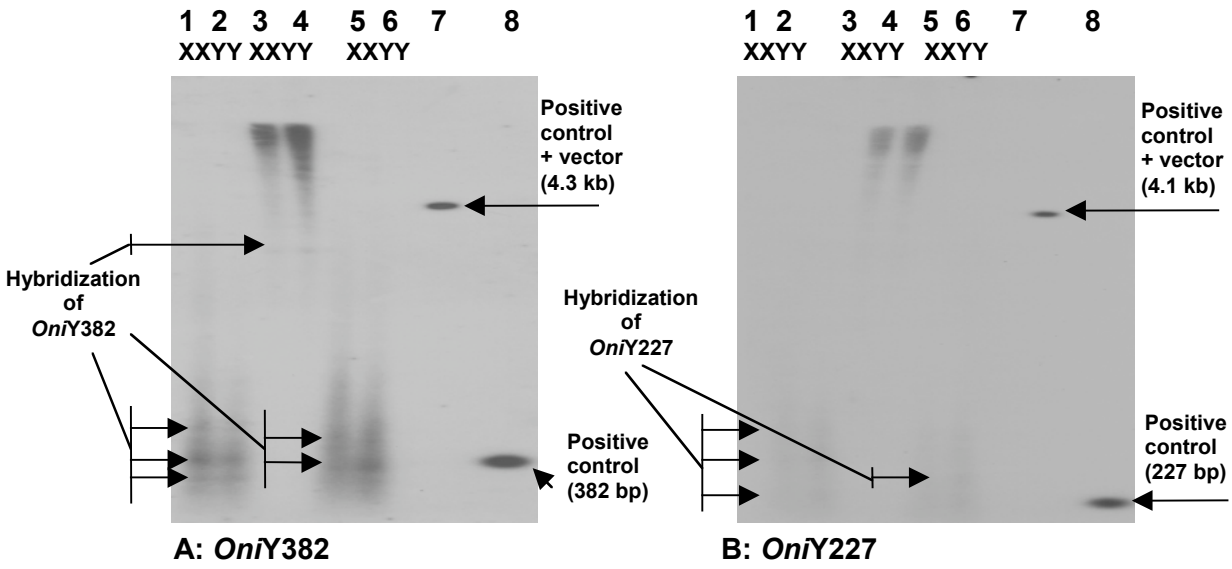


Fig. 4.8. Autoradiograpy of Southern hybridization with sex-linked AFLP marker derived probes. A: with *Oni*Y382 and B: *Oni*Y227. 1-2: *Mse*I digested samples; 3-4: *Eco*RI digested samples; 5-6: double digested samples. 1, 3 and 5 are XX females; 2, 4 and 6 are YY males. A7: 10 copies of plasmid DNA with *Oni*Y382 insert; A8: 10 copies of *Oni*Y382. B7: 10 copies of plasmid DNA with *Oni*Y227 insert; B8: 10 copies of *Oni*Y227.

When *Oni*Y227 was used as a probe, three bands were produced in the *Mse*I digested female and male samples, the smaller of which was 227 bp in size. In the double digested samples, this probe hybridized to produce one band, larger than the expected 227 bp, in both female and male samples (Fig. 4.8).

4.3.5. PCR based tests with AFLP pre-selective and internal primers (derived from SLAMs) using AFLP pre-selectively amplified PCR products as template

The results of the PCR based tests with AFLP pre-selective and internal primers (derived from SLAMs) using AFLP pre-selectively amplified PCR products as template are presented in Fig. 4.9. The results of this experiment revealed multilocus PCR amplification when the *Eco*RI pre-selective primer was used in combination with forward or reverse sex-linked AFLP marker derived primers, multiple bands were

observed in both positive and negative samples (positive means presence of Y-linked in males and negative means absence of Y-linked in female and vice versa). In contrast, the *Mse*I pre-selective primer did not amplify any products when used in conjunction with the AFLP marker derived internal primers.

PCR amplified a bright slightly smaller band (expected size band) than the original AFLP markers when the *Eco*RI pre-selective primer was used with the sex-linked AFLP marker derived internal forward primers in addition to several other larger size bands (non-specific bands) in both positive and negative samples for markers *Oni*Y425, *Oni*X420 and *Oni*Y382 (Fig. 4.9A, B and D). However, in the negative samples these smaller bright bands appeared to be slightly larger than those of the positive samples (Fig. 4.9A, B and D).

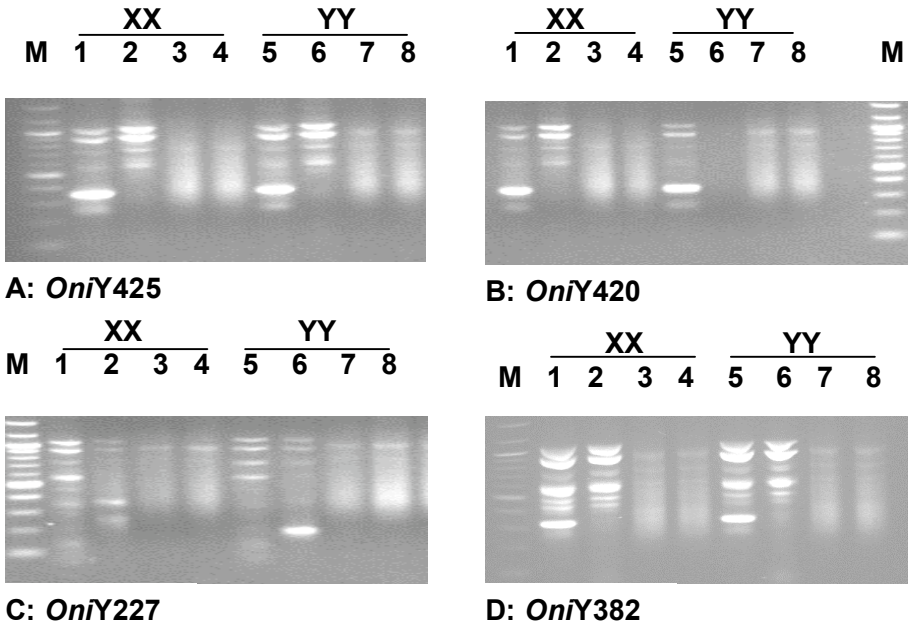


Fig. 4.9. PCR based tests with AFLP pre-selective PCR products as template. **A**: *Oni*Y425, **B**: *Oni*X420, **C**: *Oni*Y227 and **D**: *Oni*Y382. M: 100 bp ladder DNA size marker. Lanes: 1-4 XX females; 5-8 YY males; 1,2, 5 and 6: *Eco*RI pre-selective primers; 3, 4, 7 and 8: *Mse*I pre-selective primers; 1, 3, 5 and 7: sex-linked AFLP marker derived forward primers; 2, 4, 6 and 8: sex-linked AFLP marker derived reverse primers.

The same type of amplification was also observed when tested with *Oni*Y227. However, when the *Eco*RI pre-selective primer was used with the *Oni*Y227 derived internal forward primer, sex-specific amplification was obtained only in the positive sample (Fig.4.9C).

4.3.6. Linkage analysis of AFLP markers with sex locus

The sex-linked status of each SLAM was assigned on the basis of AFLP screening among the gynogenetic individuals and numbers of recombinants were determined on the basis of the segregation of markers in a group of phenotypically sexed males and females from a cross between the XY founder neofemale and XX donor neomale (described in detail in Chapter 3; Fig. 3.1). Linkage analysis was also performed for gynogenetic individuals separately and also for the combined data derived from normal cross and gynogenetic individuals.

A 2X2 chi-square contingency analysis was also performed to test male:female distribution of recombinants in pooled data derived from the normal cross progeny. The results of the 2X2 chi-square analysis are presented in Table 4.3. Significant ($P > 0.05$) differences were observed in male and female distribution of "recombinants" (apparent recombinants) in the observed pooled normal cross group data (Table 4.3).

Table 4.3. 2X2 Chi-square contingency table for number of recombinants for three SLAMs in two families of normal cross fish (pooled data). " apparent recombinant from observed data".

	♂	♀	Total	χ2, 1df
"Recombinant"	10	0	10	8.41*
Non-recombinant	19	25	44	
Total	29	25	54	

*significantly different ($P > 0.05$).

2X2 contingency chi-square analysis was also performed to test whether there are any significance differences in recombinants between normal cross and gynogenetic groups for three sex-linked markers in two families. Results indicated no significant differences ($P < 0.05$) in recombinants between these two groups in observed data for three markers in two families, but the numbers of recombinants were always higher in normal cross group than gynogenetic groups (Tables 4.4, 4.5 and 4.6.).

Table 4.4. 2X2 chi-square contingency table among gynogenetics and normal cross group for *Oni*X420/*Oni*Y425.

	Recombinants	Non-recombinants	Total	χ2, 1df
Normal cross	6	24	30	0.864**
Gynogenetics	2	24	26	
Total	8	48	56	

**not significantly different ($P < 0.05$).

Table 4.5. 2X2 chi-square contingency table among gynogenetics and normal cross group for *Oni*Y382/ -.

	Recombinants	Non-recombinants	Total	χ2, 1df
Normal cross	4	26	30	0.079**
Gynogenetics	2	24	26	
Total	6	50	56	

**not significantly different ($P < 0.05$).

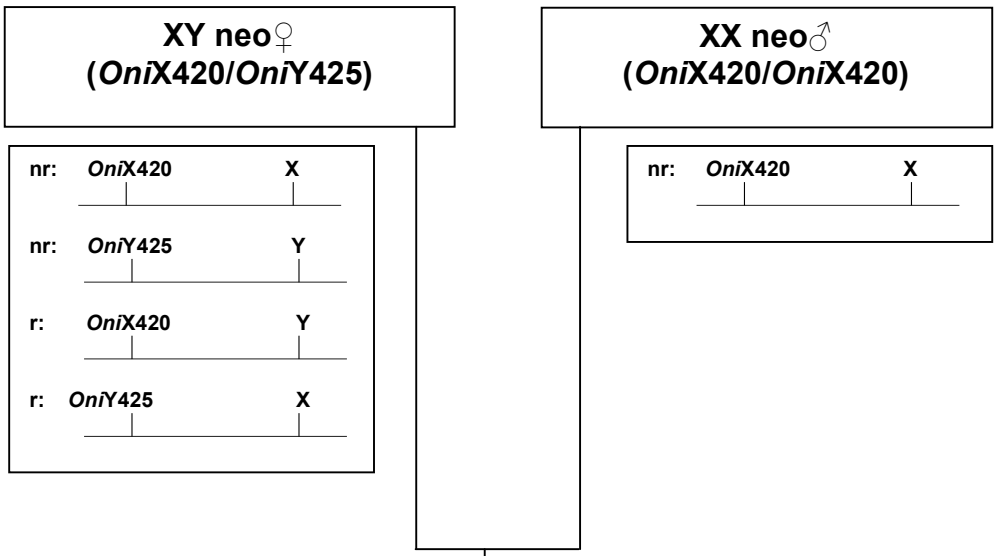
Table 4.6. 2X2 chi-square contingency table among gynogenetics and normal cross group for *Oni*Y227/ -.

	Recombinants	Non-recombinants	Total	χ2, 1df
Normal cross	4	20	24	3.50**
Gynogenetics	0	32	32	
Total	4	52	56	

**not significantly different ($P < 0.05$).

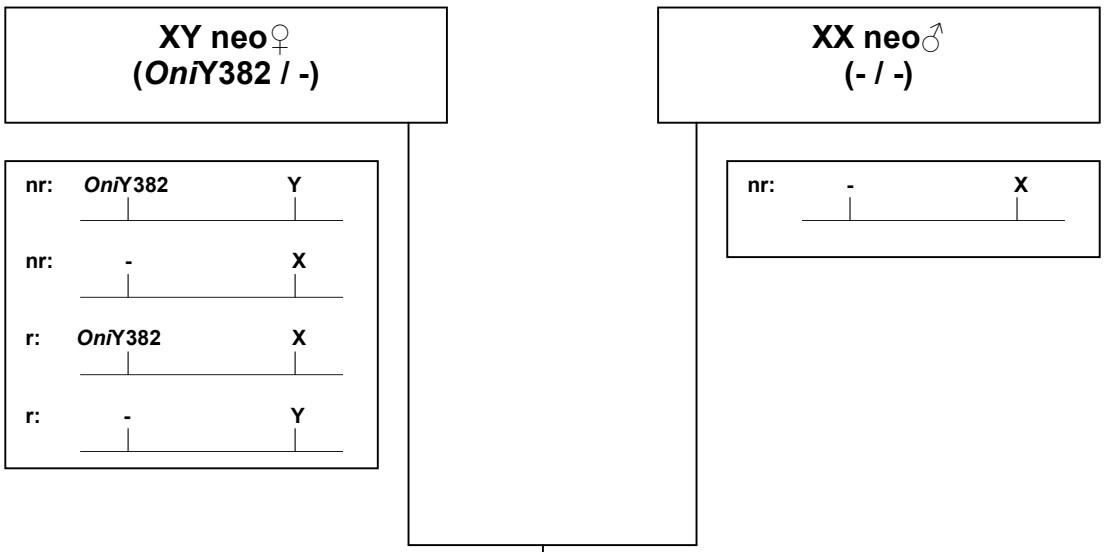
The results of the determination of the numbers of recombinants and non-recombinants observed during linkage analysis of three AFLP markers (*Oni*X420/*Oni*Y425, *Oni*Y382 and *Oni*Y227) and the sex locus in *O. niloticus* among normal cross individuals are presented in Fig. 4.10 A, B, and C, while analysis of the linkage between *Oni*X420/*Oni*Y425 and *Oni*Y382 in the normal cross progeny is presented in Fig. 10D. Summary results of the linkage analysis are presented in Tables 4.7, 4.8 and 4.9.

Among the normal cross progeny, the results revealed 6 recombinants in the segregation of marker *Oni*X420/*Oni*Y425 and sex (Fig. 10A), while four recombinants were observed in the case of *Oni*Y382 (Fig. 10B) and *Oni*Y227 (Fig. 10C). Two recombinants were also observed between markers *Oni*X420/*Oni*Y425 and *Oni*Y382 (Fig. 10D). Similarly numbers of recombinants were also scored among gynogenetic groups in two families. No recombinant was observed in case of *Oni*Y227 and sex locus in family 4, while two recombinants were observed in case of *Oni*X420/*Oni*Y425, *Oni*Y382 and sex locus in family 1 and also between *Oni*X420/*Oni*Y425 and *Oni*Y382. In case of pooled data eight and six recombinants were observed between *Oni*X420/*Oni*Y425, *Oni*Y382 and sex locus respectively in family 1, while four recombinants were observed in case of *Oni*Y227 and sex locus in family 4. Two recombinants were also observed among the pooled data between *Oni*X420/*Oni*Y425 and *Oni*Y382.



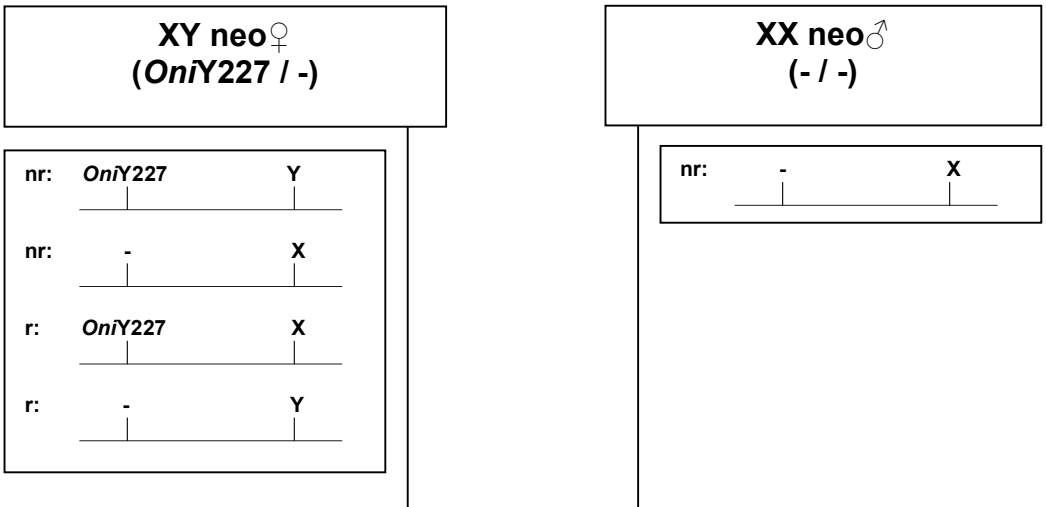
Locus	♂		♀	
	n=15	status	n=15	status
*Oni*X420/*Oni*X420	6	Recombinant	15	Non-recombinant
*Oni*X420/*Oni*Y425	9	Non-recombinant	0	Recombinant

Fig. 4.10A. Linkage analysis of X-linked *Oni*X420/ Y-linked *Oni*Y425 and Sex in normal cross group of family 1; nr: non-recombinant, r: recombinant.



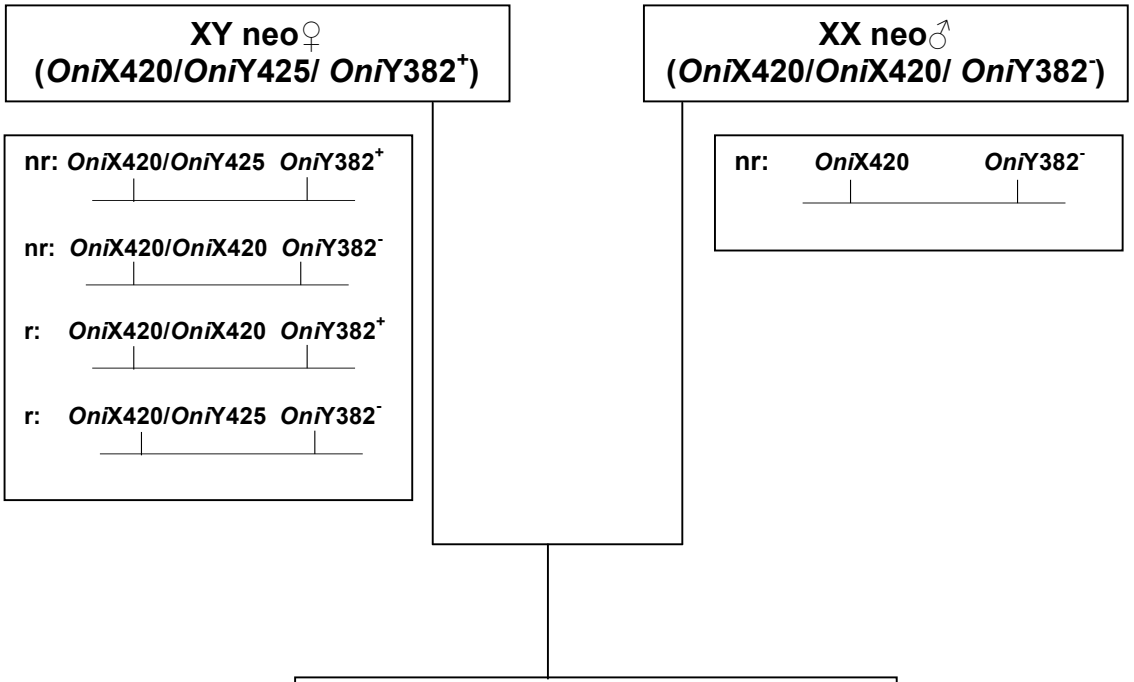
Locus	♂		♀	
*Oni*Y382	n=15	status	n=15	status
+	11	Non-recombinant	0	Recombinant
-	4	Recombinant	15	Non-recombinant

Fig. 4.10B. Linkage analysis of Y-linked *Oni*Y382 and Sex in normal cross group of family 1; nr: non-recombinant, r: recombinant.



Locus	♂		♀	
*Oni*Y227	n=14	status	n=10	status
+	10	Non-recombinant	0	Recombinant
-	4	Recombinant	10	Non-recombinant

Fig. 4.10C. Linkage analysis of Y-linked *Oni*Y227 and Sex in normal cross group of family 4; nr: non-recombinant, r: recombinant.



Genotype	♂		♀	
	n=15	status	n=15	status
*Oni*X420/*Oni*Y425/*Oni*Y382 +	9	Non-recombinant	0	Recombinant
*Oni*X420/*Oni*X420/*Oni*Y382 -	0	Recombinant	19	Non-recombinant
*Oni*X420/*Oni*X420/*Oni*Y382 +	2	Recombinant	0	Recombinant
*Oni*X420/*Oni*Y425/*Oni*Y382-	0	Recombinant	0	Recombinant

Fig. 4.10D. Linkage analysis of X-linked *Oni*X420/ Y-linked *Oni*Y425 and Y-linked *Oni*Y382 in normal cross group of family 1; nr: non-recombinant, r: recombinant.

In the next step, the information on the number of recombinants was used to determine the linkage of three AFLP markers with sex by calculating the recombinant fraction and LOD score for each marker with the sex locus. Linkage map distances for each AFLP markers with sex were also measured based on the results of recombination

fractions and the assumption of complete interference. Similarly, the map distance and LOD score between *Oni*X420/*Oni*Y425 and *Oni*Y382 were also measured and calculated and results are presented in Tables 4.7, 4.8 and 4.9. The results derived from linkage analysis are also presented diagrammatically in Fig. 4.11.

Table 4.7. Summary of linkage analysis of AFLP markers and sex locus among the normal cross individuals of *Oreochromis niloticus*. * LOD cut off of 2.0 were used; ** map distance was measured in centiMorgan (cM) where complete interference was considered as $x = \theta$.

Loci	Total number of individuals (n)	Number of Recombinants (k)	Recombination fraction (θ)	LOD score* (Zmax)	Map distance (cM)**	Family
*Oni*X420/*Oni*Y425 and Sex	30 (15♂+15♀)	6	0.2	2.51	20	1
*Oni*Y382 and Sex	30 (15♂+15♀)	4	0.13	3.91	13	1
*Oni*Y227 and Sex	24 (14♂+10♀)	4	0.16	2.52	17	4
*Oni*X420/*Oni*Y425 and *Oni*Y382	30 (15♂+15♀)	2	0.07	5.83	7	1

Table 4.8. Summary of linkage analysis of AFLP markers and sex locus among the gynogenetic individuals of *Oreochromis niloticus*. * LOD cut off of 2.0 were used; ** map distance was measured in centiMorgan (cM) where complete interference was considered as $x = \theta$.

Loci	Total number of Individuals (n)	Number of Recombinants (k)	Recombination fraction (θ)	LOD score* (Zmax)	Map distance (cM)**	Family
*Oni*X420/*Oni*Y425 and Sex	30 (15♂+15♀)	2	0.07	5.84	7	1
*Oni*Y382 and Sex	30 (15♂+15♀)	2	0.07	5.84	7	1
*Oni*Y227 and Sex	24 (14♂+10♀)	0	0	7.22	0	4
*Oni*X420/*Oni*Y425 and *Oni*Y382	30 (15♂+15♀)	0	0	9.03	0	1

Table 4.9. Summary of linkage analysis of AFLP markers and sex locus among the pooled data (both gynogenetics and normal cross individuals) of *Oreochromis niloticus*. * LOD cut off of 2.0 were used; ** map distance was measured in centiMorgan (cM) where complete interference was considered as $x = \theta$.

Loci	Total number of individuals (n)	Number of Recombinants (k)	Recombination fraction (θ)	LOD score* (Zmax)	Map distance (cM)**	Family
*Oni*X420/*Oni*Y425 and Sex	56 (28♂+28♀)	8	0.14	6.9	14	1
*Oni*Y382 and Sex	56 (28♂+28♀)	6	0.11	8.6	11	1
*Oni*Y227 and Sex	56 (30♂+26♀)	4	0.07	10.6	7	4
*Oni*X420/*Oni*Y425 and *Oni*Y382	56 (28♂+28♀)	2	0.03	13.1	3	1

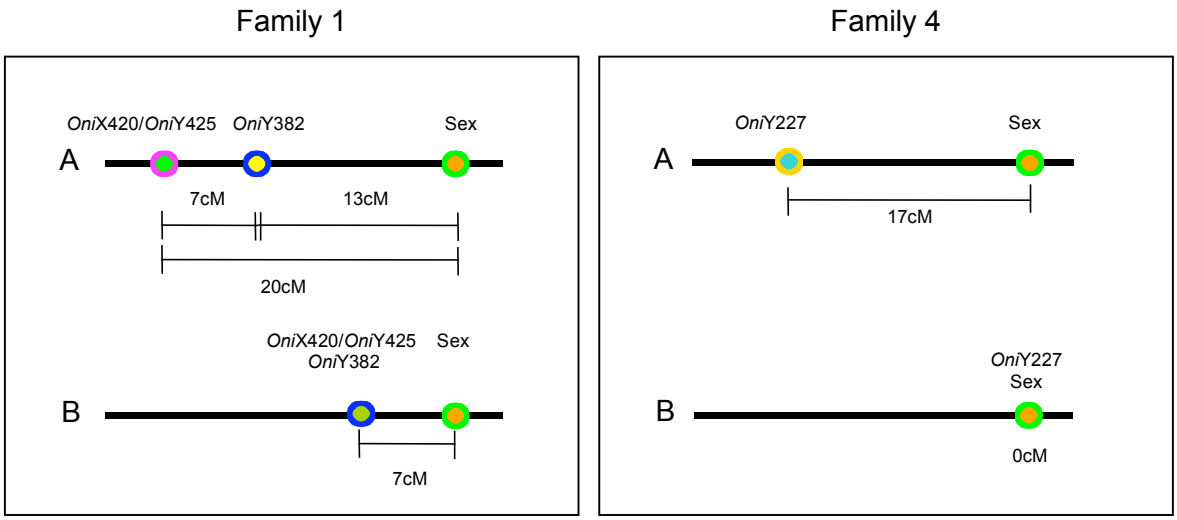


Fig. 4.11. Schematic diagram showing arbitrary locations of SLAMs in the sex chromosome of *Oreochromis niloticus* in families 1 and 4. A: in normal cross individuals and B: in gynogenetic individuals.

4.3.7. AFLP analysis of unrelated samples

Tables 4.10 a, b and c show the results of AFLP analysis of randomly selected unrelated males and females (both progeny-tested and non-tested) with primer combination E6 (*Mse*I-CTA/*Eco*RI-AAG), which generated 3 sex-linked AFLP markers in two families during the individual family set screening.

Table 4.10a. Results of AFLP screening in unrelated progeny-tested normal *Oreochromis niloticus*.

Genotypes	No. of individuals	Marker's presence		
		*Oni*Y227	*Oni*Y425	*Oni*X420
XX-♀	9	2 (22.2%)	1 (11.1%)	2 (22.2%)
XY- ♂	10	7 (70.0%)	10 (100.0%)	3 (30.0%)
YY- ♂	5	1 (20.0%)	5 (100.0%)	0 (0.0%)

Table 4.10b. Results of AFLP screening in unrelated non-progeny-tested *Oreochromis niloticus*. *: non-progeny tested putative YY gynogenetic males.

Phenotypes	No. of individuals	Marker's presence		
		*Oni*Y227	*Oni*Y425	*Oni*X420
Males*	12	10 (83.3%)	4 (33.3%)	3 (25.0%)
Females	4	1 (25.0%)	3 (75.0%)	3 (75.0%)

Table 4.10c. Summary results of AFLP screening in unrelated non-progeny-tested *Oreochromis niloticus*.

Phenotypes	Total no. of individuals	Marker's presence		
		*Oni*Y227	*Oni*Y425	*Oni*X420
Males	27	18 (67.0%)	19 (70.4)	6 (22.2%)
Females	13	3 (23.1%)	4 (31.0%)	5 (38.5%)

Summarizing the results of AFLP screening in unrelated samples, *Oni*Y227 and *Oni*Y425 correctly identified 67.0% and 70.4% of males respectively. However, those two markers were also found to be present in 23.1% and 31.0% of females respectively. The rate of identifying females by *Oni*X420 was very low (38.5%) and 22.2% of males were also found to show the presence of this marker (Table 4.10c).

A 2 X 2 chi-square contingency analysis was also performed to test the association of these markers with correct identification of sex. Results showed that markers *Oni*Y227 and *Oni*Y425 were significantly ($P < 0.05$) associated with correct identification of sex in unrelated samples. However, no such significant ($P > 0.05$) association was observed with *Oni*X420 in sex identification.

4.3.8. Single locus analysis in unrelated samples

In the next step, a small number (n = 25) of randomly selected unrelated XX, XY and YY individuals were screened using the SLAM-derived PCR primers. In all four cases, it was found that the SLAM-derived PCR primers could not determine sex in unrelated individuals (Table 4.11; Fig. 4.12).

Table 4.11. SLAM-derived PCR primer screening in unrelated samples. The table shows the number of positive PCR amplifications in non-progeny-tested unrelated females, males and YY individuals of *Oreochromis niloticus*. Numbers of individuals are in parentheses.

Phenotypes	SLAMs			
	*Oni*Y227	*Oni*Y382	*Oni*Y425	*Oni*X420
Females (10)	4	10	6	10
Males (10)	7	10	3	10
YY (5)	5	5	5	5

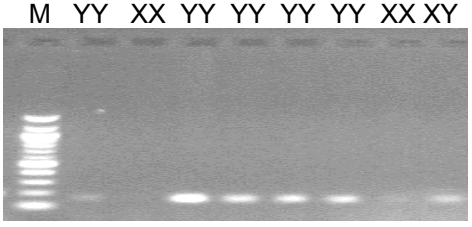


Fig. 4.12. *Oni*Y227 derived inside primer with randomly selected unrelated females, males and YY individuals; M= 100 bp ladder DNA size marker.

4.4. Discussion

The goal of these experiments was to search for and characterize sex-linked DNA markers in *Oreochromis niloticus*. For this purpose, an AFLP comparison of populations that differed only in their sex chromosome complement was undertaken. This technique has previously been used to identify sex-linked DNA markers in several species of plants and animals (Griffiths and Orr, 1998; Griffiths *et al*., 2000; Reamon-Büttner and Jung, 2000; Gadau *et al.*, 2001). In this case, four sex-linked AFLP markers were identified within two source families of *O. niloticus* by performing a fluorescent detection AFLP technique (Chapter 3).

It was found that the results could be replicated with radioisotope-labelled primers. Furthermore, isolation of the bands from the gels and subsequent reamplification with sex-linked AFLP marker-specific selective primer pairs suggested that each band represented the true sex-linked AFLP marker sequence identified earlier (Chapter 3).

4.4.1. Single locus conversion and PCR analysis of SLAMs

Successful conversion of polymorphic AFLP markers into single locus markers requires identification of true sequences of the marker responsible for a given polymorphism. Sequence analysis of all four sex-linked markers identified in the present experiment did not show any differences when sequences of several clones from each marker were compared. This result was expected as the isolated bands were reamplified successfully with the specific selective AFLP primer without any non-specific PCR amplification. However, conversion of AFLP markers into locus-specific markers is often difficult, because most AFLP markers are shorter in length, which makes it

difficult to design primers internally, which in turn increases the possibility of amplifying non-specific genomic sequences.

In many cases attempts have been made to convert poorly adapted dominant molecular markers into large-scale locus specific applications such as, co-dominant SCARs (sequence characterized amplified regions) have been developed from dominant RAPD markers (Adam-Blondon *et al.*, 1994; Williamson *et al.*, 1994; Garcia *et al.*, 1996; Naqvi and Chattoo, 1996). Some other approaches were also studied such as designing STS primers (by keeping the original restriction enzyme sequence and selective nucleotide sequences as the 5' end of the primer sequence), converting AFLP markers into RFLPs (Reamon-Büttner and Jung, 2000) or CAPs (cleaved amplified polymorphic sequences; Terauchi and Kahl, 1999). The inverse PCR technique has also been applied to convert dominant AFLP to co-dominant STS markers. However, the inverse PCR technique is technically demanding and, moreover, time consuming (Bradeen and Simon, 1998). Sometimes polymorphic AFLP markers contain sequences that are enriched with microsatellite sequences, which can be used to convert AFLP markers into locus-specific co-dominant markers (Nakamura *et al.*, 2001).

In the present experiment PCR primers designed internally from the derived sequences of sex-linked markers successfully converted three (*Oni*Y425, *Oni*X420 and *Oni*Y227) out of four sex-linked AFLP markers: showing sex-specific PCR amplification within the source family samples. This suggests that sequence differences exist between the males and females within the source family which is sufficient for sex-specific amplification of the internal primers. In one case (*Oni*Y382) STS primers were used for single locus conversion as internal primers failed to show sex-specificity,

suggesting sex-linked polymorphism in this case is most likely due to selective nucleotides at the 3' ends of the selective AFLP primers.

The other PCR-based analysis involving pre-selective PCR amplification products, there the internal and AFLP pre-selective primers, produced some interesting results. In the cases of *Oni*Y425, *Oni*X420 and *Oni*Y382, PCR amplified products in both the positive (e.g. Y-linked marker present only in the male of the source family) and negative (e.g. Y-linked marker absent in the female within the source family) (Fig. 4.9) samples. However, in the case of *Oni*Y227, sex-specific amplification was observed when the internal forward primer was used with the *Eco*RI pre-selective primer, which is more likely due to a high level of sequence dissimilarity among the positive and negative samples. Therefore, it is most likely that in the case of *Oni*X420, *Oni*Y425 and *Oni*Y382, there is significant sequence homology between males and females, and only minor differences. However in the case of *Oni*Y227, sequence differences between males and females are probably greater compared to the other three markers which could be reproduced by PCR as a positive/negative situation, both in the original AFLP analysis and as single locus amplification.

4.4.2. Linkage analysis in unrelated samples

In many fish species a high level of chiasma interference has been observed during cross over, resulting for most chromosomes in only one chiasmata event per meiosis (Allendorf *et al*., 1986; Hussain *et al*., 1994a). During linkage analysis of AFLP markers and the sex locus and also between two markers (*Oni*X420/*Oni*Y425, *Oni*Y382),

complete interference was assumed following the evidence of earlier studies in this species, as well as in some other fish species.

Results of the AFLP screening revealed that recombinants were only observed among male individuals not only in the gynogenetic groups but also in the normal cross groups for all sex-linked markers. However the numbers of recombinants were lower in the gynogenetic groups than normal cross groups. As normal cross fish are only sexed phenotypically, some of these phenotypic males could actually be sex-reversed neomales produced more likely due to autosomal or environmental influences.

The results of the 2 X 2 chi-square analysis showed significant ($P > 0.05$) differences in recombinants between the male and female groups. These results suggest that some of the recombinant males are actually sex-reversed XX neomales and thus we might have overestimated the recombination frequency during linkage analysis for normal cross as well as for the combined data. If so, these markers would show more tight linkage with the sex locus. However, most of the gynogenetic individuals were progeny tested and normal cross individuals were phenotypically sexed. It is also found that among the gynogenetics in family 1, two recombinants were observed for the two sex-linked markers (*Oni*420/*Oni*Y425 and *Oni*Y382), however same two fish were found to be recombinant for those two sex-linked markers. One of these two YY gynogenetics was not progeny tested, while the other produced 2% females during progeny testing.

The analysis of linkage between three AFLP markers (*Oni*X420/*Oni*Y425, *Oni*Y382 and *Oni*Y227) and the sex locus in *O. niloticus* revealed moderate to tight linkage of these markers with the sex locus in all three groups (normal cross groups, gynogenetics and pooled data) tested. Two of the markers (*Oni*X420/*Oni*Y425 and *Oni*Y382) were

also closely linked to each other. As chi-square analysis showed significant differences in male:female distribution of recombinants among the pooled normal cross individual therefore, it is better to be cautious during interpreting the results of linkage analysis in normal cross group as well as during combined linkage analysis where "apparent recombinant" might interfere in actual linkage condition by the misidentification of false recombinants.

Appearances of unexpected males in *O. niloticus* is well documented in various crosses and has been explained as consequences of autosomal or environmental (mainly temperature) influences or combination of both (Mair *et al*., 1991a; Hussain *et al*., 1994a; Sarder *et al*., 1999; Kwon, 2000; Chapter 7 of this thesis). Kwon (2000) studied the sex ratios of progeny produced from crosses between the same XX neomale used in this experiment with six different XX normal females. He observed 8% - 59% males with an average of 18% males in the progeny. In another experiment, the same XX neomale was used on one occasion with an XX female and 11% males were observed among the progeny (Sayema Sayeed: personal communication). Male-skewed sex ratios among the progeny were also observed when this XX neomale was used with XY neofemales (described in Chapter 2 of this thesis). Similar phenomenon of autosomal sex reversal of XX females was also suggested in common carp (Komen *et al*., 1992).

4.4.3. Southern analysis of SLAMs

In order to determine the nature of the polymorphisms that resulted in the sex-linked AFLPs identified in Chapter 3, the genomic distribution of two sex-linked AFLP markers was investigated by Southern hybridization. Southern hybridization was not

performed for *Oni*X420 and *Oni*Y425, because they appeared to be allelic from sequence analysis. This analysis aimed to determine if the polymorphisms in the other two SLAMs (*Oni*Y382 and *Oni*Y227) were a consequence of sequence differences (such as restriction site differences) or the presence or absence of the entire sequence between the sexes. The results showed more than one (up to three) hybridization bands in genomic DNA digested with the same restriction endonucleases used in the original AFLP experiments (*Mse*I and *EcoR*I) in both male and female samples. The most likely explanation is that there are multiple copies of similar sequences in both males and females, only one of which was detected during AFLP screening due to the consequences of differences in selective nucleotide sequences at the 3' end of AFLP selective primers.

Sex-specific hybridization is also unlikely to be detected in Southern analysis if there is very little sequence dissimilarity between male and female linked alleles as in the case of *Oni*X420 and *Oni*Y425, where four SNPs and five indels were observed between males and females. In *Asparagus officinalis* sex-linked AFLP markers were found to consist of low-copy to highly repetitive sequences (Reamon-Büttner and Jung, 2000). FISH analysis of sex-linked AFLPs in this species found that the sequences were present on all of the chromosomes (Reamon-Büttner *et al.*, 1999). This kind of non-specific hybridization of sex-linked AFLP markers in asparagus was explained by the fact that the AFLP marker polymorphisms were defined by nine nucleotides at *Eco*RI end and seven nucleotides at the *Mse*I end and the fragment size (Reamon-Büttner and Jung, 2000). However, single copy AFLP sequences have been reported in some species of plant, such as potato (Meksem *et al.*, 1995) and in rice (Cho *et al.*, 1996).

4.4.4. AFLPs and PCR analysis in unrelated samples

Both AFLP and PCR analysis with SLAMs-derived primers in randomly selected unrelated samples across the species failed to show any sex specificity. This result is more likely to be expected as we observed that recombination is occurring between SLAMs and the sex locus (see linkage analysis). It is more likely that considering the recombination of these markers and the sex locus, the linkage phases of these markers in unrelated individuals are random. However, further study is required involving randomly selected progeny tested individuals to monitor the segregation of these markers across the species.

4.4.5. Summary and conclusion

This section summarizes all the marker development work described in Chapter 3 and Chapter 4.

AFLP is a dominant marker system, detecting polymorphism usually as a presence/absence situation. Therefore allelic conditions cannot be detected readily. Due to the nature of the AFLP technique such presence/absence type polymorphisms are generated by (a) the complete absence of one AFLP band specific sequence in one individual which is present in the other; (b) mutations in AFLP regions such as indels and SNPs; (c) restriction endonuclease site differences between the individuals; or (d) the number and arrangement of selective nucleotide sequences at the 3' ends of AFLP selective primers.

In the present experiment, based on the evidence of sequence analysis, *Oni*Y425 and *Oni*X420 appeared to be allelic with four SNPs and five indels. The PCR analysis with

internal primers designed against the polymorphic regions of the sequences were able to show sex-specificity within the source family, indicating five indels (and also four SNPs, although not tested specifically) were the most likely cause of sex-linked polymorphisms.

In the case of *Oni*Y382, the results of Southern and PCR analyses (both single locus and pre-selective/internal primers) suggested that the three selective nucleotides at the 3' ends of the selective AFLP primers were the reason for sex-linked polymorphism. The failure of internal primers to show sex-specificity even within the source family samples, the success of STS primers and the presence of similar Southern hybridization patterns support this.

Although *Oni*Y227 failed to show sex specificity in Southern hybridization, sex-specific PCR amplification (within the source family) with internal primers (without knowing the allelic situation) is an indicator of comparatively larger sequence dissimilarity between the sexes compared to the other markers. Even the PCR experiment involving pre-selective AFLP product and primers with internal primers showed sex-specificity. Therefore, from the above discussion, these three sex-linked (considering *Oni*X420/*Oni*Y425 as allelic) AFLP markers can be ranked as *Oni*Y227 → *Oni*X420/*Oni*Y425 → *Oni*Y382 considering the efficiency in different experiments.

It has been reported that sex chromosomes contain large numbers of repetitive sequences, some of which are not found on other chromosomes of a given species. It has also been demonstrated that AFLP fragments which are repetitive sequences could be used as probes for FISH analysis (Reamon-Büttner *et al*., 1999). Sex-chromosome specific probes which appear to differ in copy numbers between the X and Y

chromosomes have been developed in *O. niloticus* by microdissection and subsequent degenerate oligonucleotide primed PCR (DOP-PCR) (Harvey *et al*., in press).

The sex-linked markers identified in the present experiment have also been used to screen an *O. niloticus* BAC (Bacterial Artificial Chromosome) library. Positive clones were identified and subsequently used in FISH (fluorescence *in situ* hybridization) to metaphase chromosome spreads. The initial results suggested specific hybridization of some of the positive BAC clones containing these markers to the sex chromosomes (Chuta Boonphakdee: personal communication). Therefore these sex-linked markers appear to be of potential use in the physical mapping of the sex chromosomes in *O. niloticus*. These markers could also help to find more efficient sex-linked markers in this species, and hopefully sex determining genes. The subsequent FISH using positive BAC clones could also be used to generate more markers located closer to the sex determining locus.

The goal of this research was to develop a fast and inexpensive PCR-based marker system that could be used in screening for sex identification of *O. niloticus* without having to rely only on progeny testing. Although four sex-linked markers were identified which were sex-linked within two source families in the present experiment, they failed to show sex specificity across the species. However, with these sex-linked markers, the sex of most progeny within the source family could easily be identified. The X-linked marker, identified in this experiment, could also be used to distinguish XY from YY individuals within the progeny derived from the YY male of this family and will eventually help make monosex culture more efficient. Although the present study failed to develop sex-specific markers in this species, the sex-linked markers which were

developed could be used to understand the processes underlying sex determination in this species and could also be used in map-based cloning of the sex determining gene(s).

Chapter 5

Genetic Markers For The Identification Of Parentage And Gynogenetic Status Of *Oreochromis niloticus*

5.1. Introduction

Large numbers of molecular genetic markers have been developed in the last two decades. These markers can be classified on the basis of their transmission and dynamics (Park and Moran, 1994). Nuclear markers, such as, RFLPs (restriction fragment length polymorphism), RAPDs (randomly amplified polymorphic DNA), VNTRs (variable number of tandem repeats; mini- and microsatellites), AFLPs (amplified fragment length polymorphisms), biochemical marker (such as allozymes), and mitochondrial DNA (mtDNA) markers have been applied to solve various problems of fish conservation and management (Ferguson and Danzmann, 1998). In fisheries research these genetic markers have been applied to three broad areas: stock structure analysis, aquaculture and taxonomy/systematics (Ward and Grewe, 1994) with varying degrees of success (Carvalho and Hauser, 1994).

5.1.1. Molecular markers for parentage analysis

Parentage analysis in various fish species is of importance not only for proper management of captive populations but also for better understanding of mating patterns in the natural environment (O'Connell and Wright, 1997). Parentage assignment can be achieved using any type of genetic marker provided that there is a sufficient degree of polymorphism (Gerber *et al.*, 2000). A variety of molecular techniques have been applied in various fish species to elucidate inheritance in populations, and generally provide more efficient tools than of phenotypic markers.

Analysis of inheritance in genetic manipulation studies, such as gynogenesis and androgenesis, is important to evaluate the success of the procedure. Phenotypic markers

can be used if the trait is based on a recessive allele (Galbusera *et al.*, 2000) and such characteristics have been used to assess inheritance in gynogenetic fish such as carp (Nagy *et al.*, 1978) and tilapia (Don and Avtalion, 1988; Varadaraj, 1990a). However, such morphological markers are rare, and, even if available, often require confirmation using biochemical or molecular markers to allow the unambiguous identification of inheritance.

Biochemical markers such as allozyme loci have been applied in several fish species to monitor genetic manipulation. Such species include chinook salmon (Levanduski *et al.*, 1990), ayu (Han *et al.*, 1991), carp (Yousefian *et al.*, 1996) and tilapia (Hussain *et al.*, 1993; Müller-Belecke and Hörstgen-Schwark, 1995; Sarder *et al.*, 1999; Karayucel, 1999). Allozyme analysis has also revealed high levels of heterozygosity at a number of loci in meiotic gynogenesis (Hussain *et al.*, 1993, Sarder *et al.*, 1999). This technique can thus be an efficient tool in discriminating meiotic gynogenetic individuals from mitotic gynogenetic individuals (Hussain *et al.*, 1993; Sarder *et al.*, 1999). However, low levels of genetic variability are often observed using this technique, which limits its application (Ferguson and Danzmann, 1998).

The recent advances in DNA technology have provided many abundant genetic markers and often result in the detection of high levels of genetic variation. Multilocus DNA fingerprinting using VNTRs (Tautz and Renz, 1984; Jeffreys *et al.*, 1985a,b) has been used in inheritance analysis studies of genetically manipulated populations of tilapia (Carter *et al.*, 1991; Sarder *et al.*, 1999; Karayucel, 1999; Jenneckens *et al.*, 1999) and African catfish (Volckaert *et al.*, 1994).

The PCR-based fingerprinting technique of amplified fragment length polymorphisms (AFLPs) has been shown to detect polymorphism in both simple and complex genomes (Vos *et al.*, 1995). As described in Chapter 3, the main principle of the AFLP technique is the selective amplification of restricted DNA fragments from a digest of total genomic DNA using PCR. This technique has been successfully applied to the study of maternal inheritance in meiotic gynogenetic sea bass (Felip *et al.*, 2000). However, as AFLPs generate dominant markers, it is difficult to identify the level of heterozygosity in meiotic gynogenesis.

For any molecular marker, heterozygosity for a particular locus is important to discriminate meiotic from mitotic gynogenetic populations. For this, a genetic marker should ideally be co-dominant. Allozyme markers have been found to be useful (Linhart *et al.*, 1987; Hussain *et al.*, 1993), but microsatellites are superior considering the level of polymorphism and the information content of a single locus, as well as the sample requirement and sampling method (Ferguson *et al.*, 1995). Therefore, microsatellites would be a better choice than allozyme markers. It has also been suggested that the average microsatellite locus has a greater probability of detecting differentiation than an allozyme locus (Ferguson and Danzmann, 1998).

5.1.2. Microsatellites

The isolation of satellite DNA by buoyant density gradient centrifugation and its characteristic tandemly arranged motifs with highly repetitive DNA sequences was one of the major breakthroughs for the generation of the present day molecular markers (Tautz and Renz, 1984; Epplen *et al.*, 1997). Satellite DNA motifs are usually very long,

with repeat units of hundreds of base pairs and have been found to be mostly confined to the heterochromatin regions of the chromosomes (Epplen *et al.*, 1997). Another class of satellite DNA termed minisatellites or VNTRs was discovered in the human genome (Wyman and White, 1980; Jeffreys *et al.*, 1985a). Analysis of these has shown them to be among the most mutable sequences known. Minisatellites are much smaller in total length compared to the satellite DNA, with repeats units ranging from 7-100 bp, arranged in tandem and organized in many loci. Many of them are found to be clustered near the telomeric regions of the chromosomes. Probably the most powerful Mendelian marker was discovered in 1984 and was termed as microsatellite (Tautz and Renz, 1984; Litt and Luty, 1989; Weber and May, 1989). These are also tandemly repeated with a repetitive unit of 1-6 bp and are shorter in length than minisatellites. Satellites, minisatellites and microsatellites can be highly variable and thus form excellent tools for genetic characterization. Their variability is most often due to particular arrays on a given chromosome having different repeat numbers in different individuals. Thus, they form allelic variants and for a number of mini- and microsatellites, almost every individual is heterozygous.

A common property of mini- and microsatellite tandem arrays is that identical or related motifs occur at multiple genomic sites, i.e. these sequences share the properties of both tandemly repeated as well as interspersed DNA. Moreover, different mini- and microsatellites often occur intermingled with each other in a particular stretch (Armour *et al.*, 1989, 1992; Weber, 1990). The intermingling of different types of repeats together with the accumulation of point mutations within the repeat units may result in DNA

sequences which are cryptically simple (Tautz *et al.*, 1986) i.e. their repeat structure is more or less concealed (Weising *et al.*, 1994).

Microsatellite sequences are very abundant and well dispersed in the eukaryotic genomes and can account for up to 1% of the genome. They are mainly found within intron regions but can be found within expressed regions (Edwards *et al.*, 1998). They are also found in prokaryotes (Field and Wills, 1998) and in viruses (Davis *et al.*, 1999). Their repeat motif size usually ranges from 1-6 bp. However, the most commonly studied microsatellites are di- (CA)n, tri- (CCTCCT)n and tetra- (GATAGATA)n nucleotide repeats. The arrangement of these repeats in a genome can be pure (e.g. CACACACACACACACA), compound (e.g. CACACACAGAGAGAGAGA) or interrupted (e.g. CACACACAGGGCACACA) (Tautz and Renz, 1984; Litt and Lutty, 1989).

The variability in microsatellites is derived from mutation, and understanding the mutational process in microsatellite loci is important in developing statistical procedures for inter-population comparisons (Di Rienzo *et al.*, 1994). Various studies have been conducted to analyse the mechanisms causing microsatellite mutations (Levinson and Gutman, 1987; Henderson and Petes, 1992; Schlötterer and Tautz, 1992; Weber and Wong, 1993). One of the two major theories of the origin and evolution of microsatellites is slipped strand mispairing (SSM), with an association with the mismatch repair system (Levinson and Gutman, 1987; Schlötterer and Tautz, 1992; Gordenin *et al.*, 1997; Eisen, 1999). The other major theory is unequal crossing over between the sister chromatids. However, this is as not convincing for microsatellites as it is for satellite sequences (Levinson and Gutman, 1987).

Two models of mutation have also been proposed to describe variation at microsatellite loci, the infinite allele mutation model (IAM) and the stepwise mutation model (SMM). The IAM model predicts that mutation will generate only new allelic conditions which may involve any number of repeat units. In contrast, the SMM model predicts that mutation occurs through the gain or loss of a single repeat unit. This means that some mutations will generate alleles that already exist in the population (reviewed by O'Connell and Wright, 1997). Most observed mutations at microsatellite loci involve single repeat unit increasing or decreasing in copy number (Hatsbacka *et al.*, 1992; Henderson and Petes, 1992; Kwiatkowski *et al.*, 1992; Mahtani and Willard, 1993; Oudet *et al.*, 1993; Weber and Wong, 1993), with occasional changes involving several repeats (Weber and Wong, 1993). Although the SMM model assumes all changes take place in the repetitive region, allelic size data across species suggests changes also occur in the flanking region (Rico *et al.*, 1996; Angers and Bernatchez, 1997).

The rate of mutational changes at microsatellite loci is vary variable and known to range from $10^{-5} – 10^{-2}$ per generation (Jarne and Lagoda, 1996). It has also been reported that the rate of slippage is dependent on the structure of the repeat sequence, for example, longer and GC-rich repeats show lower rates of mutational changes than shorter and AT-rich repeats (Schlötterer and Tautz, 1992; Brinkmann *et al.*, 1998; Schlötterer *et al.*, 1998). It has also been reported that the tetra-nucleotide repeat microsatellites have a higher mutational rate than the di-nucleotide repeats (Weber and Wong, 1993).

5.1.2.1. Abundance of microsatellites in fish genomes

Based on the screening of size-selected genomic libraries from several species, microsatellites appear to be abundant in the genomes of teleost fish. Arrays of (GT)n repeats were reported to occur, on average, every 7 kb in Atlantic cod (Brooker *et al.*, 1994), 12 kb in zebra fish (Goff *et al.*, 1992), 11-56 kb in Atlantic salmon (McConnell *et al.*, 1995b), and 23 kb in brown trout (Estoup *et al.*, 1993). Estoup *et al.* (1993) also screened for (CT)n microsatellites and found that they were about one-third as abundant as (GT)n arrays. The proportion of (GT)n to (CT)n is surprisingly similar to that reported for rats and humans (3:1) (Beckmann and Weber, 1992). In addition, considering the frequency and dispersion of microsatellites throughout the teleost genome, and the high level of variability (Estoup *et al.*, 1993; Brooker *et al.*, 1994; Nielsen *et al.*, 1995; McConnell *et al.*, 1995a, b), these sequences promise to be ideal for genome mapping in fish (Wright, 1993). Further, the almost unlimited number of potential markers makes it possible to accumulate assemblages of compatible loci well suited for various general applications. These marker systems in fish, comprising several highly variable loci, could be developed for pedigree and parentage assessment. Also systems employing less variable loci could be developed for population applications, where increased numbers of alleles make analyses more powerful, necessitating larger sample sizes (Carvalho and Hauser, 1994; McConnell, 1995a, b; Shaklee and Bentzen, 1998; Arnegard *et al*., 1999).

5.1.2.2. Microsatellites and their application in fisheries research

Microsatellites are versatile and almost ideal Mendelian genetic markers for studies of ecology, evolution and conservation (reviewed by O'Reilly and Wright, 1995; Jarne and Lagoda, 1996; O'Connell and Wright, 1997; Neff *et al.*, 2000). These markers have been widely used in many areas of fisheries research such as to assess the effective population size of stocks (Garcia deLeon *et al*., 1997; Shaklee and Bentzen, 1998; Arnegard *et al*., 1999), stock identification (Shaklee and Bentzen, 1998), levels of inbreeding (Tessier *et al.*, 1997), population structure and gene flow (Garcia deLeon *et al.*, 1997; Arnegard *et al.*, 1999), parentage (Knight *et al*., 1998; Galbusera *et al*., 1996, 2000; Jones and Avise, 1997; Peruzzi and Chatain, 2000), quantitative traits (Jackson *et al.*, 1998) and linkage mapping (Kocher *et al*., 1998; Sakamoto *et al*, 2000). In *O. niloticus,* microsatellites have been used to construct a genetic linkage map (Kocher *et al.*, 1998; McConnell *et al.*, 2000). Phylogenetic relationships have also been studied in several species of the tribe Tilapiini (Sobolewska, 1999). The species composition of tilapia strains has also been investigated using microsatellite loci (Costa-Pierce and Doyle, 1997). Microsatellites have been used in marker-assisted selective breeding of *O. niloticus* (Agresti *et al.*, 2000). Polymorphic microsatellite loci have also been used to study paternal transmission in genetically manipulated populations, such as gynogenesis in African catfish (Galbusera *et al.*, 2000) and in European seabass (Peruzzi and Chatain, 2000).

Microsatellite loci are usually amplified by PCR as single loci, a process which is time-consuming and not very cost effective. Co-amplification of two or more microsatellite loci in a single PCR, or PCR multiplexing, is a powerful technique, which

considerably reduces the time and cost associated with microsatellite genetic assays (Chamberlain *et al.*, 1988; Neff *et al.*, 2000). Since its description (Chamberlain *et al.*, 1988), PCR multiplexing has been successfully applied in many areas of DNA analysis (reviewed by Henegariu *et al.*, 1994). However, optimization of multiplex PCR is often difficult and time-consuming. Several protocols for PCR multiplexing using either fluorescent labels with automated detection and on radioisotope labelling have been published (Edwards *et al.*, 1991; Kimpton *et al.*, 1993; Oetting *et al.*, 1995; Paetkau *et al.*, 1995; Henegariu *et al.*, 1997; Ricciardone *et al.*, 1997; Fishback *et al.*, 1999; Neff *et al.*, 2000) and, associated problems and solutions have been discussed.

In the present study, attempts were made to use microsatellite loci in multiplex reactions for parentage analysis in genetically manipulated as well as normal *O. niloticus*. Seven microsatellite loci were screened for heterozogysity (as mentioned in section 2.1.2.4) in founder female and donor male used in the production of mitotic gynogenesis. To identify gynogentic status of the offspring, i.e. to eliminate the paternal genome transmission in the gynogentic progeny and success of female diploidization, sveral microsatellite loci which were heterozygous in both founder female and donor male with clearly different allele sizes were selected and used in multiplex PCR. Based on the recent development of molecular technology and the availability of powerful molecular marker systems, the present study was also investigated amplified fragment length polymorphisms (AFLPs) to verify mitotic gynogenesis in Nile tilapia *Oreochromis niloticus*. In addition, several microsatellite loci were also screened to discriminate meiotic from mitotic gynogenetics.

5.2. Materials and methods

5.2.1. Fish

Both meiotic and mitotic gynogenetic *Oreochromis niloticus* and their founder females, donor male and corresponding controls were used in this experiment. The technique for the production of mitotic gynogenetic *O. niloticus* was described in Chapter 2. The meiotic gynogenetics were produced in the same way as mitotic gynogenetics except that they were produced by the retention of the second polar body instead of preventing first mitosis. The eggs were fertilized with UV irradiated sperm as described in Chapter 2 and a heat shock of 41-42°C was applied for 4 mins, starting 5 mins after fertilization (Sarder *et al.*, 1999). Controls were produced by normal crosses using eggs fertilized with normal sperm.

Parentage analysis was performed on four families of mitotic gynogenetics and two families of meiotic gynogenetics using microsatellite loci. AFLP was also performed on two families to elucidate parentage inheritance in mitotic gynogenesis.

5.2.2. DNA extraction

Blood or fin samples from larger fish or whole fry were used to extract DNA. The procedure for DNA extraction and analyses of quality and quantity of DNA were described in Chapter 2.

5.2.3. Microsatellite primers

Seven di-nucleotide repeat microsatellite loci were selected from those isolated from *O. niloticus* (Lee and Kocher, 1996). Primers were selected mainly on the basis of

degree of heterozygosity. Four of those (UNH189, UNH197, UNH203 and UNH208) were selected from an earlier experiment carried out in Tilapiines at the Institute of Aquaculture (Sobolewska, 1999). The heterozygosity information about the other loci (UNH127, UNH211 and UNH228) was kindly provided by Professor Tom Kocher (personal communication).

Table 5.1. Microsatellite primers used in the parentage analysis.

Loci name	Primer sequences and labelling			%	Allele size	EMBL
	Forward primer 5' - 3'	ABI dye	Reverse primer 5' - 3'	heterozygosity	range (bp)	accession no.
UNH127	TCTAATGGTTCTTATTATCTCA	6-FAM	CTATACATGATTGTGCAATAAATAA	100	126-128	G12280
UNH189	ATCGATGCTTTAAGAATCAG	6-FAM	TTCTCTGACATTTTTCAGC	73	144-181	G12341
UNH197	CAGGATGGTGAGATGTTT	HEX	TTAAGTGGAAGAAGTCAATG	62	158-209	G12348
UNH203	CACAAAGATGTCTAAACATGT	TET	GAATTTGACAGTTTGTTGTTTAC	95	86-107	G12354
UNH208	CTTCTTGGCCTACAATTT	FAM	CAGATGGGTGATAGCAA	77	86-106	G12359
UNH211	GGGAGGTGCTAGTCATA	TET	CAAGGAAAACAATGGTGATA	100	130-160	G12362
UNH228	ACACCTTCACACAACTACG	TET	GATAATAATGATAATGTGGCCT	100	214-216	G12379

All primers were synthesized at 0.01 μmol scale by MWG-Biotech (UK Ltd.). The forward primers were labelled at the 5′ end with three different ABI fluorescent dyes (FAM, HEX and TET) for fragment analysis in an ABI PRISM™ 377 DNA sequencer (PE Applied Biosystems). The forward primers were labelled with ABI fluorescent dyes on the basis of the allele size range. Primers with overlapping allele size were labelled with different coloured dyes to avoid ambiguity during data analysis (Table 5.1; Fig. 5.1). The forward primers were diluted in $TE_{0.1}$ to 100 μM stock and aliquoted as 10 μM working solutions.

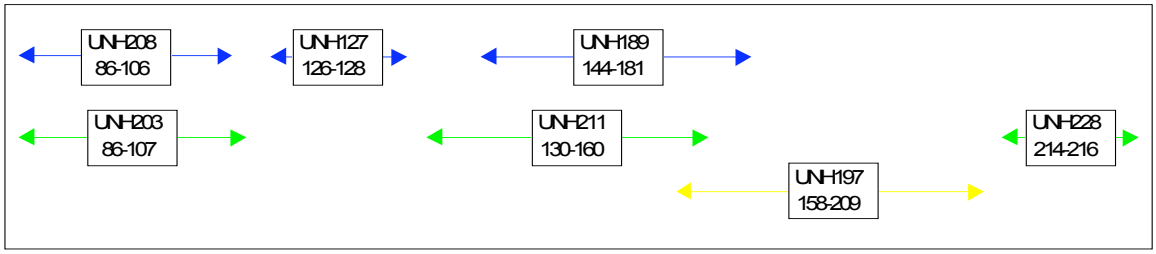


Fig. 5.1. Schematic representation of allele size range and ABI fluorescent dye labelled microsatellite loci used in the present experiment.

Reverse primers were resuspended in $TE_{0.1}$ (10 Mm Tris, pH 8.0; 0.1 mM EDTA) to 100 µM stock solution and aliquoted into several sterile tubes as 10 µM working solutions. Both stock and working primer solutions were stored at -20°C. ABI dye labelled forward primers were kept in dark vials as well as boxes, and shielded from excessive light to prevent photoactive degradation.

5.2.4. Single locus PCR optimisation for microsatellite loci

All seven microsatellite primer sets were first optimized as single locus PCRs. A temperature gradient PCR was performed (T gradient thermocycler; Whatman Biometra) to optimize the PCR conditions for ten different annealing temperatures and three different $MgCl_2$ concentrations for a reaction volume of 10 µl.

5.2.5. Multiplex PCR optimization for microsatellite loci

On the basis of single locus analysis, multiplexing PCR was designed for four families of mitotic gynogenetics of *O. niloticus*. A touchdown PCR programme was used for the multiplex amplification of different loci in all four families of mitotic gynogenetics in a volume of 10 µl PCR.

Using microsatellites, parentage analysis was performed in four families (1, 3, 4 and 5) of mitotic gynogenetics. Two families (1 and 3) of meiotic gynogenetics were also screened to determine the ability of these markers to differentiate mitotic and meiotic gynogenetic individuals. Parentage analysis in both mitotic and meiotic gynogenetic groups was performed in a panel comprising founder female, donor male, randomly selected mitotic or meiotic gynogenetics and respective control progeny in multiplex. To distinguish meiotic from mitotic gynogenetics, single locus screening was performed for all seven loci.

5.2.6. AFLP primers

For identification of mitotic gynogenetic pedigrees, 12 different AFLP primer combinations were investigated in two families (1 and 4) of mitotic gynogenetics. Eight AFLP primer combinations were investigated in each family (see Table 3.4. for AFLP primer combinations). Primer combinations A2, A7, C6, D7, E2, E6, H7 and H8 were investigated in family 1 and A7, B1, B2, C6, D4, E6, H1 and H8 in family 4. Four of the AFLP primer combinations (A7, C6, E6, H8) were common in both families. The AFLP technique was performed as described in Chapter 3. A similar panel of individuals described earlier was also used for AFLP analysis.

5.2.7. Fragment analysis on ABI automated sequencer and data analysis

Fragment analysis for both microsatellites and AFLPs were performed as described in Chapter 3 except for that of microsatellites GeneScan™-350 [TAMRA] size standard was used and electrophoresis was performed using filter set C for 2.5 hrs.

5.2.8. Statistical analysis

Chi-square analysis was performed to test the segregation of AFLP markers in normal cross and gynogenetic progeny.

5.3. Results

5.3.1. Microsatellites and parentage

5.3.1.1. Single locus PCR optimization

Single locus PCR was performed in 10 µl reaction volume containing 1X PCR buffer, 1.0, 1.5 or 2.0 mM $MgCl_2$, 200 µM of dNTPs, 0.75 µM of each primer and 0.5 units of *Taq* DNA polymerase (Advanced Biotechnologies, UK) and 40-60 ng of DNA template. All seven loci were optimized separately first in two DNA samples. The gradient PCR condition and the block temperature profile for ten different lanes are summarized in Table 5.2. Lanes 2 to 11 were used on the PCR block (Table 5.2).

Table 5.2. Conditions for gradient PCR. NT= not tested, T= tested.

Steps	Temperature (°C)	Duration (minutes:seconds)	Number of cycles
Initial denaturation	96	3:00	-
Denaturation	95	0:50	
Annealing*	49-69	0:50	5
Extension	72	1:10	
Denaturation	94	0:50	
Annealing*	49-69	0:50	26
Extension	72	1:10	
Final extension	72	7:00	-
	60	30:0	-
*annealing gradient			

Lane number											
1	2	3	4	5	6	7	8	9	10	11	12
49.0	49.5	50.9	53.0	55.4	57.8	60.2	62.6	64.9	67.1	69	69.0
NT	T	T	T	T	T	T	T	T	T	T	NT

After gradient PCR, products were electrophoresed on 1.5% agarose gel with 0.5X TAE running buffer to check amplification. Successfully amplified products were also analysed on a 5% Long Ranger® (Biowhittaker Molecular Applications, ME, USA) gel using an ABI PRISM™ 377 DNA sequencer.

Once single locus PCR was optimized for all seven loci, another round of single locus PCR was performed on a panel of brood stock, comprising all founder females and the donor male used in the gynogenesis experiments. The results of single locus PCR on the broodstock were analysed and a multiplex PCR plan was designed on the basis of allelic variation between the founder females and the donor male.

Single locus optimization for all seven microsatellite loci was performed for ten temperatures and three different $MgCl_2$ concentrations (1.0, 1.5 and 2.0 mM) using a temperature gradient PCR machine. Single locus amplification was successful for all seven loci and annealing temperatures optimized with 1.5 mM $MgCl_2$ concentration are summarized in Table 5.3.

Table. 5.3. Single locus annealing temperature optimization of all seven loci with 1.5 mM $MgCl_2$ concentration.

Loci	Annealing temperature (gradient)											
	49.0	49.5	50.9	53.0	55.4	57.8	60.2	62.6	64.9	67.1	68.5	69.0
UNH127	NT	+	+	+*	+	+	-	-	-	-	-	NT
UNH189	NT	+	+	+	+*	-	-	-	-	-	-	NT
UNH197	NT	+	+	+	+*	+	-	-	-	-	-	NT
UNH203	NT	+	+	+	+	+*	-	-	-	-	-	NT
UNH208	NT	+	+	+	+*	-	-	-	-	-	-	NT
UNH211	NT	+	+	+	+	+*	+	-	-	-	-	NT
UNH228	NT	+	+	+	+*	+	-	-	-	-	-	NT

* optimized annealing temperature based on GeneScan™ fragment analysis; NT: not tested

Results revealed that PCR amplified products in a range of different temperatures for all loci. Annealing temperature for each locus was selected on the basis of fragment analysis by electrophoresis on 5% denaturing LongRanger® gel using an ABI automated sequencer, with subsequent data analysis by GeneScan™ (V. 2.1) and Genotyper™ (V. 2.1) analysis software (PE corporation). The annealing temperatures which generated the most defined bands with the fewest stutter bands, were selected for

later PCR amplification. Based on the Genotyper™ and GeneScan™ analysis, 55°C annealing temperature was selected for UNH189, UNH197, UNH208 and UNH228, 53°C for UNH127 and 58°C for UNH203 and UNH211 (Table 5.3).

Considering the $MgCl_2$ concentrations, concentrations of 1.0 and 1.5 mM appeared to be best for all loci with fewer false and stutter bands in comparison to reactions with 2 mM $MgCl_2$ concentration. Moreover, reactions using 2.0 mM concentration required a higher annealing temperature than those with 1.0 and 1.5 mM concentrations (Fig. 5.2). Therefore, 1.5 mM $MgCl_2$ concentration was selected for this experiment.

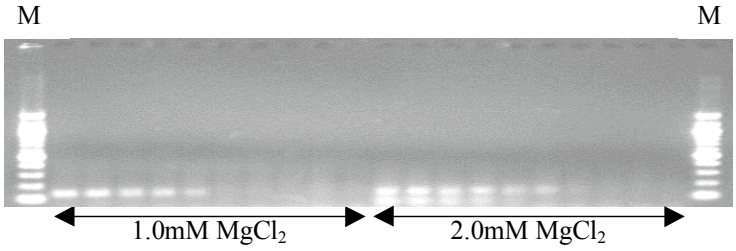


Fig. 5.2. Single locus optimization of locus UNH127 for ten different annealing temperatures (49-69°C, left to right) and two different $MgCl_2$ concentrations (1.0 mM and 2.0 mM) for one DNA sample. M: 100 bp ladder DNA size marker.

The results of the single locus screening of the brood stock with all seven microsatellite loci are presented in Tables 5.4 and 5.5. Five females and two males were screened for all seven loci optimized earlier in two DNA samples. The allelic variation and heterozygosity in all founder females were analysed and compared to that of the donor male to find suitable loci for parentage analysis.

Table 5.4. Single locus optimization for seven microsatellite loci in broodstocks.

Loci	Brood stock identification numbers and observed allele size (in bp)						
	10FE	3EE6	327	365	591	817	OA23**
UNH127	124/124*	124/154	124/124*	124/124*	124/124*	126/128	126/126*
UNH189	153/168	158/168	162/168	162/168	143/153	153/178	143/143*
UNH197	181/190	156/181	166/181	166/176	185/185*	166/166*	166/166*
UNH203	83/116	86/116	86/86*	86/89	86/89	83/89	86/86*
UNH208	100/111	100/103	100/100*	100/100*	86/86*	93/103	103/103*
UNH211	133/136	130/136	133/153	133/133*	130/130*	126/136	126/126*
UNH228	214/224	214/224	214/224	214/224	224/224	214/224	224/224*

* homozygous; ** spontaneously sex reversed XX clonal male

Five microsatellite loci (UNH189, UNH197, UNH203, UNH208 and UNH211) appeared to be informative on the basis of the single locus screening on these broodstock fish. Based on the evidence of allelic variation data between the founder females and the donor male these five loci were selected for parentage analysis in the four gynogenetic families (Table 5.4.).

The heterozygosity and the number of alleles produced by all the seven loci screened on seven individuals are summarized in Table 5.5. The observed heterozygosity in these seven loci ranged from 29-86%. The number of alleles observed per locus ranged from 2-6. The highest heterozygosity (86%) was observed in UNH189, with six different alleles (Table 5.5).

Table 5.5. Summarized results for single locus broodstock screening with seven microsatellite loci. Published allele size ranges are in parentheses.

Loci	Number of individuals	Number of heterozygotes	Number of alleles	Observed allele size range (bp)	% heterozygosity
UNH127	7	2	4	124-128 (126-128)	29
UNH189	7	6	6	143-178 (144-181)	86
UNH197	7	4	6	156-190 (158-209)	57
UNH203	7	5	4	83-116 (86-107)	71
UNH208	7	3	5	86-111 (86-106)	43
UNH211	7	4	5	130-153 (130-160)	57
UNH228	7	5	2	214-224 (214-216)	71

UNH197 and UNH208 were found to be informative for parentage analysis of mitotic gynogenesis in family 1. Allelic variation was observed between family 1 founder female (10FE) and the donor male (817) for these two loci and they were selected for duplex PCR analysis. Similarly, UNH189, UNH197 and UNH203 were selected for a triplex PCR in family 3 (3EE6 and 817). Based on the evidence of allelic variation between the founder female and donor male (817), UNH189, UNH203, UNH208, UNH211 and UNH189, UNH208 were selected for a tetraplex and duplex PCR analysis in families 4 (327) and 5 (365) respectively (Tables 5.4 and 5.5).

5.3.1.2. Multiplex PCR for microsatellite loci

A duplex (family 1 and family 5), a triplex (family 3) and a tetraplex (family 4) PCR were designated for the parentage analysis in four different families of mitotic gynogenetics based on the single locus optimization and allelic variation between the founder females and donor male as described earlier (Table 5.6).

Table 5.6. Multiplex PCR plan based on single locus optimization.

Founder female (XY) tag number	Donor male (XX) tag number	Family	Loci optimized for multiplex PCR	
			number	name
00-013E-10FE	005-117-817	1	2	UNH197 UNH208
00-013E-3EE6	005-117-817	3	3	UNH189 UNH197 UNH203
002-046-327	005-117-817	4	4	UNH189 UNH203 UNH208 UNH211
000-366-365	005-117-817	5	2	UNH189 UNH208

A touchdown PCR programme was designed for the multiplex amplification of different loci in all four families of mitotic gynogenetics (Table 5.7). This touchdown programme was used for multiplexed PCR of two, three or four microsatellite loci in all four families.

Table 5.7. Touchdown programme applied for multiplexed PCR of microsatellites in *Oreochromis niloticus*.

Steps	Temperature (°C)	Duration (mins:secs)	Number of cycles
Initial denaturation	96	3:00	
Denaturation	95	0:50	
Annealing	60	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	59.5	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	59	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	58.5	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	58	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	57.5	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	57	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	56.5	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	56	0:50	1
Extension	72	1:10	
Denaturation	95	0:50	
Annealing	55.5	0:50	1
Extension	72	1:10	
Denaturation	94	0:50	
Annealing	55	0:50	25
Extension	72	1:10	
Final extension	72	7:00	
	60	30:00	

A reaction volume of 10 µl PCR was prepared as described earlier except that the primer concentrations were: for the duplex reaction 0.4 µM of each primer; for the triplex 0.3 µM of each primer; and for the tetraplex 0.2 µM of each primer. The PCR products were first electrophoresed on 2% agarose gels with 0.5X TAE running buffer, before running on 5% LongRanger® gel in an ABI PRISM™ 377 DNA sequencer for fragment analysis.

The touchdown PCR programme designed to amplify products in a multiplex PCR was successful. The parentage inheritance in four families of mitotic gynogenesis revealed the success of gynogenesis. The results are summarized in Table 5.8 and an example of a GeneScan™ (V. 2.1) electropherogram is presented in Fig. 5.3. Although on some occasions artifacts were observed, these were easily resolved by comparison with the results of single locus optimization.

In family 1, the duplex PCR of UNH197 and UNH208 successfully revealed that the mitotic gynogenetics inherited only one of the maternal alleles. Control animals inherited one of the alleles from the founder female and either one from the donor male. All the mitotic gynogenetics tested for UNH197 inherited the maternal 190 bp allele. In contrast, for UNH208 the inheritance of each of the maternal alleles among the mitotic gynogenetics was equal. Although the sample size was small, the inheritance among the control individuals was more or less equal for both loci.

Table 5.8. Microsatellite multiplex in four families of mitotic gynogenetics used for parentage analysis. NT= not tested, *: number of individuals screened were variable in different families.

Loci	Family	Sample set* (allele size in bp)													
		Founder	Donor	Mitotic gynogenetics								Controls			
		Female	Male	1	2	3	4	5	6	7	8	1	2	3	4
UNH 197	1	181/190	166/166	190/190	190/190	190/190	190/190	190/190	190/190	NT	NT	166/181	166/181	166/190	NT
UNH 208		100/111	93/103	111/111	111/111	111/111	100/100	100/100	100/100	NT	NT	93/111	93/100	93/100	NT
UNH 189	3	158/168	153/178	158/158	158/158	158/158	NT	NT	NT	NT	NT	153/158	153/168	153/158	NT
UNH 197		156/181	166/166	181/181	181/181	181/181	NT	NT	NT	NT	NT	156/166	156/166	166/181	NT
UNH 203		86/116	83/89	86/86	86/86	116/116	NT	NT	NT	NT	NT	86/89	83/116	83/116	NT
UNH 189	4	162/168	153/178	162/162	162/162	168/168	162/162	168/168	168/168	168/168	168/168	153/162	153/168	NT	NT
UNH 203		86/86	83/89	86/86	86/86	86/86	86/86	86/86	86/86	86/86	86/86	83/86	86/89	NT	NT
UNH 208		100/100	93/103	100/100	100/100	100/100	100/100	100/100	100/100	100/100	100/100	93/100	100/103	NT	NT
UNH 211		133/153	126/136	133/133	133/133	153/153	133/133	133/133	133/133	133/133	153/153	136/153	126/133	NT	NT
UNH 189	5	162/168	153/178	162/162	168/168	162/162	162/162	162/162	NT	NT	NT	168/178	162/178	168/178	153/162
UNH 208		100/100	93/103	100/100	100/100	100/100	100/100	100/100	NT	NT	NT	93/100	93/100	93/100	93/100

UNH189, UNH197 and UNH203 successfully revealed only maternal inheritance in mitotic gynogenetics as triplex in family 3. All mitotic gynogenetics tested for these three loci were homozygous for one of the maternal alleles, while the controls were heterozygous, inheriting one maternal and one paternal allele. The inheritance of only one maternal allele in mitotic gynogenetics was observed at loci UNH189 and UNH197. Tetraplex PCR at loci UNH189, UNH 203, UNH 208 and UNH 211 in family 4 and duplex at loci UNH189 and UNH 208 in family 5 also revealed only maternal inheritance in mitotic gynogenetic offspring (Table 5.8).

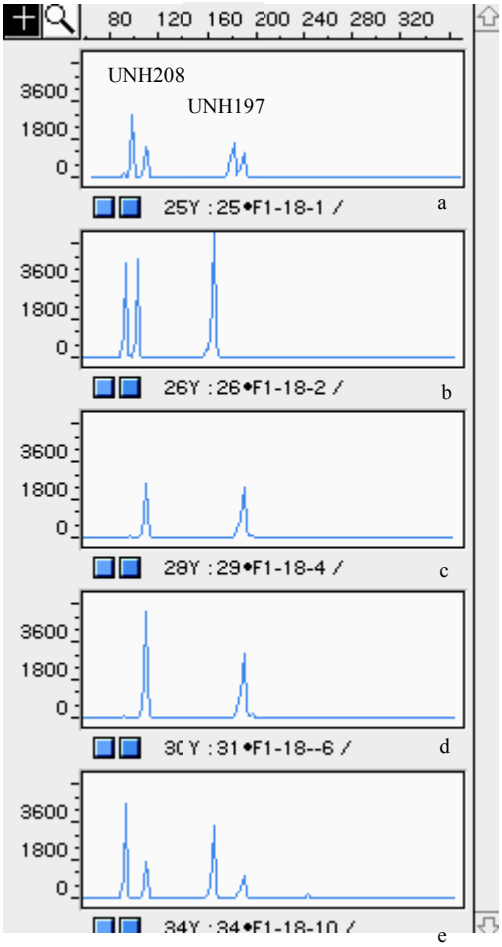


Fig. 5.3. Electropherogram generated by GeneScan™ (V. 2.1) software showing an example of a duplex PCR with UNH197 and UNH208 loci in *Oreochromis niloticus*. a: founder female of family 1; b: donor male; c-d: mitotic gynogenetic individuals and e: control.

5.3.1.3. Identification of meiotic gynogenetics

Six microsatellite loci were screened on two families (family 1 and 3) to identify and differentiate the pedigree of meiotic gynogenetics. This experiment was designed to look for loci which could discriminate between mito- and meio- gynogenetics through high gene-centromere recombination rates in the meiotic gynogenetics (close to 100%, like

*ADA** locus in *O. niloticus*; Hussain *et al*., 1994a). The results are presented in table 5.9. An example of GeneScan™ electropherogram is presented in Fig. 5.4.

The allelic inheritance was scored for all six loci in a panel comprising founder female, donor male and randomly selected meiotic gynogenetic individuals. First a panel comprising founder female, donor male, seven randomly selected meiotic gynogenetic individuals, several mitotic gynogenetics and controls was screened for all six loci. The data were analysed and another round of screening was performed with more individuals but with only those loci that generated a high level of heterozygosity among the meiotic gynogenetic progeny.

Table 5.9. Observed allele size range and percent heterozygosity generated by six microsatellite loci in two families (family 1 and 3) of meiotic gynogenetic *Oreochromis niloticus*; (n): total number of individuals screened; * homozygous.

Loci	Observed alleles (bp) in founder XY neo♀ and XX donor neo♂			Number of heterozygous meiotic gynogenetics		% Heterozygosity Observed		average
	♀ (10FE)	♀ (3EE6)	♂ (817)	♀ (10FE)	♀ (3EE6)	♀ (10FE)	♀ (3EE6)	
UNH 127	124/124*	124/154	126/128	0 (7)	5 (7)	0	71	n/a
UNH 189	153/168	158/168	153/178	17 (18)	12 (12)	94	100	97
UNH 197	181/190	156/181	166/166*	17 (18)	3 (7)	94	43	68.5
UNH 203	83/116	86/116	83/89	17 (18)	1 (7)	94	14	54
UNH 208	100/111	100/103	93/103	1 (7)	3 (7)	14	43	28.5
UNH 211	133/136	130/136	126/136	18 (18)	5 (7)	100	71	85.5

The observed heterozygosity generated by all six loci tested in the two families ranged from 0-100% in family 1 and 14-100% in family 3. All the meiotic gynogenetic progeny were heterozygous in family 1 when screened at UNH211. A high percentage of heterozygosity (94%) was also observed in meiotic gynogenetic progeny in this family when screened at UNH189, UNH197 and UNH203. All the meiotic gynogenetic

individuals in this family were found to be homozygous at UNH127 as this locus was homozygous in the founder female.

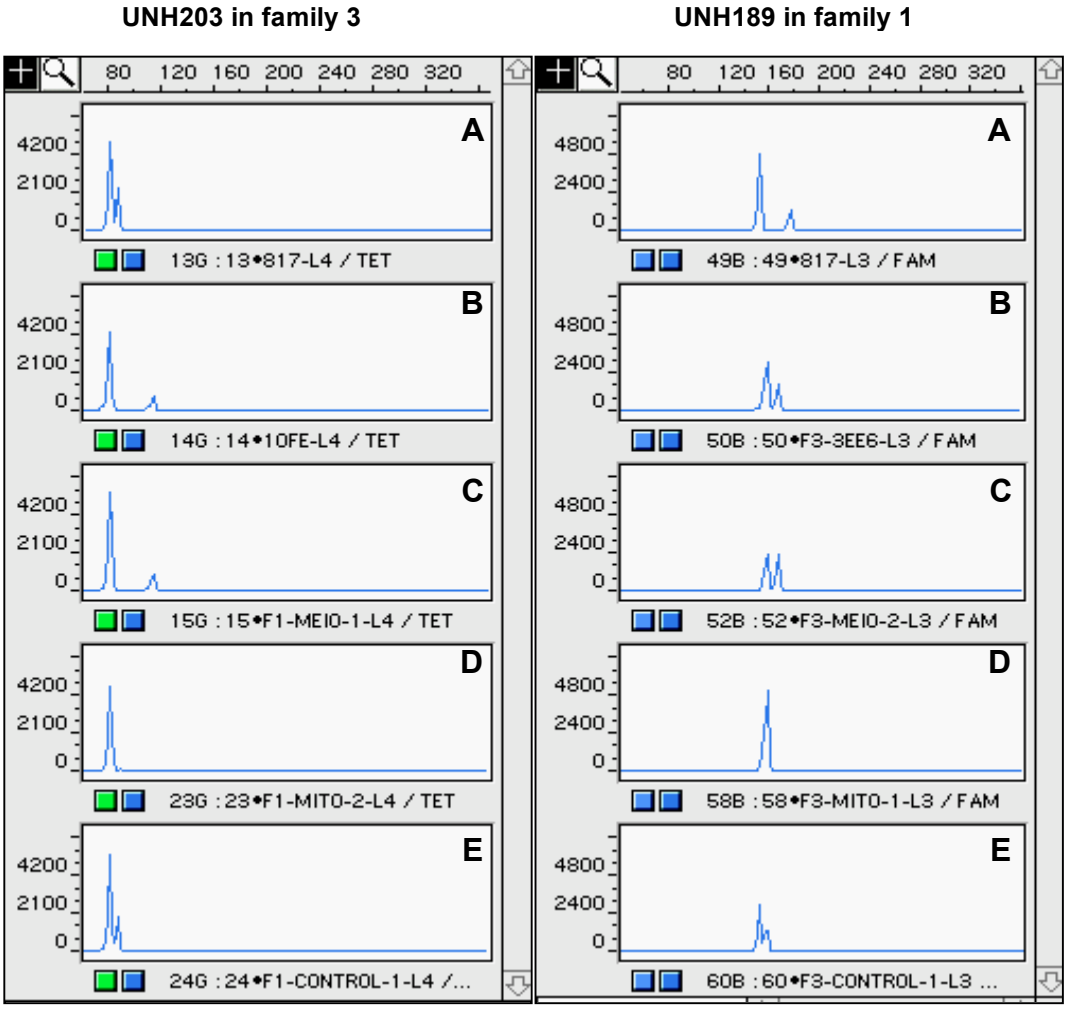


Fig.5.4. Electropherogram generated by GeneScan™ (V. 2.1) software showing the heterozygosity of meiotic gynogenetics of *Oreochromis niloticus* for UNH203 and UNH189 in family 3 and 1 respectively. a: donor male; b: founder female; c: meiotic gynogenetic individual; d: mitotic gynogenetic individual and e: control.

In the case of family 3, UNH189 revealed 100% heterozygosity among the meiotic gynogenetic individuals. High levels of heterozygosity (71%) were also observed at UNH127 and UNH211. In this family only one out of seven meiotic gynogenetic individuals tested was found to be heterozygous at UNH203 (Table 5.9).

The observed average heterozygosity for all the six microsatellite loci among the two families of meiotic gynogenetics ranged from 54 to 97%. The highest average heterozygosity was observed at UNH189. UNH127 showed 71% heterozygosity in family 3, while all the meiotic individuals screened for that locus were homozygous in family 1 due to the homozygous condition of that locus in the founder female (Table 5.9).

5.3.2. AFLP fingerprinting and parentage

Twelve out of sixty four AFLP primer combinations (Table 3.4 and Table 5.10) were screened for parentage analysis in two families (1 and 4) of *Oreochromis niloticus* involved in the production of mitotic gynogenetics. Interpretations of AFLP fingerprints were based on the absence of donor male-specific bands (diagnostic bands) in founder females as well as in the gynogenetic progeny. Founder female-specific bands which were absent in the donor male have not been considered, because the absence of a band in some progeny could represent the product of a maternal locus in which the founder female was heterozygous (Haley, 1991).

Four of these 12 AFLP primer combinations were common among the two families and each gynogenetic family was screened for eight AFLP primer combinations. A panel of individuals comprising founder female, donor male and randomly selected 10 putative gynogenetic and 20 control progeny was screened for the AFLP primer combinations described. The results of AFLP screening are summarized in Table 5.10 and an example GeneScan™ electropherogram is presented in Fig. 5.5.

The frequency of diagnostic bands ranged from 1-3 per primer combination in both the families. In total 1,387 bands were generated by the 12 AFLP primer combinations and 26 diagnostic bands were screened, revealing the success of gynogenesis in both families. No diagnostic polymorphisms were observed for primer combination D7 in family 1 and H1 in family 4 (Table 5.10).

Table 5.10. Paternal inheritance of AFLP markers in two families of mitotic gynogenetic *Oreochromis niloticus*.

Gynogenetic Family number	Primer combination	Total number of bands	Inheritance of donor male specific markers in progeny					
			number	size (bp)	Gynogenetic (n = 10)		Control (n = 20)	
					present	absent	present	absent
1	A2	116	2	350	0	10	8	12
				368	0	10	20*	0
	A7	83	2	307	0	10	20*	0
				365	0	10	20*	0
	C6	82	1	429	0	10	7	13
	D7	100	0	-	-	-	-	-
	E2	80	3	223	0	10	13	7
				241	0	10	16*	4
				246	0	10	5	15
	E6	70	3	215	0	10	20*	0
				230	0	10	10	10
				345	0	10	10	10
	H7	86	1	309	0	10	13	7
	H8	98	2	195	0	10	8	12
				232	0	10	10	10
	Family1 total	715	14					
4	A7	87	1	292	0	10	11	9
	B1	88	1	61	0	10	13	7
	B2	101	1	305	0	10	8	12
	C6	79	3	294	0	10	7	13
				298	0	10	18*	2
				429	0	10	13	7
	D4	72	1	183	0	10	11	9
	E6	61	3	230	0	10	12	8
				298	0	10	20*	0
				345	0	10	8	12
	H1	93	0	-	-	-	-	-
	H8	91	2	195	0	10	8	12
				232	0	10	10	10
	Family4 total	581	12					
Grand total (F1 + F4)		1387	26					

* significantly different from 1 : 1 inheritance ratio

In total, 715 and 581 AFLP markers were generated by eight different AFLP primer combinations in families 1 and 4 respectively. Among them 14 diagnostic AFLP bands were detected in family 1 and 12 diagnostic AFLP bands were detected in family 4 which successfully discriminated the gynogenetic fish in these two families. The highest

numbers of diagnostic bands (3) were generated by AFLP primer combinations E2 and E6 in family 1 and C6 and E6 in family 4.

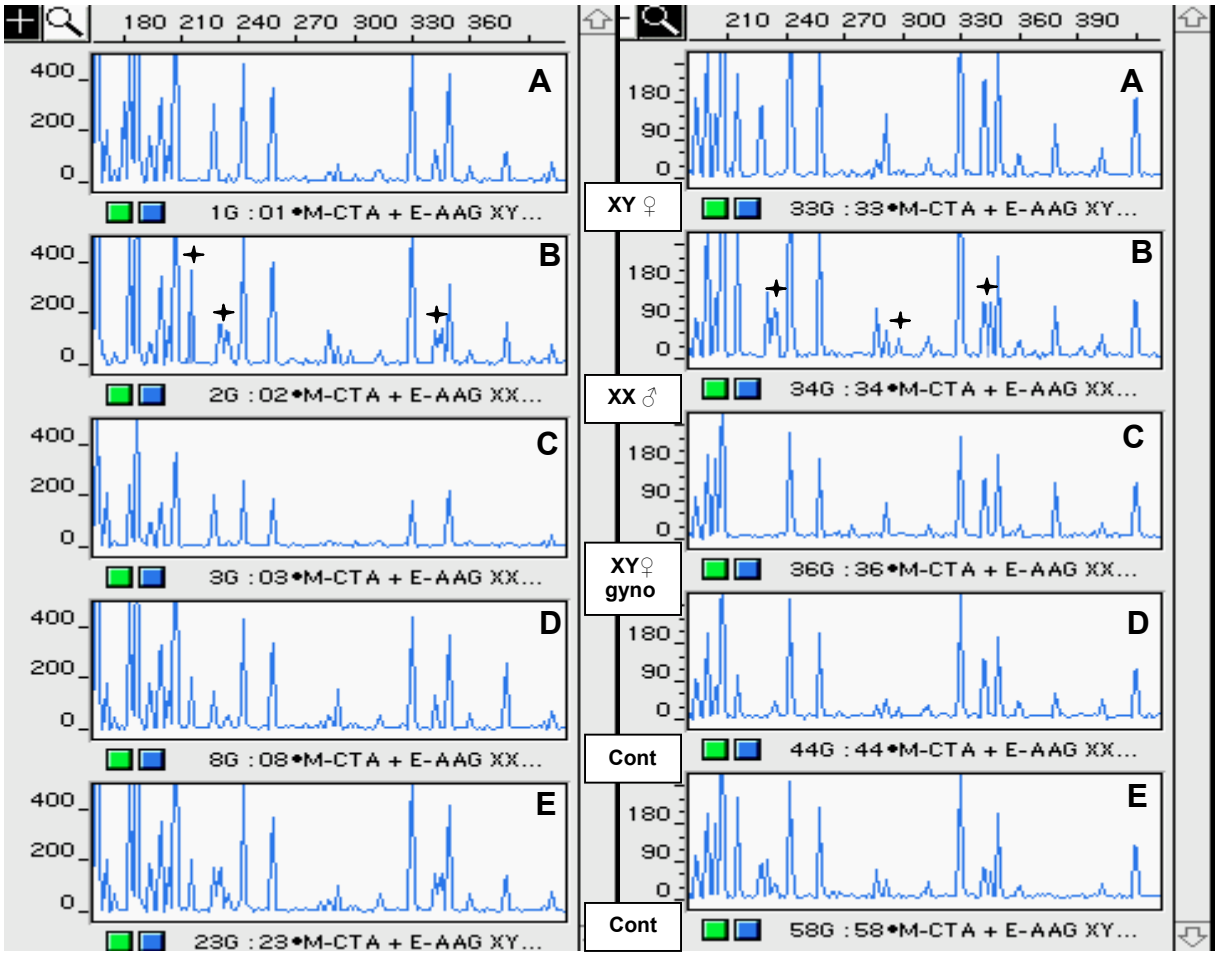


Fig. 5.5. Electropherogram generated by GeneScan™ (V. 2.1) software showing an example of fingerprint pattern produced from two different AFLP primer combinations for evaluating gynogenetic status in *Oreochromis niloticus*. A: XY founder mother; B: XX donor father; C: XX gynogenetic female; D and E: control fish. ✦ showing the male specific diagnostic bands.

Ten randomly selected gynogenetic individuals were screened in each family for eight AFLP markers. In addition, the AFLP primer combination E6 was also screened on 26 and 32 new mitotic gynogenetic individuals in families 1 and 4 respectively. No paternal inheritance was observed in any of these gynogenetic progeny, i.e. male specific

diagnostic bands were absent in all the gynogenetic individuals. This result also suggested 100% success of the gynogenesis procedure in both families (Table 5.10).

The segregation pattern of donor male specific diagnostic markers in families 1 and 4 is presented in Table 5.10 and the frequency distribution of the presence of all 26 AFLP diagnostic markers in the controls of both gynogenetic families are presented in Fig. 5.6.

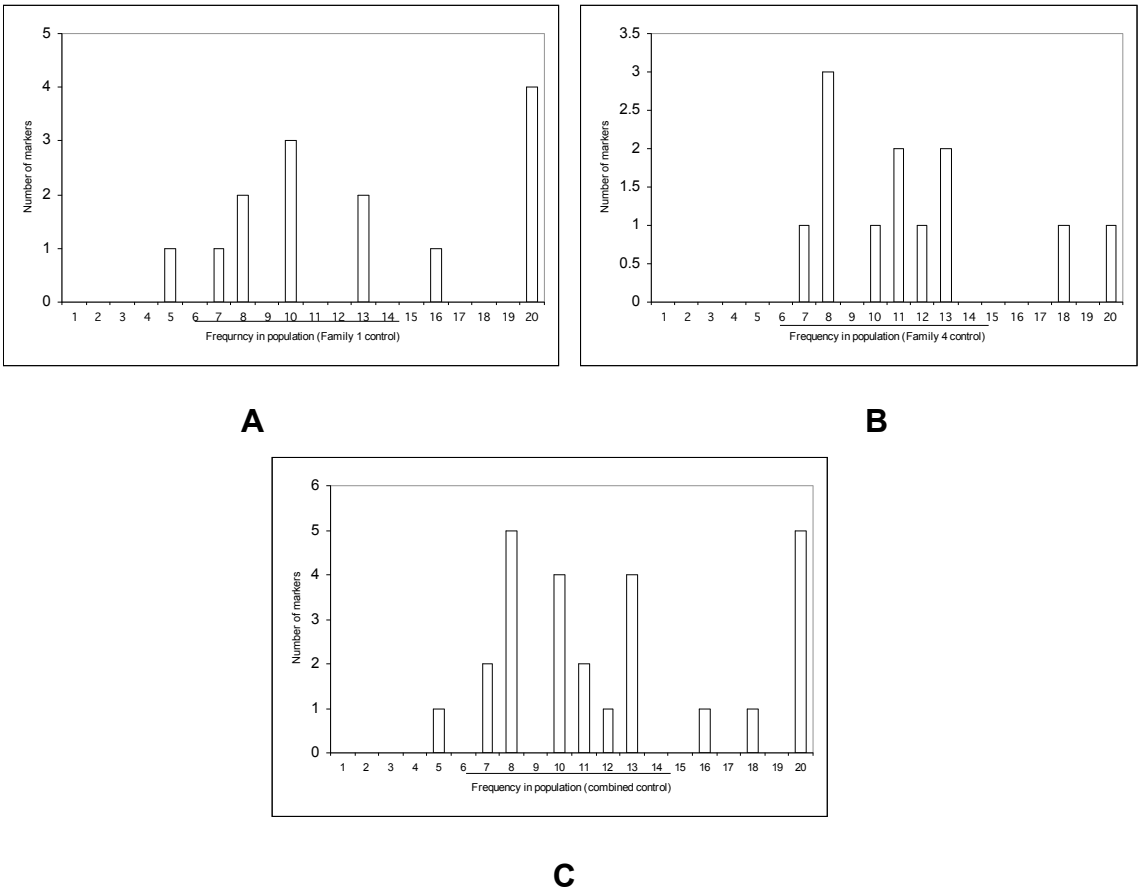


Fig. 5.6. Frequency distribution of the presence of AFLP donor male specific diagnostic markers in the control group; A: Family 1; B: Family 4; C: Combined. Underlined shows the range of heterozygosity ($P < 0.05$).

The presence of all 26 donor male specific diagnostic AFLP markers tested in the control groups for the two gynogenetic families revealed a percent frequency range from 25-100% (Fig. 5.5). Four markers were found to be present in all control progeny of

family 1 while one marker was present in all control progeny of family 4 (Fig. 5.6). However, no significant ($P < 0.05$) deviation from 1:1 inheritance ratio was observed for the rest of the markers among the control progeny in both gynogenetic families (Table 5.10).

5.4. Discussion

5.4.1. Parentage, gynogenesis and microsatellites

Parentage assignment can be achieved with any type of genetic marker provided that it is sufficiently polymorphic and for that reason microsatellites have been preferred to isozymes (Gerber *et al.*, 2000). Although several studies have been conducted using polymorphic microsatellites for paternity analysis in both plants (Dow and Ashley, 1998; Streiff *et al.*, 1999) and animals (Moran and Garcia-Vazquez, 1998; O'Reilly *et al.*, 1998), few studies have analyzed parentage assemblage in genetically manipulated populations (Galbusera *et al.*, 2000; Peruzzi and Chatain, 2000).

The polymorphic microsatellite loci used in this experiment verified gynogenetic inheritance in *O. niloticus*. The observed level of heterozygosity for all loci used in this experiment were different from those previously reported (Sobolewska, 1999; Kocher: personal communication). This may be due to the limited sample size in these experiments. However, a sufficient level of heterozygosity was observed to fulfil the requirements of the present study. Most earlier experiments concerning the inheritance in genetically manipulated fish have involved the application of multilocus DNA fingerprinting with various minisatellite probes, randomly amplified polymorphic DNA or analysis with enzymatic markers (e.g. Carter *et al.*, 1991; Hussain *et al.*, 1993; Sarder *et al.*, 1999; Volckaert *et al.*, 1994; Van Eenennaam *et al.*, 1996). However, Galbusera *et al.* (2000) and Peruzzi and Chatain (2000) successfully used polymorphic microsatellite loci to verify maternal inheritance in gynogenetic populations of African catfish and European seabass respectively.

The multiplexing PCR involving two, three and four loci was also successful in revealing the success of gynogenesis in four families. Multiplexing PCR is often difficult, requiring specific primer design and optimization of conditions (Neff *et al.*, 2000). Since its initial description (Chamberlain *et al*., 1988) PCR multiplexing has been applied in a wide variety of applications in many different organisms including human, to study mutations (Shuber *et al*, 1993), deletion analysis (Henegariu *et al*. 1994), polymorphisms (Mutirangura *et al*., 1993; Rithidech *et al*., 1997), genetic analysis (Wenburg *et al*., 1996; Leneuve *et al*., 2001), population and parentage (Herbinger *et al*., 1995; Fishback *et al.*, 1999; Shikano and Taniguchi, 2002) and pathology (Alegre *et al*., 2001; Buerger *et al*. 2001). However, in the present study, based on the single locus optimization in gynogenetic founder animals, duplex, triplex and tetraplex PCR using microsatellites was successful for all cases. The interpretation of the data, particularly in tetraplex reactions, was often complex, with one of the main problems being the overlapping of two alleles of equal sizes labelled with two different ABI dyes. However, earlier information about each locus obtained from the single locus optimization was used as a guide to resolve this problem.

Multiplex PCRs with two, three and four loci in four different gynogenetic families were successful. No paternal transmission was observed in any of the gynogenetic individuals, as observed by Galbusera *et al.* (2000) and Peruzzi and Chatain (2000). In the present experiment, no heterozygosity was observed in any microsatellite loci screened among mitotic gynogenetic progeny, a phenomenon that has also been reported in putative mitotic gynogenetic African catfish (Galbusera *et al.*, 2000).

As only a small number of individuals were screened to study the maternal inheritance among the gynogenetic and control individuals, the inheritance of maternal alleles did not always closely fit to the Mendelian ratio in both the gynogenetic and control groups. However, the overall results confirmed that microsatellite markers can be used to monitor the success of genetic manipulation in *O. niloticus*.

It is well established that induced meiotic gynogenesis produces a proportion of heterozygous individuals due to crossing over occurring during meiosis between a gene and its centromere. Discrimination of meiotic from mitotic gynogenetic individuals is important and necessary. For this purpose, it is necessary to choose suitable genetic marker(s) which have the following basic criteria. The locus or loci must be located distal to the centromere with comparatively high recombination frequency and must be heterozygous in the founder parent. Allelic variation between the founder and donor parents must also be present. To find a genetic marker with such criteria is often difficult. However, a heterozygous donor parent with one common allele or a donor parent homozygous for one allele present in the founder parent can also be considered where no other suitable markers are available.

In the present experiment, six polymorphic microsatellite loci were screened singly in two families to determine if these loci could discriminate meiotic from mitotic gynogenetic individuals. Two microsatellite loci (UNH211 in family 1 and UNH189 in family 3) were observed to produce 100% heterozygosity among the meiotic progeny, suggesting high rates of recombination for those two loci. High rates of recombination were also observed at three other loci (UNH189, UNH197 and UNH203) in family 1 and two loci (UNH127 and UNH 211) in family 3 (Table 5.9).

The highest average rate of recombination was observed at UNH189. UNH211 also showed a high average recombination frequency. Although UNH127 showed a reasonable rate of recombination in family 3, this locus generated all homozygous meiotic gynogenetics in family 1, demonstrating the homozygosity of that locus in the founder female. Galbusera *et al.* (2000) used microsatellite loci in a similar study and observed 71-86% recombination at two microsatellite loci in meiotic gynogenetic progeny of African catfish.

Earlier Hussain *et al.* (1994a) estimated the recombination of several isozyme loci in a genetically manipulated populations of *O. niloticus*, and observed 100% recombination rates at the *ADA** locus. Due to this high recombination frequency, the *ADA** locus has been successfully used to discriminate meiotic from mitotic gynogenetics in this species (Sarder *et al.*, 1999; Karayucel, 1999).

The overall versatility of microsatellites clearly indicates their general superiority over other forms of genetic markers. Compared to allozymes, microsatellites are a non-lethal technique, allowing selection and preservation of parental types and the maintenance of progeny for further investigation. When compared to the Southern hybridization-based multilocus fingerprinting techniques, PCR-based microsatellites are more sensitive, require less DNA and are quicker. For each cross, microsatellites do not require identification of parent-specific bands as is the case when using RAPD markers, and therefore, direct identification of parent-specific alleles is possible without prior progeny testing (Ferguson *et al.*, 1995; Peruzzi and Chatain, 2000). Microsatellites also have high reproducibility (Lynch and Milligan, 1994).

In summary, the application of polymorphic microsatellite loci for the parentage analysis of genetically manipulated population of *O. niloticus* was able to verify the success of this technique. Once optimized, the approach of amplification of several microsatellite loci in multiplex PCR was also found to be beneficial considering the time, efforts and costs. Although no single microsatellite locus consistently showed 100% gene-centromere recombination, when >1 loci with high levels of recombination (>75% heterozygosity) are used as a multiplex PCR, they could be used to study parentage in normal and genetically manipulated populations, as well as to discriminate meiotic from mitotic gynogenetics.

5.4.2. Parentage, gynogenesis and AFLPs

The AFLP fingerprinting technique as described by Vos *et al.* (1995) was successful in analysing the success of gynogenesis in *O. niloticus*. Since its description, this technique has been widely adopted to generate fingerprints in both eukaryotes and prokaryotes. In the present experiment, ten out of twelve primer combinations generated polymorphisms which were able to detect parental inheritance in two different families of mitotic gynogenetic *O. niloticus*. These primers usually generated between 61 and 116 AFLP bands. However, the number of donor male-specific diagnostic markers was low, only 1-3 diagnostic markers for each primer combination. Lower numbers of diagnostic markers reflect the low level of genetic variation between the parents. However, it is very difficult to interpret this low variation without large-scale population screening with this marker system. Although a low number of diagnostic markers were detected in this experiment, they efficiently detected the gynogenetic individuals.

AFLP fingerprinting usually generates many different highly polymorphic loci; therefore it is difficult to determine which alleles belong to which loci. Thus zygosity at a particular locus usually cannot be determined. However, the zygosity conditions of donor male-specific diagnostic markers can be inferred by analysing their segregation among control individuals. In the present experiment, five out of 26 diagnostic markers showed 100% inheritance among the control progeny, indicating the homozygotic conditions of those loci in the donor males.

Therefore, in summary it can be stated that the AFLP technique can be adopted as an efficient tool for parentage analysis in genetically manipulated populations. In *O. niloticus*, this technique was successful in assessing the success of gynogenesis. Although for this experiment the broodstock were not screened prior to the experiment, such screening could be of help in identifying primer combinations with high numbers of polymorphisms (although difficult in inbred laboratory populations), allowing screening of more individuals by reducing time and effort.

In the present experiment both AFLPs and polymorphic microsatellite loci were efficient in parentage assignment in both normal and gynogenetic progeny of *O. niloticus*. However, present experiment confirmed again that microsatellite loci are more appropriate since microsatellites are codominant markers, so all genotypes can be observed, unlike AFLPs which tend to have a dominant expression. In addition, some microsatellite loci showed a high level of gene-centromere recombinations suitable for discriminating meiotic from mitotic gynogenetics.

Chapter 6

Spontaneous Diploidization Of The Maternal Chromosome Set In *Oreochromis niloticus*

6.1. Introduction

Spontaneous diploidization of the maternal chromosome set (SDM) is a rare, but well known natural event in fish and has been found to occur in a number of species. Several species from the family Poeciliidae and some populations of crucian carp (*Carassius auratus*) reproduce naturally by SDM. In general, SDM is the mechanism by which all-female populations are produced. In naturally occurring all-female populations, males from another species commonly act as sperm donors, triggering the fertilization of the eggs without contributing genetic material to the zygote (reviewed by Gold, 1979; Cherfas, 1981; Thorgaard, 1983).

Among non-SDM species, this phenomenon can be detected in artificial gynogenesis experiments by the appearance of normal diploid progeny among the haploid control group in which no diploidization treatment has been applied. The occurrence of such spontaneous normal diploid embryos among gynogenetic haploids, produced after fertlization of intact eggs with genetically inactivated sperm, is extremely rare (Cherfas *et al.*, 1995).

The SDM phenomenon has also been reported in a number of wild populations of pure species as triploids, among laboratory hybrid progenies and also in gynogenetic experiments in triploid rainbow trout (Cuellar and Uyeno, 1972), triploid California roach (Gold and Avise, 1976), coho salmon and brook trout hybrids (Uyeno, 1972), in hybrids within the Pleuronectidae (Purdom and Lincoln, 1974), hybrid and gynogenetic grass carp and common carp (Stanley, 1976), the hybrid between *Ctenopharyngodon idella* and *Hypophthalmichthys nobilis* (Marian and Krasznai, 1978), in gynogenesis of common carp (Nagy *et al.*, 1978; Hollebecq *et al.*, 1986; Linhart *et al.*, 1986) and in

plaice (Thompson *et al.*, 1981). High rates of SDM have also been detected by the appearance of a high proportion of triploid fish in hatchery stocks of rainbow trout and tench (Thorgaard and Gall, 1979; Flajshans *et al.*, 1993) and high proportions of spontaneous diploid embryos in gynogenetic haploid progeny of ornamental (koi) common carp (Cherfas *et al.*, 1991).

Several hypotheses have been proposed regarding the diploidization of maternal chromosome sets in a population that normally shows natural reproduction. Cherfas *et al.* (1995) suggested that some accidental internal or environmental factors (e.g. egg over-maturation, sudden temperature changes) might affect the process of egg fertilization, causing diploidization of maternal chromosome sets, which could be the result from the suppression of the second meiotic division. This hypothesis has been partly confirmed by another study involving the genetic analysis of naturally occurring gynogenetic plaice (Thompson *et al.*, 1981). For some other cases genetic predisposition to diploidization of maternal chromosome sets has been suggested, but no strong evidence has been presented (Cuellar and Uyeno, 1972; Thorgaard and Gall, 1979; Flajshans *et al.*, 1993).

6.1.1. Identification of SDM progeny

The SDM progeny can be identified in the same way as identification of maternal inheritance in induced gynogenetic progeny. As described in Chapters 2 and 5, external morphology, biochemical and molecular markers have been used in several fish species to identify individuals of maternal origin, such as external morphological markers in rainbow trout (Chourrout, 1980) and zebrafish (Streisinger *et al.*, 1981), biochemical

markers such as allozyme polymorphisms in rainbow trout (Purdom *et al.*, 1985), in Nile tilapia (Mair *et al.*, 1987; Hussain *et al.*, 1993; Müller-Belecke and Hörstgen-Schwark, 1995; Sarder *et al.*, 1999; Karayucel, 1999) and in loach (Suwa *et al.*, 1994). DNA level polymorphisms have been used to identify maternal inheritance in tilapia (Carter *et al.*, 1991; Sarder *et al.*, 1999; Jenneckens *et al.*, 1999; Karayucel, 1999), in African catfish (Galbusera *et al.*, 2000) and in seabass (Peruzzi and Chatain, 2000; Felip *et al.*, 2000).

6.1.2. Objectives

This chapter reports for the first time the spontaneous diploidization of the maternal chromosome set (SDM) in *O. niloticus*, and verification of this by multilocus fingerprinting using Jeffreys' (Jeffreys *et al.*, 1985a) human derived 33.15 minisatellite probe. This chapter will also discuss the survival and sex ratios of SDM fish in *O. niloticus*.

6.2. Materials and methods

6.2.1. Fish

Fish stocks used were as described in Chapter 2. Eleven XY neofemale *O. niloticus* were used as broodstock in this experiment. They were collected from the XY neofemale stock produced and maintained in the re-circulating tropical aquarium facility of the Institute of Aquaculture, University of Stirling. At the beginning of the experiment they were brought to the spawning facility from the stocking unit. General management, maintenance and spawning techniques were as described in Chapter 2.

6.2.2. SDM progeny

In the present experiment, SDM fish were produced as a by-product of induced gynogenesis experiments as described in Chapter 2 from the UV control groups. Eggs from XY neofemales were fertilized with UV irradiated sperm collected from XX neomales. The UV-irradiation technique to inactivate sperm genetic material was performed as described in Chapter 2. Females were hand stripped and eggs were divided into three batches. One batch of eggs was fertilized with non-irradiated sperm as a control group, the second batch was fertilized with UV irradiated sperm and the third batch was also fertilized with UV irradiated sperm and subjected to heat shock for induced diploid gynogenesis (described in Chapter 2).

After fertilization eggs were incubated in a temperature controlled (27±1°C) re-circulatory incubation unit. Fertilization and survival of the embryos were checked at pigmentation, hatching and yolk sac absorption stages. After yolk sac absorption, the fry

were released to the rearing tanks. After one month of rearing, fry were transferred to larger tanks for growing (described earlier in Chapter 2).

The sex ratios of the control group were obtained by sexing fish using gonad squashing and aceto-carmine staining (Guerrero and Shelton, 1974) at 8-12 weeks post fertilization. The SDM progeny were reared to maturity and phenotypic sex was determined, based on external morphology of the genitalia.

6.2.3. Sample collection for DNA extraction

Blood samples were collected for DNA extraction from the control group by cutting the caudal peduncle of freshly killed fish and collecting blood in a sterile tube containing Cortland's-EDTA. From the SDM progeny, a 0.2-0.8 ml blood was collected from the caudal vein using a 1 ml syringe with a 25G X 5/8″ needle, with Cortland's-EDTA used as an anti-coagulant. After collection, samples were centrifuged briefly, the supernatant was removed and the tube was immediately dipped into a flask containing liquid nitrogen. Blood samples were then kept at -20°C. The same procedures were followed for DNA extraction, quantity and quality assessment as described in Chapter 2.

6.2.4. Multilocus DNA fingerprinting

Multilocus DNA fingerprinting was performed by digestion of high molecular weight total genomic DNA with the *Hinf*I endonuclease, Southern transfer on to nylon membranes and subsequent hybridization with Jeffreys' 33.15 human-derived minisatellite (Jeffreys *et al.*, 1985a) probe. This protocol was performed as described in Chapter 2.

6.2.5. Chromosome karyotyping

Both newly hatched or one-day-old, and blood from the adult fish were used for karyotyping of metaphase chromosome spreads.

6.2.5.1. From newly hatched larvae

Newly hatched embryos were first transferred to a Petri dish containing 0.002 – 0.005% colchicine solution and left for 4-6 hrs at 25°C. After colchicine treatment, embryos were transferred to a Petri dish containing chilled 0.7% NaCl solution and the head and yolk sac were removed with a surgical needle. The tissues were then transferred to distilled water for 8-12 mins. Tissues were fixed in 3:1 methanol: acetic acid and maintained in fixative for between 30 mins and 6 weeks at 4°C.

For slide preparation the embryonic tissues were removed from the fixative and were placed in a cavity on a Perspex slide containing 2-3 drops of 50% glacial acetic acid. The tissues were ground with a glass rod for about one min. After ten minutes the cell suspensions were dropped from 30-40 cm height with a capillary tube onto a clean slide placed on a hot plate (45°C). After 8-10 seconds any remaining fluid was removed. Once dry, slides were removed from the hot plate.

Slides were stained with 10% Giemsa (prepare in 0.1 M phosphate buffer, pH 7.0) for 20 mins, rinsed in distilled water, air dried, and mounted with a cover slip using DPX (BDH, Ltd.). Metaphase chromosome spreads were examined using an Olympus binocular microscope.

6.2.5.2. From blood culture

Blood samples were collected from caudal vein following the procedure describe earlier, using a heparin treated syringe (rinsed with 4 mg/ml ammonium heparin). Whole blood was centrifuged for 5 mins at 1000 g. The Buffy coat (white cell layer) was removed and added to 10 ml of PB-max karyotyping medium (Gibco-BRL), gently mixed by inversion and incubated at 28º C for 4 days.

For chromosome preparation colcemid was added to a final concentration of 0.1 μg ml^{-1} (for 10 ml, add 100 μl of 10 μg/ml solution). The sample was incubated at 28º C for a further hr and then centrifuged at 250 g for eight mins and the supernatant was discarded (leaving the pellet as dry as possible). The pellet was resuspended in 10 ml of 75 mM KCl and incubated at 28ºC for 8-10 mins. One ml of fresh fixative (3:1 methanol:acetic acid) was added, the sample centrifuged at 400 g for 10 mins and the supernatant discarded. The pellet was resuspended very slowly in 10 ml of fixative (added drop by drop), centrifuged at 400 g for 8 mins and the supernatant discarded. This step was repeated at least another 3 times. Final resuspension was in a small volume of fixative and the quality was checked by making a slide as described in 6.2.5.1.

6.2.6. Statistical analysis

For analysis of survival rates Kruskal-Wallis tests were performed to compare the survival at different stages of embryonic development between the control and mitotic gynogenetics using Minitab (V. 13) statistical analysis software. To interpret the sex ratios, chi-square analysis was performed on expected ratios.

6. 3. Results

6.3.1. Survival and sex ratios of SDM and control *O. niloticus*

Eleven XY neofemale *O. niloticus* were used in this experiment. The SDM phenomenon was observed in progeny of only one female (00-013E-3EE6; see Chapter 2 for details of the broodstock). No significant differences ($P < 0.05$) in survival at the different stages of embryonic development were observed between the SDM and the diploid control group (Table 6.1.). The survival of SDM individuals and the control group at the yolk sac absorption stage ranged from 12-47%.

Table 6.1. Survival at different stages of development in SDM, diploid control groups and induced mitotic gynogenetics from *Oreochromis niloticus* XY neofemale 00-013E-3EE6; number of eggs fertlized are in parentheses.

Trials	% Survival at pigmentation			% Survival at hatching			% Survival at YSA		
	Control	Individuals with SDM	Mitotic Gynogenetics	Control	Individuals with SDM	Mitotic Gynogenetics	Control	Individuals with SDM	Mitotic Gynogenetics
1	62.0 (34)	62.0 (37)	41.0 (823)	56.0	57.0	8.01	47.0	47.0	5.22
2	64.0 (204)	53.0 (96)	12.0 (1599)	61.0	40.0	0.08	25.0	34.0	0.03
3	64.0 (183)	31.0 (154)	12.0 (781)	56.0	15.0	0.04	23.0	12.0	0.02
Mean	63.33	48.66	21.66	57.66	37.33	2.71	31.66	31.0	1.75
Stdev.	1.15	15.94	16.74	2.88	21.12	4.58	13.31	17.69	2.99

Mitotic gynogenetic individuals were also produced from the same female. However, significant ($P < 0.05$) differences in survival rates in all three different stages were observed among the mitotic gynogenetic, SDM and control groups (Table 6.1).

Sex ratios obtained from the control and SDM progeny are presented in Table 6.2. In both the control and SDM, sex ratios were significantly skewed towards males ($P < 0.05$). Among the SDM group 30 individuals were sexed phenotypically with only two females observed (Table 6.2).

Table 6.2. Sex ratios in control and SDM progeny of *Oreochromis niloticus*.

	Total no. of fish sexed	No. of females	No. of males	% female	% male	$\chi 2$ (1:1)
Control	143	50	93	35	65	12.93*
SDM	30	2	28	7	93	22.53*

* significantly different from 1:1 sex ratio (P<0.05).

The results from the observation of several (n=5 from each group) randomly selected individuals from both control and SDM groups revealed all diploid chromosome complements with 2n = 44. No triploid individuals were observed (Fig. 6.1).

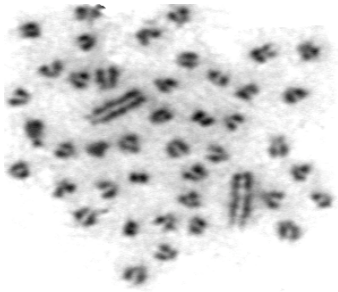


Fig. 6.1. Metaphase chromosome spread of a normal control *Oreochromis niloticus* individual showing diploid chromosome complement (2n = 44).

6. 3. 2. Multilocus DNA fingerprinting

Twelve randomly selected SDM progeny were tested to check inheritance using multilocus DNA fingerprinting. Jeffreys' 33.15 probe revealed only maternal inheritance in the SDM progeny, producing individual-specific multilocus fingerprints (Fig.6.2).

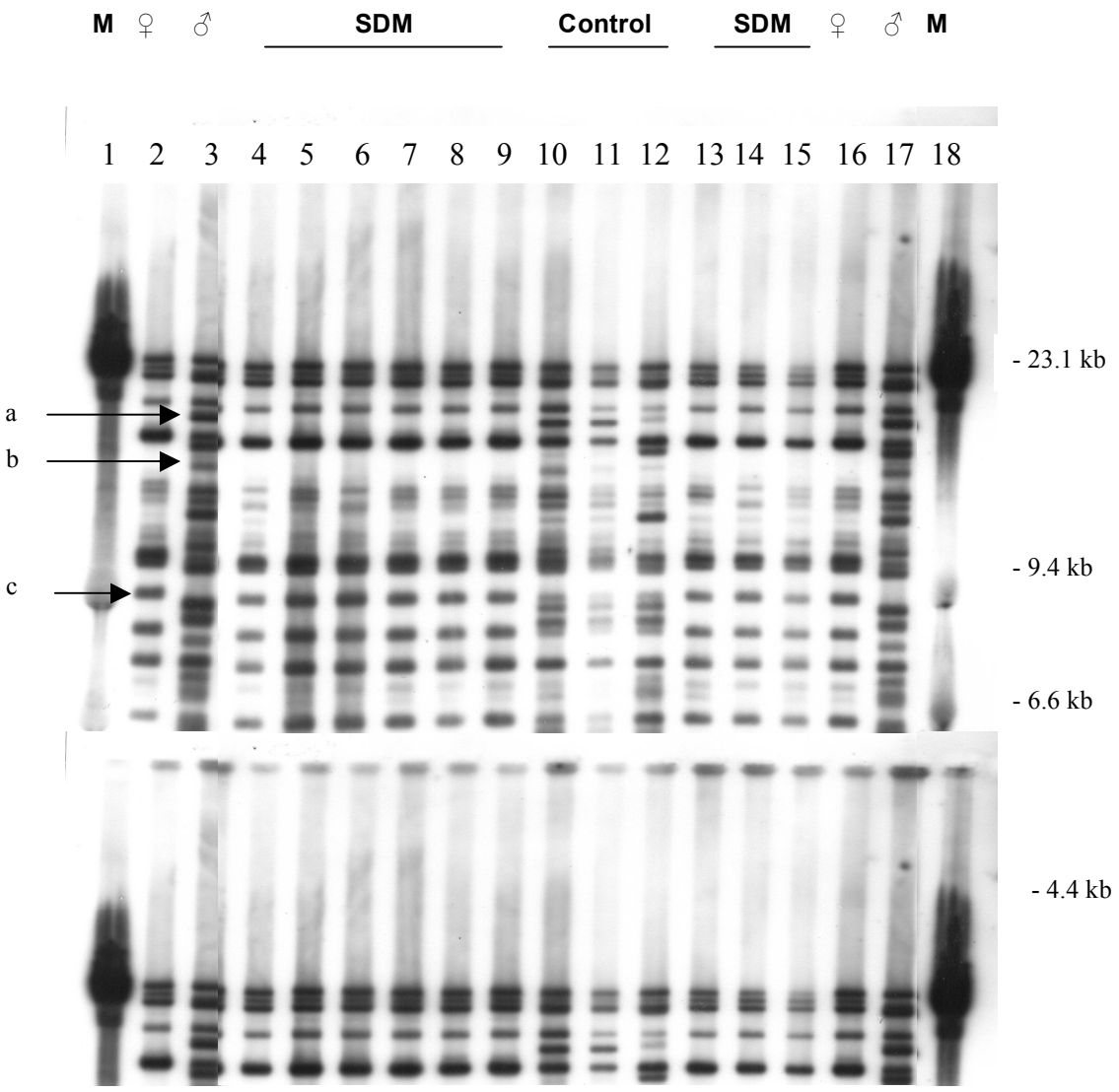


Fig. 6.2. Autoradiograph generated by Jeffreys' 33.15 probe for identification of SDM progeny in *Oreochromis niloticus*. Lane 1 and 18: λ DNA-*Hind*III digest size marker, 2 and 16: founder female; 3 and 17: donor male; 4-9 and 13-15: spontaneous gynogenetic individuals; 10-12: control individuals. a and b: examples of paternal-specific bands and c: a maternal-specific band.

All the SDM progeny tested by fingerprinting inherited only maternal-specific bands, with the absence of paternal-specific bands, indicating that SDM had occurred. In contrast, the control progeny inherited both maternal and paternal specific bands as expected.

6.4. Discussion

In the present experiment, the SDM phenomenon has been confirmed in *O. niloticus* by the appearance of diploid progeny from treatments which should have given only gynogenetic haploid fish. The evidence from the DNA fingerprinting also indicates inheritance of the maternal genome only among the SDM progeny.

Although the SDM phenomenon is a mode of reproduction in some species of fish with all-female populations, it has also been reported to occur in some bisexual species, but is normally rare with a frequency not exceeding 1-5% (Cherfas *et al*., 1995). However, in the present experiment, a high proportion of SDM progeny (mean 31% survival) was observed in one of the XY neofemale *O. niloticus*. Cherfas *et al.* (1991) observed 19% (absolute) SDM (intact eggs X genetically inactivated sperm) and 36% (absolute) in amphimictic triploid embryos (intact eggs X intact sperm) in a control progeny of one normal female ornamental (koi) carp. Thorgaard and Gall (1979) and Flajshans *et al.* (1993) also observed high rates of SDM by the appearance of high proportions of triploid fish in hatchery stocks of rainbow trout and tench respectively.

Based on the evidence of the published information, Cherfas *et al*., (1995) summarised three types of cytological transformations producing SDM in fish. They include: (i) premeiotic endoreduplication of the chromosome set followed by two meiotic divisions, (ii) suppression of the first meiotic division (ameiotic type of maturation) and (iii) suppression of the second meiotic division. The first two types of cytological transformation are normally displayed by the naturally occurring gynogenetic fish (e.g., the molly, *Poecilia formosa*). The process of endoreduplication is

also observed in some artificial interspecific hybridizations (e.g. hybrid between crucian carp and common carp).

It is very difficult to interpret the cytological cause behind SDM, i.e. does it happen due to the suppression of meiosis-I (endoreduplication) or meiosis-II. The earlier reports on SDM suggested the suppression of both meiosis –I and –II. In rainbow trout (Quillet *et al*., 1991) and in African catfish (Galbusera *et al*., 2000), SDM was proposed to be a consequence of the suppression of meiosis –I, however, in common carp Komen *et al*. (1991) suggested meiosis-II non-disjunction as the cause of SDM.

Segregation of marker genes in SDM progeny as well as the induced meiotic gynogenetic progeny were studied in plaice (Thompson *et al*., 1981) and in common carp (Cherfas *et al*., 1995) and suppression of meiosis –II was suggested as the probable reason of SDM. 100% heterozygosity was observed in five polymorphic protein genes in SDM of koi carp and was proposed to be produced due to high recombination of these genes and their centromere and by the suppression of meiosis-II (Cherfas *et al*., 1995). However, in a separate study, Galbusera *et al*. (2000) studied the segregation of several polymorphic microsatellite loci in a group of putative mitotic gynogenetic progeny. They observed high levels of heterozygosity among some of the individuals for one microsatellite locus and suggested that those individuals were produced spontaneously by the suppression of meiosis-I. They proposed that a high level of heterozygosity for a polymorphic locus would be expected if SDM occurred by the inhibition of meiosis-I without recombination. On the other hand if SDM occurred by the inhibition of meiosis-I after recombination, 50% heterozygosity would be expected.

The sex ratio of the *O. niloticus* SDM progeny was significantly skewed towards maleness. It is known that there is a high rate of recombination between the sex determining region and its centromere in *O. niloticus*. Mair *et al*., (1991a) observed 4.1% males in a progeny of meiogynogenetics produced from XX females. Thus it is expected that SDM (SDM ≡ meiotic gynogenetics, if produced by the suppression of meiosis-II) would give a high level of XY males.

From the present experiment we would predict:

Number of XX = number of ♀♀ = 2 (non-recombinants)
Number of YY = number of XX ♀♀ = 2 (non-recombinants)
Number of XY = number of ♂♂ (XY+YY) - number of ♀♀ (= number of YY)
= 28- 2 = 26 (recombinants)
Frequency of centromere - sex determining region recombination

$$= \frac{26}{30} \times \frac{100}{1} = 86.7\%$$

However, the slightly skewed male biased ratio among the control group could suggest that males showed higher survival than females, so the value calculated above may overestimate a skewed sex ratio.

The karyological experiment was conducted to determine the ploidy status in control and SDM progeny. However, this did not reveal any triploid progeny. Thus, no SDM mechanism was operating when eggs were fertilized with viable sperm. This female therefore only produced SDM progeny when eggs were activated with genetically inactivated sperm. The actual mechanism of SDM in this particular female remains unknown.

The present experiment reports for the first time the SDM phenomenon in *O. niloticus*. However, further investigations involving normal XX females are needed to

study this phenomenon in this species. The heritable nature of this phenomenon could also be studied by setting up experiments with the surviving YY SDM males.

Chapter 7

Linkage Between The Putative Autosomal Sex Reversal Locus And The Locus Controlling Red Body Colouration In *Oreochromis niloticus*

7.1. Introduction

The influence of autosomal gene(s) on sex determination has been hypothesised due to the occurrence of atypical males and females in populations with genetically defined sex determination systems (e.g. occurrence of XX males and XY females in an XX/XY male heterogametic system). Primary sex determination in mammals is mainly governed by the gene Sry (sex determining region Y gene) on the Y chromosome (Sinclair *et al.*, 1990; Koopman *et al.*, 1991; Nagai, 1996). However, several autosomal genes such as Sox9 (Sry related HMG box: Koopman *et al.*, 1991; Nagai, 1996), WT1 (Wilm's tumor 1 gene: Kreidberg *et al.*, 1993; Toyooka *et al.*, 1998), SF1 (steroidogenic factor 1: Luo *et al.*, 1994; Shen *et al.*, 1994), MIS (Mullerian inhibiting substance: Haqq *et al.*, 1994), SIPs (Sry interacting proteins: Zhang *et al.*, 1999) and the X chromosome linked gene DAX1 (Swain *et al.*, 1998; Goodfellow and Camerino, 1999) are also associated with sex determination. Most of these genes have been identified from analysis of XX males or XY females.

Sex determination in teleost fish is governed by a remarkable variety of environmental and genetic (sex chromosomal and autosomal) mechanisms (Baroiller *et al.*, 1999). Autosomal influence on sex determination has been proposed in a number of different fish, including several *Oreochromis* species to explain unexpected sex ratios observed in both inter- and intra-specific crosses (Avtalion and Hammerman, 1978; Kallman, 1984; Majumdar, 1984; Mair *et al.*, 1990, 1991a,b; Wohlfarth and Wedekind, 1991).

In addition to its popularity as a aquaculture species, for many years the Nile tilapia, *Oreochromis niloticus*, has been regarded as a good laboratory species for the study of

sex determination and differentiation. *O. niloticus* has an XX/XY sex determination system (Jalabert *et al.*, 1974; Mair *et al.*, 1991a; Carrasco *et al.*, 1999) but sex ratios quite frequently show departures from the expected (e.g. males in XX neomale x XX female crosses, or among gynogenetic progeny from XX females). Both environmental factors (Baroiller *et al.*, 1995; Abucay *et al.*, 1999; Kwon, 2000) and secondary genetic factors (Mair *et al.*, 1991a; Hussain *et al.*, 1994a; Sarder *et al.*, 1999; Karayucel, 1999) have been reported to influence sex determination and differentiation in this species, resulting in such deviations from expected sex ratios.

An autosomally (AA or aa) influenced, three gonosomal (W, X, Y where Y=Z) model of the sex-determining system was proposed on the basis of interspecific studies in different *Oreochromis* species (Avtalion and Hammerman, 1978; Hammerman and Avtalion, 1979). According to this model, *O. mossambicus* and *O. niloticus* were described as AAXX females and AAXY males, whereas *O. aureus, O. macrochir* and *O. urolepsis hornorum* were described as aaWY females and aaYY males. The assumption of this gene balance model was that if the sum effects of both the sex chromosomes and the autosomes exceeds a certain threshold, an individual would develop as a male, if not then as female (Hammerman and Avtalion, 1979). However, this model fails to explain a number of the sex ratios observed in intraspecific crosses.

The possibility of an autosomal, recessive, sex modifying gene, epistatic to the major sex determining factors W and Z has also been proposed in *O. aureus* to explain the sex ratios obtained in intraspecific crosses (Mair *et al.*, 1991b). From analysis of meiotic and mitotic gynogenetics in *O. niloticus*, the existence of an autosomal locus (*SDL-2* with two alleles *SR* and *sr*) epistatic to the gonosomal locus has been proposed, with this

locus causing female to male sex reversal when recessive homozygous (Hussain *et al.* 1994a). Later, Sarder *et al.* (1999) suggested partial penetrance of an autosomal sex reversal locus, while Karayucel (1999) suggested linkage of an autosomal sex reversal locus to the red colour locus in this species. However, the possible involvement of several autosomal sex modifying genes was also proposed in this species as a way to explain the occurrence of females in the progeny from crosses between YY males and YY females (Mair *et al.*, 1997). Mair *et al.* (1997) suggested that more than one autosomal modifying gene influences sex determination in this species.

The differences between the sexes in fish are frequently associated with body colour variations. The linkage of this attribute with sex has been studied in several species including platyfish, guppy and medaka (reviewed by Kallman, 1984; Purdom, 1993). Sex-linked colour variants were used to elucidate sex determination in the medaka (reviewed by Yamamoto, 1961). Interaction between the recessive autosomal body colour gene and the Y-linked body colour gene in guppies has also been reported (Phang *et al.*, 1999).

Most red tilapia strains were developed by hybridization using red mutants that originally occurred in *O. mossambicus* (Penman and McAndrew, 2000). In *O. niloticus*, in addition to the wild type body colour (grey) and a dominant red colour mutation, another mutant body colour termed 'blond' has also been reported (Scott *et al.*, 1987). No published report has been found regarding sex-linked inheritance of any of these three colour variants in *O. niloticus*. However, a recent report suggested genetic linkage of red body colour with a putative autosomal sex determining locus in this species (Karayucel, 1999).

The idea of the present study was based on several earlier studies conducted in the reproduction and genetics group at the Institute of Aquaculture (IoA) and various other earlier reports on atypical sex ratios in this species. This Chapter focuses on the possibility that the red body colour linked putative sex determining locus as reported by Karayucel (1999) is the same as the putative autosomal locus with partial penetrance proposed by Sarder *et al.* (1999).

7.1.1. Objectives

Sarder *et al*. (1999) and Karayucel (1999) independently described an autosomal locus with partial penetrance as an explanation of XX female to XX male sex reversal in gynogenetic and clonal lines of *O. niloticus*. The objectives of the present study were to follow up those experiments of Sarder *et al.* (1999) and Karayucel (1999) with a view to testing the following two alternative hypotheses:

Hypothesis A: The autosomal sex reversal loci identified by Sarder *et al*. (1999) and Karayucel (1999) are the same, with partial penetrance and linkage with the gene for red body colouration.

Hypothesis B: These are different, unlinked autosomal loci with partial penetrance, one of which is linked to the gene responsible for red body colouration.

7.2. Materials and methods

7.2.1. Experimental fish stock

The *O. niloticus* used in this experiment came from the Tilapia reference collection at the IoA, University of Stirling, Scotland, UK. Homozygous red females and spontaneously sex reversed clonal neomales were used in this experiment.

Homozygous red females were collected from different family groups in the stock maintained and propagated at the IoA tropical aquarium facilities. The origin of this red tilapia has been described by McAndrew *et al.* (1988). The spontaneously sex-reversed clonal neomales were collected from a separate stock of clonal *O. niloticus*. These clonal neomales (clonal line 002-046-539) were produced during an earlier project (Sarder *et al*., 1999). Spontaneous clonal neomales were tested for progeny sex ratios and their XX neomale status was identified and described by Sarder *et al.* (1999).

The brood fish were transferred from the stock system to the spawning facilities at the beginning of the experiment. The same procedure and techniques were followed for maintenance of brood stock, spawning, hatching and rearing as described in Chapter 2.

7.2.2. Experimental plan

Two spontaneously sex reversed wild type colour clonal neomales and three homozygous red *O. niloticus* females were used to produce six families of first (F1) generation progeny. A randomly selected sample from the F1 generation of each of the six families would be sexed at 12-16 weeks post-fertilization, using gonad squashing and aceto-carmine staining (Guerrero and Shelton, 1974). If this F1 generation produced all red females, then it would be assumed that the present females were RR and also did not

carry any recessive sex reversal alleles at the same locus/i as the clonal line neomales. The remaining fish from the F1 generation would be grown to maturity for the production of a backcross generation with the male parents to study the linkage of autosomal sex modifying gene(s) with body colour. Progeny from these crosses would also be sexed at 12-16 weeks post-fertilization as described earlier. If there were some males in at least some F1 crosses, it would be assumed that these red female parents were recessive homozygous or heterozygous at an autosomal sex determining locus/i. In this case, F1 progeny from these females would not be included in the present experiment to produce backcross progeny.

In this Chapter, two alternative hypotheses are tested. Hypothesis A is based on a single putative autosomal sex reversal locus. In the case of hypothesis A, S and s are denoted as two alleles of a putative sex reversal locus and R and r as the two alleles for the red body colouration locus in *O. niloticus*. In earlier reports SR and sr have been used for the putative sex-determining locus in the clonal lines used here (Sarder *et al*., 1999). However, in this chapter only S and s will be used instead of SR and sr to avoid confusion between the red body colour locus (alleles R and r) and the putative autosomal sex-determining locus. In the case of hypothesis B, two different autosomal sex determining loci, each with two alleles (one dominant and one recessive) are denoted by S1s1 and S2s2.

A schematic diagram of the possible genotypes expected to be generated from the present experiment under hypotheses A and B is presented in Fig. 7.1a,b. In Fig. 7.1a (hypothesis A, 7.1.1) the sex determining loci described by Sarder *et al*. (1999) and Karayucel (1999) are assumed to be the same locus (SS and ss in the present thesis) with

partial penetrance and linkage with red body colouration. In Fig. 7.1b (hypothesis B, 7.1.1) two autosomal sex determining loci (S1s1 and S2s2 in the present thesis) were considered (both with partial penetrance), one of which is linked to the red body colour gene(s).

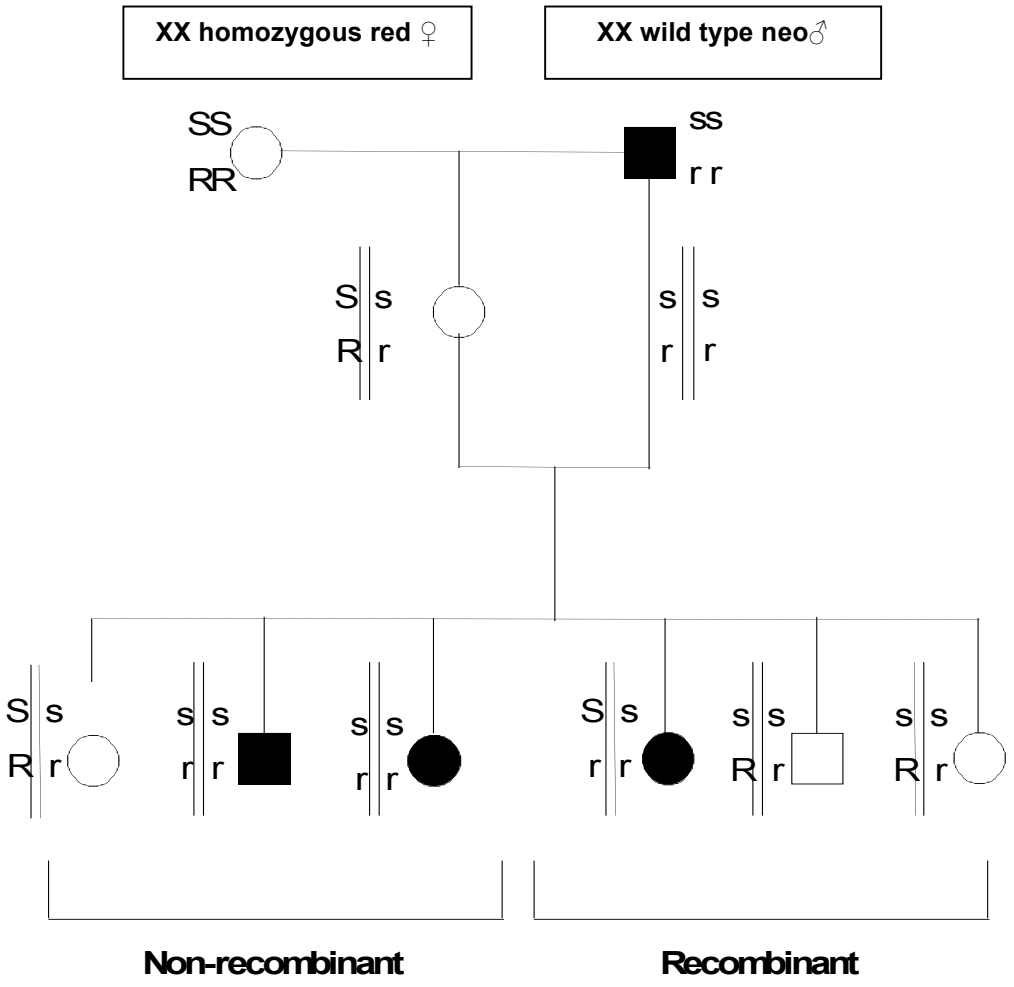


Fig. 7.1a. Schematic diagram of possible genotypes and phenotypes resulting in the backcross generation using XX SsRr females with XX ssrr spontaneously sex-reversed clone neomales in *Oreochromis niloticus* (Hypothesis A, 7.1.1); assumes partial penetrance, here ss generates both male and female phenotypes.

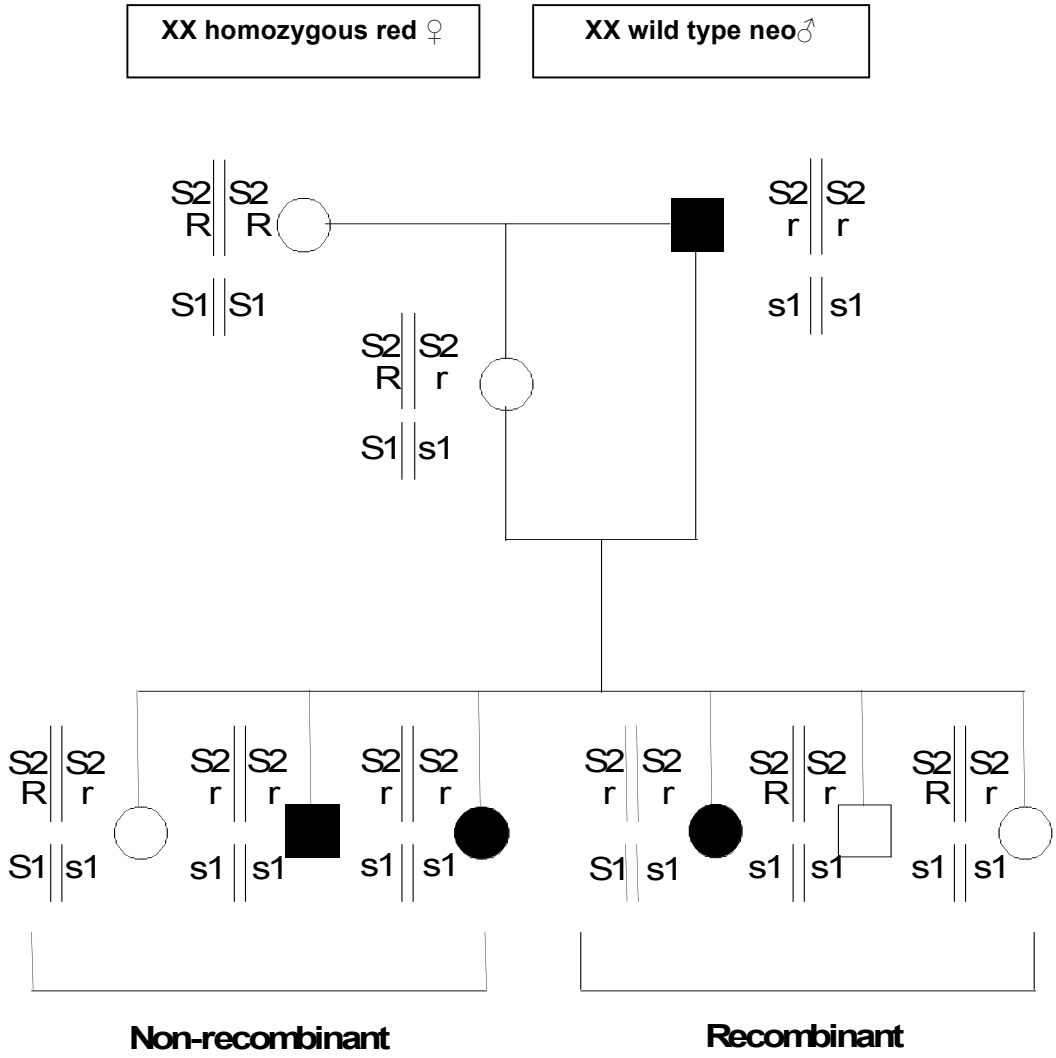


Fig. 7.1b. Schematic diagram of possible genotypes and phenotypes resulting in the backcross generation using XX S2S2S1s1Rr females with XX S2S2s1s1rr spontaneously sex-reversed clone neomales in *Oreochromis niloticus* (Hypothesis B, 7.1.1); assumes partial penetrance, here s1s1 generates both male and female phenotypes.

Four different genotypic combinations were expected under Hypothesis A from backcrossed generations produced by crossing F1 generation females (XXSsRr) to spontaneously sex reversed clonal neomales (XXssrr). The frequencies of occurrence of

these genotypes would depend on recombination between the putative autosomal sex determining locus and the locus controlling red body colouration. If these two loci were tightly linked and no recombination occurred between the putative sex determining locus and the red body colour, both wild and red type females would be found but males would be only wild type. In this situation, the non-recombinant genotype XX SsRr would produce only red type females and the non-recombinant genotype XX ssrr would produce wild type males and females (due to partial penetrance of the ss genotype) (Fig. 7.1a).

On the other hand, if there are two different sex reversal loci and some recombination occurred between the red and autosomal sex determining loci, red males would also be observed. In this case, the recombinant genotype XX S1s1rr produces only wildtype females and the other recombinant, XX s1s1Rr, produces red males and females (Fig. 7.1b). If the two loci were unlinked, then all four genotypes would be produced at equal frequencies (1:1:1:1) and the frequency of males would be equal in red and wild type colours.

7.2.3. Statistical analysis

Homogeneity chi-square analyses were performed on pooled data from different crosses and chi-square analyses were also performed in various occasions for testing different progeny sex ratios against different hypotheses. Kruskal-Wallis tests were also performed (using Minintab, V.13 statistical analysis software) to compare the survival rates at different stages in the F1 and backcrossed generation.

7.3. Results

7.3.1. Survival of the F1 progeny

The survival of the F1 progeny produced from the crosses between homozygous red females and spontaneously sex-reversed clonal neomales at different stages of development are presented in Table 7.1.

Table 7.1. Survival of F1 progeny at different stages of development. P: pigmentation; H: hatching; YSA: yolk sac absorption; 12 weeks: at the age of 12 weeks.

Red♀	Family	Clonal neo♂	% survival			
PIT tag number		PIT tag number	P	H	YSA	12 weeks
00-0121-163F	A	00-013C-AFA5	66.66	53.10	41.52	36.44
00-0121-163F	B	00-013E-OA23	51.92	33.48	18.89	15.36
00-012E-8039	C	00-013C-AFA5	80.55	70.13	56.18	46.95
00-012E-8039	D	00-013E-OA24	83.51	69.41	58.6	54.39
00-0134-36B0	E	00-013C-AFA7	72.94	65.09	52.94	47.84
00-0134-36B0	F	00-013E-OA25	67.25	59.26	47.09	43.43

7.3.2. Sex ratios and body colour in the F1 generation

Six families of *O. niloticus* were produced from crosses between three homozygous red females and two spontaneously sex reversed clonal neomales. The sex ratios and body colour inheritance data from the F1 generation are presented in Table 7.2.

Table 7.2. Sex and body colour of F1 generation produced from crosses between three homozygous red *Oreochromis niloticus* females and two wild type colour spontaneously sex reversed clonal neomales from fully inbred clonal line 002-046-539. R: red, WT: wild type colour.

Red♀	Clonal neo♂	Phenotypes of progeny			
PIT tag number	PIT tag number	R♀	WT♀	R♂	WT♂
00-0121-163F	00-013C-AFA5	74	0	0	0
00-0121-163F	00-013E-OA23	37	0	0	0
00-012E-8039	00-013C-AFA5	85	0	0	0
00-012E-8039	00-013E-OA23	91	0	0	0
00-0134-36B0	00-013C-AFA5	100	0	0	0
00-0134-36B0	00-013E-OA23	100	0	0	0
Total		487	0	0	0

A total of 487 individuals were sexed to reveal the progeny sex ratios in F1 generation. Sexing data showed 100% female progeny in the F1 generation. The observation of body colour also revealed that all of the F1 generation were red (Table 7.2).

7.3.3. Survival of backcrossed progeny

The survival of backcrossed progeny produced from the crosses between F1 females and spontaneously sex-reversed clonal neomales at different stages of development are presented in Table 7.3 and the relative survival rate at different stages of development in F1and backcrossed progeny are summarized in Fig. 7.2.

Table 7.3. Survival at different stages of development in backcrossed progeny.

F1♀	Family	Clonal neo♂	% survival			
PIT tag number		PIT tag no.	P	H	YSA	12 weeks
00-01DC-DFD3	1.1	00-013E-OA23	45.62	25.53	13.71	3.30
00-01DC-EE12	1.2	00-013C-AFA5	54.02	31.49	18.85	5.05
00-0135-A666	2.1	00-013E-OA23	40.60	14.42	3.69	0
00-01E0-3EEE	2.2	00-013E-OA23	68.34	51.44	35.72	13.58
00-01E0-A875	2.3	00-013C-AFA5	61.65	30.09	22.57	9.70
00-01E0-A875	2.3	00-013E-OA23	40.58	35.01	23.07	11.67
00-01E0-3CB2	3.1	00-013C-AFA5	56.13	46.69	27.35	4.71
00-01E0-3CB2	3.1	00-013E-OA23	68.35	41.79	15.23	4.29
00-01DF-59E9	4.1	00-013C-AFA5	58.54	19.23	6.41	0
00-01DF-59E9	4.1	00-013E-OA23	41.22	27.77	11.40	3.21
00-01DC-EC38	5.1	00-013E-OA23	71.32	54.74	34.45	4.65
00-01E0-36B0	5.3	00-013E-OA23	50.15	35.86	23.40	6.07
00-012E-5DDD	6.1	00-013E-OA23	47.67	40.92	15.61	8.43
00-012C-227E	6.3	00-013E-OA23	66.66	54.08	31.67	4.28

No significant differences were observed in relative survival at pigmentation stage between the F1 and backcrossed progeny. However, at hatching, yolk sac absorption and 12 weeks of age, the relative survival of the backcrossed generation was significantly lower than that of the F1 progeny (Kruskal-Wallis test, $P < 0.05$) (Table 7.1 and 7.3).

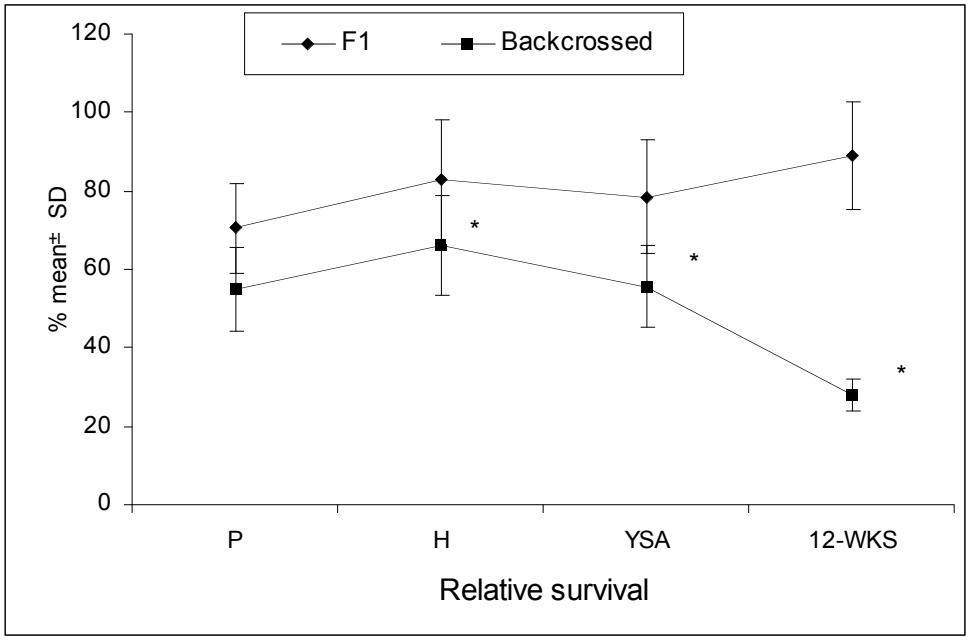


Fig. 7.2. Percent mean relative survival of F1 and backcrossed progeny during different stages of development; P: pigmentation; H: hatching; YSA: yolk sac absorption; Wks: weeks, *: significantly different ($P < 0.05$) from F1 generation.

In the F1 generation, no noticeable level of deformity was observed among the progeny. However, in the backcrossed generation a high level of deformity was observed among the progeny, with the most common deformity being the lack of one or both eyes. All the deformed fish died at the yolk sac absorption stage.

Low fertilization efficiency was observed in all backcrosses due to poor sperm quality of both males. Sperm were analysed and counted and very low proportions of

motile sperms were observed, explaining the poor fertilization rates. Low sperm concentration was also observed in one of the XX neomales and the other XX neomale only produced viable sperm for a limited period of time.

7.3.4. Linkage between the putative autosomal sex reversal locus and the red body colouration

Linkage between the putative sex reversal locus and red body colouration in the Nile tilapia (*Oreochromis niloticus*) was studied from the inheritance of body colour and sex ratios among the backcrossed generation produced from 12 different females from six F1 families. The results are presented in Tables 7.4 and 7.5.

A total of 335 individuals were analysed for sex and body colour. Of the 12 F1 females tested, only one female (female no.10) produced all female progeny. However, the data pooled from the 12 females were homogeneous (homogeneity χ^2 value = 5.078, 11 d.f., $P > 0.5$). The summarized results also revealed a proportion (10.74%) of males among the progeny. The overall sex ratio was significantly different from a 1:1 ratio (χ^2 value = 206.47, 1 d.f., $P < 0.00001$). The results of overall colour inheritance among the progeny were not significantly different from a 1:1 colour ratio ($\chi^2 = 0.0326$, 1 d.f., $P > 0.5$) (Table 7.4 and 7.5).

Table 7.4. Sex and body colour of backcross generation produced from 12 heterozygous red *Oreochromis niloticus* F1 females from 6 families, crossed to two wild type colour males from fully inbred clonal line 002-046-539; R: red, WT: wild type colour.

Female number	Progeny phenotypes			
	R♀	WT♀	R♂	WT♂
1	5	7	2	0
2	9	10	1	2
3	37	34	2	4
4	17	23	3	1
5	15	20	4	1
6	7	10	2	2
7	4	5	0	1
8	6	4	0	1
9	12	10	1	2
10	8	12	0	0
11	7	10	1	2
12	17	10	2	2
Total	144	155	18	18

When body colour ratios were analysed within sex, no significant (P >0.05) difference was observed from a 1:1 ratio. Equal proportions of males and females were observed in each body colour type (Table 7.5).

Table 7.5. Summarized results of sex and body colour in backcrossed progeny of *Oreochromis niloticus* (for sex, % ratios are in parentheses and for body colour, % males are in parentheses); R: red, WT: wild type colour.

Sex		Body colour	
WT ♂: R ♂	WT ♀: R ♀	WT ♀: WT ♂	R ♀: R ♂
18: 18	155: 144	155: 18	144: 18
(50:50)*	(51.8: 48.2)*	(10.40)**	(11.11)**

* not significantly different from 1:1 body colour ratio (P < 0.05)

** significantly different from 1:1 sex ratio (P > 0.05)

On the other hand, when sex ratios were analysed within each body colour type, small proportions of males (10.40% in wild type and 11.11% in red) were observed in each body colour group. However, the sex in each body colour type was not significantly different from a 1:1 ratio (Table 7.5).

7.4. Discussion

This study revealed no linkage between the putative autosomal sex-determining locus of Sarder *et al.* (1999) and the red body colour locus in *Oreochromis niloticus*.

In the F1 generation 100% female progeny with red body colour were produced in crosses between homozygous red females and spontaneously sex reversed wild type colour clonal neomales. This strongly indicated heterozygosity for the red body colour locus and absence of homozygosity for autosomal sex reversal allele(s) in the F1 generation. This result also revealed that the red parents were homozygous for the red locus (RR) and indicated that they were not carrying any sex reversal alleles at the putative sex reversal locus/i responsible for sex reversal in clonal line parent. Hussain *et al.* (1994a) hypothesized the recessive expression of such a locus as an autosomal sex inversion factor from female to male. Later Sarder *et al.* (1999) and Karayucel (1999) also supported and extended the idea by hypothesising partial penetrance, as indicated by the appearance of a small proportion of males in progeny groups.

However, Kwon (2000) in a separate study on parental influence on progeny sex ratio in this species used the same males as in this study. The author performed a series of testcrosses between these two males and eight normal females. He found 3.4-17.9% male progeny in five out of eight females; the other three gave 100% female offspring. He concluded the phenomenon was a consequence of parental influences, most likely autosomal or environmental factors. It is possible that some of the females he used in his experiment were heterozygous or homozygous recessive for autosomal sex determining locus/i, resulting in males in the progeny, or it could have happened due to environmental influences. Again the 3.4-17.9% males he observed in his study would

also indicate a partial penetrative influence as suggested by Sarder *et al.* (1999) and Karayucel (1999), and also observed in the present experiment (F2 generation).

The results of the backcrossed generation suggested the occurrence of all four genotypes in the progeny as described earlier in Hypothesis B. The inheritance of body colour in the backcrossed generation followed the expected Mendelian ratio. No significant (P<0.05) deviation from 1:1 body colour ratio was observed between the wild type and red in any progeny group.

The sex ratio data revealed up to 19% males in the backcrossed progeny (Table 7.4). Only one female out of 12 females tested produced all female progeny. The overall percentage of males produced in these experiments was 10.74%, which was significantly different from a 1:1 sex ratio (P<0.05). A similar phenomenon of "unexpected" male progeny was also observed in different *Oreochromis* species by Avtalion and Hammerman (1978), Hammerman and Avtalion (1979), Mair *et al.*, (1990, 1991a,b and 1997), Hussain *et al.* (1994a), Sarder *et al.* (1999), Karayucel (1999), Kwon (2000) and in common carp by Komen and Richter (1993). In all cases, influences of one or more autosomal genes were suggested to explain the phenomenon.

In the present experiment, the linkage between the putative sex determining locus and the locus controlling the red body colour was also studied. No noticeable linkage was observed between these two loci as equal proportions of males were observed in both colour phenotypes (Table 7.5). This result was not in agreement with that of Karayucel (1999), where strong linkage was observed between these two loci, as evidenced by virtually all males being red in a population of meiotic gynogenetics produced from nine different heterozygous red females. There was one exceptional wild type male. The

autosomal sex reversal locus/i found in Sarder *et al.*'s (1999) clonal line is not linked to the red colour locus, and is thus not linked to the gene which caused sex reversal in Karayucel's (1999) gynogenetics. Therefore, Hypothesis A (7.1.1) can be rejected. So, Hypothesis B is correct i.e. more than one autosomal sex reversal locus is operating in *O. niloticus*. According to Hypothesis B, two autosomal loci can be considered as the simplest explanation: locus 1 with alleles S1/s1 which was described by Sarder *et al*. (1999) (although it is possible that there are more than one such loci in the clonal line with homozygous recessive genotype or these could have been a mutation in a sex determining gene in the sex chromosome), and locus 2 with alleles S2/s2 as described by Karayucel (1999) in meiotic gynogenetics, which is linked to the gene responsible for red body colour. The occurrence of more than one autosomal sex reversal locus has also been suggested by Mair *et al.* (1997) while describing unexpected YY females in progeny of all YY males.

Depending on the nature and linkage, evidence for influences of autosomal genes in sex reversal of *O. niloticus* can be put into three broad categories: (i) bi-directional (male to female and vice-versa) influence of autosomal sex reversal locus/i (ii) influence of autosomal sex reversal locus/i linked to genes responsible for red body colour and (iii) bi-directional environmental (correlated to rearing temperature) influence of autosomal sex reversal locus/i.

(i) Progeny testing results of several families of XX and YY mitotic gynogenetic individuals produced from XY neofemales described in Chapter 2 of this thesis can be provided as an example of this category. The progeny of three of the four gynogenetic families conformed almost exactly to the expected

100 % females (in progeny of XX gynogenetic females: no males out of 305 progeny) and 100 % males (in progeny of YY gynogenetic males: two females out of 580 progeny), while in the family 5, progeny testing of both sexes of gynogenetics produced departures from the expected sex ratios (5 females out of 178 progeny of XX females; 30 males out of 255 progeny of YY males). Sex reversal thus occurred in both directions within the progeny of one family, while it was almost completely absent in the other three families. This also suggests that the controlling factor in family 5 (see Chapter 2) was autosomal - unlikely to be sex linked since both XX and YY were affected. The same locus/i could be involved in sex reversal in this category.

(ii) The study of Karayucel (1999) is an example of this category, where very tight linkage of genes of autosomal sex reversal and red body colour was presented. In her experiment she found 10% red males from a progeny of 546 meiotic gynogenetics produced from 9 different XY heterozygous red females. Only one wild type male was observed in one female out of 278 progeny. This study indicated the tight linkage of red body colour genes with autosomal sex determining genes. It is difficult to predict if this locus only causes female to male sex reversal, as the experiment described by Karayucel (1999) did not test the converse.

(iii) In this category, autosomal genes are responsible for bi-directional sex reversal. The results of Abucay *et al*. (1999) suggested bi-directional sex reversal with rearing temperatures. High temperature causes both female to

male sex reversal (in XX genotypes) and male to female sex reversal (in YY genotypes and to a lesser extent in XY genotypes) (Abucay *et al*., 1999; Kwon *et al*., 2002). A significant correlation was observed between the percentages of males and females with rearing temperatures (Abucay *et al.*, 1999; Penman: personal communication) suggesting high rearing temperature may enhance the expression of the effect of genetic sex factors (autosomal sex modifying genes). Abucay *et al*. (1999) subjected several groups of putative all-female progeny to high and low rearing temperatures. They observed a significant correlation in the percentage of males produced between treatment and control groups. Those groups which had high percentages of males in control (28°C, below TSD threshold) groups, also showed higher percentages of males in the treatment groups. From this result they hypothesized that extreme conditions such as high temperatures might enhance the expression of autosomal sex modifying genes resulting in higher percentage of males in the treatment groups. However, it is difficult to predict whether the same locus/i described earlier is involved in this category or not.

The simplest explanation of the results of the present experiment is that two different, unlinked autosomal loci with partial penetrance can cause female to male sex reversal, and that one of theses is closely linked to the gene which causes red body colour. This supports hypothesis B as described earlier in section 7.1.1.

Although evidence is presented here for the existence of (at least) two separate "sex reversal" loci (at least one of which is autosomal), nothing is known about the nature of

these genes and their normal function. It may be that their normal function is involved in the process of sexual differentiation and it seems likely that the genotypes of these loci which cause sex reversal do so by destabilisation of this process at key stages, leading to low and variable frequencies of sex reversal (partial penetrance). Therefore, the above discussion suggested, in favour of the Hypothesis B as mentioned in the objectives that more than one autosomal sex reversal loci exists, one of which is linked to red body colour in *O. niloticus*.

Chapter 8

General Discussion

This thesis describes an analysis of sex determination in the Nile tilapia *Oreochromis niloticus*, by a combination of chromosome set manipulations, molecular genetic techniques and linkage analyses. Moreover, molecular genetic techniques were applied for the parentage assignment of normal and genetically manipulated populations with a view to establish a suitable detection system for the rapid screening of such individuals for both research and commercial purposes. This thesis also reports for the first time the occurrence of spontaneous diploidization of the maternal chromosomes in this species.

8.1. Chromosome set manipulation and the analysis of sex determination

As discussed earlier the Nile tilapia *Oreochromis niloticus*, primarily has a genetic sex determination system (GSD) with influences of autosomal sex modifying genes and environmental factors. Sex determination in tilapia species has been the subject of intensive research for the last four decades (e.g. Hickling, 1960; Chen, 1969; Jalabert *et al.*, 1974; Avtalion and Hammerman, 1978; Penman *et al.*, 1987a; Mair *et al.*, 1991a,b; Hussain *et al.*, 1994a; Mair *et al.*, 1997; Carrasco *et al.*, 1999; Sarder *et al.*, 1999; Guan *et al.*, 2000; Kwon *et al.*, 2001; Harvey *et al.*, in press). Six different approaches have been applied for the analysis of sex determination in this species: i) analysis of the sex ratios in various crosses involving inter-specific hybridization, ii) progeny testing of sex-reversed fish iii) by chromosome set manipulations, iv) by studying the chromosomal morphology in both metaphase and meiotic stages, v) chromosome microdissection of putative sex chromosomes (Harvey *et al.*, in press) and vi) by molecular analysis (Bardacki *et al*., 1994; McConnell *et al*., 1996). All of these approaches concluded that

sex is primarily determined by a single genetic factor, with low levels of influence from autosomal and environmental factors.

Because of the influence of these other factors, phenotypic sex does not always reflect the genetic sex in this species. Therefore, genetically defined homozygous populations, which theoretically differ from each other only by their sex chromosome complement, were produced by mitotic gynogenesis for use in these experiments (Chapter 2).

Gynogenesis has already proved to be efficient at producing homozygous individuals in a shorter time than a conventional breeding programme. The technique has also proved to be an efficient way of revealing the sex determining mechanisms of many fish species.

Multilocus DNA fingerprinting (using human derived Jeffreys' 33.15 hypervariable minisatellite probe) and progeny testing confirmed the success of the gynogenesis and proved that all mitotic gynogenetics tested, were either XX or YY. The survival of the mitotic gynogenetic individuals was poor compared to that of the control in this experiment. However, poor survival among gynogenetic progeny has already been reported in this species and several other species (Chourrout and Itskovich, 1983; Mair *et al.*, 1987; Hussain *et al.*, 1993). The poor survival of gynogenetic progeny is the result of the deleterious effects of lethal genes in homozygous conditions (Don and Avtalion, 1988; Varadaraj, 1990b; Sarder *et al.*, 1999) and of mechanical damage to the eggs during hand stripping and shock treatments.

One of the XY neofemales (founder of family 3) used in the production of mitotic gynogenetics consistently produced diploid individuals in UV control group by the

spontaneous duplication of maternal chromosomes (SDM). This SDM phenomenon is rare but not unknown among fish (reviewed by Gold, 1979; Cherfas, 1981; Thorgaard, 1983; Cherfas *et al.*, 1995). However, this phenomenon has not been reported previously in *O. niloticus*. The sex ratio of the spontaneously diploid offspring was also highly biased toward males, as might be predicted for retention of the second polar body (Chapter 6). Further study is needed in this species regarding the heritable nature of this SDM population.

8.2. Amplified fragment length polymorphisms (AFLPs) and sex linkage

AFLP analysis is a fingerprinting technique which provides the capacity to investigate any genome in a comparatively shorter time than most of the other available molecular marker systems. Despite its obvious advantages, it is a dominant marker system, therefore, polymorphisms are only evident on a presence or absence basis, rather than through allelic variations. However, this technique has gained importance in a wide range of genetic applications to find a locus or loci linked to a gene or genes or trait of interests, and has been used in numerous applications for both eukaryotic and prokaryotic genomes (Vos *et al*., 1995; reviewed by Blears *et al*., 1998).

In the present experiment, XX and YY homozygous *O. niloticus* generated by mitotic gynogenesis (described in Chapter 2) were compared to find the differences at the level of sex specific sequences using AFLPs. Table 8.1 shows the summary of AFLP screening.

Table 8.1. Overall summary of AFLP screening for sex-linked markers in *Oreochromis niloticus*.

	Grand pool	Family pool		Individual family set	
		Dam1	Dam4	Dam1	Dam4
AFLP primer combinations used	64	31	31	8	8
Total no. of AFLP markers screened	3138	1704	1757	538	586
Average no. of markers per combination	49.03	54.97	56.67	67.25	73.25
Number of sex-linked AFLP markers (SLAMs)	0	14	16	3	1
Percent of SLAMs	0	0.82	0.91	0.55	0.17

64 primer combinations were used in this experiment, with an average of 49 markers produced per combination. No sex-linked markers were identified among the grand pools of XX and YY. However, 30 putatively sex-linked markers were identified in two family pools of XX and YY homozygous individuals. Some of these putative sex-linked markers were discarded in individual family set screening on the basis of their segregation inside the source family samples and by statistical significance analysis (chi-square with one sided Fisher's exact test). Finally, four sex-linked markers (sex-linked within the two source families) were identified, suggesting that only limited differences exist between the sex chromosomes in this species. This further supports the hypothesis that the sex chromosomes of this species are at an early stage of differentiation (Carrasco *et al*., 1999) with minimum sequence dissimilarity in sex chromosomes.

Because of the nature of polymorphism generated by the AFLP technique, it is often difficult to convert the actual AFLP polymorphisms into single locus PCR based markers which are very useful for the rapid screening of large populations. In most cases, each polymorphic AFLP band is represented by several bands of the same size (such as, one strong band in the positive pool and some faint bands present in both positive and negative pools), possibly sharing the same restriction sites but differing in sequence. Most of the AFLP markers are small, between 200 and 500 bp, which is a

limiting factor for primer designing. Therefore, it is critical to identify the true polymorphic AFLP bands for successful conversion of AFLP markers into single locus markers.

In the present experiment, internal primers were found to be successful in converting the original AFLP sex-linked polymorphic bands for three out of the four sex-linked markers. In the other case (*Oni*Y382), STS primers were designed which were able to detect the sex-linked polymorphism. The AFLP technique has been applied successfully to generate markers tightly linked to sex and other traits of interest in several species of plants and animals (Reamon-Büttner *et al.*, 1998; Griffiths and Orr, 1999; Griffiths *et al.*, 2000; Reamon-Büttner and Jung, 2000; Nakamura *et al*., 2001). The AFLP technique in the present experiment was not able to generate a universal sex-linked marker in *O. niloticus*. However, isolation of markers which were sex-linked within the source families is a major breakthrough, because no sex-linked DNA level marker has been published in this species before. Thus, these markers will help to understand sex determination in this species better. In addition, this research should facilitate the physical mapping of the sex determining gene(s). Hopefully this will allow the isolation of universal markers for sex identification in the future. Moderate linkage was observed between these markers and the sex locus i.e. a low level of recombination is occurring between the sex determining region and the sex-linked markers identified in this study.

8.3. Parentage analysis using molecular techniques

Parentage assignment is important for the proper management of captive populations as well as to monitor the reproductive patterns in natural populations. The use of molecular markers for parentage analysis depends on the nature of the marker system, such as the degree of polymorphism; and the purpose of the study. The principal objective of the experiment described in Chapter 5 was to establish a suitable and effective system to investigate parentage in the normal and genetically manipulated population of *O. niloticus*. For this purpose three different marker systems were investigated, multilocus DNA fingerprinting using hypervariable probes, AFLPs and polymorphic microsatellite loci. All of these methods were successful. However, given the degree of homozygosity among the manipulated populations, it was possible to monitor them adequately only by using co-dominant marker systems such as polymorphic microsatellite loci.

In the present experiment, microsatellite loci were also investigated to study the rate of recombination in polymorphic loci among the meiotic and mitotic gynogenetic populations. Meiotic gynogenetic individuals are heterozygous for some loci due to crossing over, however, mitotic gynogenetics are homozygous for every gene locus. The polymorphic microsatellite loci used in this study were investigated in two families of meiotic gynogenetics of *O. niloticus*. Several microsatellite loci showed high degrees of gene-centromere recombinations suitable for discriminating meiotic from mitotic gynogenetics, however further study is required involving larger sample sizes to study gene centromere-recombination rates. The present experiment therefore shows that microsatellite loci can be applied to investigate the success of genetic manipulation as

well as to differentiate meiotic and mitotic gynogenesis, in addition to more general parentage assignment applications.

8.4. Linkage analysis of autosomal sex determining genes and body colouration

As already discussed in Chapter 7, more than one gene on the autosomes takes part in the determination of genetic sex in *O. niloticus*. Evidence of autosomal genes has been reported earlier in several studies and the possibility that more than one gene affects sex determination has been suggested (Hussain *et al*., 1994a; Mair *et al*., 1991a,b; Sarder *et al*., 1999). The linkage of these autosomal genes with red body colouration was also investigated. That no linkage could be established in the present experiment strongly suggested that more than one autosomal gene is operating in the determination of genetic sex in *O. niloticus*, one linked with red body colouration and the other(s) completely unlinked to that gene, with partial penetrance.

8.5. Conclusions

In conclusion, it can be stated that sex determination in Nile tilapia *Oreochromis niloticus*, is primarily governed by a monofactorial genetic system (XX/XY) with some degree of influence of autosomal genes and earlier reported environmental factors. Therefore, the following concluding remarks can be put forward:

- This present investigation failed to show fixed sequence differences between the sex chromosomes in this species, but has demonstrated the presence of polymorphic genetic markers linked to the main sex determining region (XX/XY) in this species.
- These markers can also be used to provide an initial series of physical or genetic markers for the more precise location of the genes primarily responsible for sex determination. Physical mapping of these markers using BAC library inserts is underway. The initial results showed hybridization of BACs containing these markers in the sex chromosome, adding to the linkage evidence for these markers being located in the sex chromosome and providing further evidence of GSD in this species.
- The present investigation also demonstrated that more than one autosomal sex modifying gene with partial penetrative nature is operating in this species, responsible for autosomal sex reversal, one of which is linked to red body colouration.
- The phenomenon of spontaneous diploidization of the maternal chromosome set has also been demonstrated in this species. However, further study is required.

- This study also revealed that AFLPs, hypervariable minisatellite probes and polymorphic microsatellite loci are useful in evaluating parentage in normal and gynogenetic progeny. Particularly, microsatellite loci were more efficient and confirmed their efficiency over dominant markers such as AFLPs; multilocus DNA fingerprinting with minisatellite probes and RAPDs, where allelic variation cannot be detected readily. Some microsatellite loci also showed high level of gene-centromere recombination, discriminating meiotic from mitotic gynogenetics.

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