MARKER-ASSISTED SELECTION IN ENHANCING GENETICALLY MALE NILE TILAPIA (*Oreochromis niloticus* L.) PRODUCTION

MOHD GOLAM QUADER KHAN

MARKER-ASSISTED SELECTION IN ENHANCING GENETICALLY MALE NILE TILAPIA (*Oreochromis niloticus* L.) PRODUCTION

A thesis submitted for the Degree of Doctor of Philosophy

Mohd Golam Quader Khan

M. Sc. (Fisheries Biology and Genetics)

Institute of Aquaculture, University of Stirling
Stirling, Scotland, UK

UNIVERSITY OF
STIRLING

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DECLARATION

I declare that this PhD thesis has been composed by myself based on my own research. It has not been submitted for any other degrees. The nature and extent of any work carried out by, or in conjunction with others has been acknowledged by reference.

Signature of Candidate ……..

Signature of Supervisor……..

Signature of Co-supervisor…….

Date ……

This work is dedicated to the departed soul of my mother

ABSTRACT

All-male fry are preferred to prevent uncontrolled reproduction before harvest in intensive Nile tilapia (*Oreochromis niloticus*) aquaculture. Males also grow faster than females. An alternative approach to direct hormonal masculinisation of tilapia fry is to produce fry that are genetically male. However, sex determination system in tilapia is fairly complex. Recent developments have resulted in a linkage map and genetic markers that can be used to analyse the sex determination system. To analyse the genetic sex determination mechanism and to develop marker-assisted selection in the Stirling Nile tilapia population, a fully inbred line of clonal females (XX) was verified using test crosses and DNA markers (mostly microsatellites) to use as a standard reference line in sex determination studies. A series of crosses were performed involving this line of females and a range of males. Three groups of crosses were selected (each group consisted of three families) from progeny sex ratio distributions, and designated as type 'A' (normal XY males x clonal XX females), type 'B' (putative YY males x clonal XX females) and type 'C' (unknown groups of males x clonal XX females), for sex linkage study. For type 'A', inheritance of DNA markers and phenotypic sex was investigated using screened markers from tilapia linkage group 1 (LG1) to confirm the LG1-associated pattern of inheritance of phenotypic sex and the structure of LG1. Screened markers from LG1, LG3 and LG23 were used to investigate the association of markers with sex in families of type 'B' and 'C'. In addition, a genome-wide scan of markers from the other 21 LGs was performed to investigate any association between markers and sex, in only families of cross type 'B'. LG1 associated pattern of inheritance of phenotypic sex was confirmed by genotype and QTL analyses in families of cross type 'A'. Analyses of genotypes in families of type 'B' and 'C' showed strong association with LG1 markers but no association with LG3 and LG23 markers. Genome wide scan of markers from all other LGs did not show any significant association between any markers and the sex. The allelic inheritance of two tightly linked LG1 markers (UNH995 and UNH104) in families of type 'B' and 'C' identified polymorphism in the sex determining locus: one of the alleles was associated mostly with male offspring whereas another allele was associated with both progeny (mostly males in type 'B' families, and approximately equal numbers in type 'C' families). This knowledge was used to identify and separate supermales ('YY' males) that should sire higher proportions of male progeny, reared to become sexually mature for use as broodstock. Two of them were crossed with XX females (one clonal and one outbred) to observe the phenotypic expression of the strongest male-associated allele in progeny sex. The observations of 98% male (99 males out of 101 progeny) and 100% male (N=75) from these two crosses respectively, suggest that a marker-assisted selection (MAS) programme for genetically male Nile tilapia production could be practical. This study also suggests that the departures from the sex ratios predicted using a "simple" XX/XY model (i.e., YY x XX should give all-male progeny) were strongly associated with the XX/XY system, due to multiple alleles, rather than being associated with loci in other LGs (e.g., LG3, LG23). This study also tentatively names the allele(s) giving intermediate sex ratios as "ambivalent" and emphasizes that the presence and actions of such allele(s) at the same sex-determining locus could explain departures from predicted sex ratios observed in some earlier studies in Nile tilapia.

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Chapter 1. General Introduction

This chapter gives an account of the historical background and production trends of tilapia, importance of monosex (male) production in the species of tilapia and possible ways of producing all males with an emphasis on genetically male tilapia production. The sex determination systems in tilapia are also described with complexities and challenges and compared with those in other species along with the importance of using molecular markers to unveil the sex determination mechanism. Marker-assisted selection in commercially important fish species are given with monosex production approach in Nile tilapia, and finally the objectives of the present research work are mentioned.

1.1 Historical background, farming potential and production trends of tilapia

Tilapia, sometimes referred to as St. Peter's fish (since it is thought to be the fish caught by St. Peter in the Sea of Galilee), have been important sources of food for man since recorded history began. Drawings of tilapia farming have been found on Egyptian tombs dating back to 2,500 B.C (Bardach et al., 1972). Tilapia consist of some 80 species, including three main genera (Trewavas 1983); *Oreochromis* (about 30 species), *Sarotherodon* (over 10 species) and *Tilapia* (about 40 species). However, only a few of these are of major importance in aquaculture: the Nile tilapia *Oreochromis niloticus* Linnaeus, the blue (or Jordan) tilapia *O. aureus* Steindachner, the Mozambique tilapia *O. mossambicus* and to a lesser extent the Sabaki tilapia *O. spilurus spilurus* Gunther, the three-spotted tilapia *O. andersonii* Castelnau, the blackchin tilapia *S. melanotheron* Ruppell and the redbreast tilapia *T. rendalli* Boulenger. Originating from Africa, where they and other cichlids dominate freshwater lakes, they are part of the large cichlid family also found in South and Central America, the Indian subcontinent, and the Middle East.

Throughout the 20th century, mostly for commercial purposes, introduction of tilapia spread all over Asia, Europe and the USA (Keenleyside, 1991). Farming of tilapia is now in a dynamic state of expansion to satisfy both domestic and international markets and they are cultured in 85 countries currently according to FAO (2009) and consist predominantly (~75%) Nile tilapia.

There are a good number of advantages that favour Nile tilapia farming. They can be cultured in freshwater, brackishwater and even in full-strength seawater and can tolerate poor water quality and environmental fluctuations. They may grow well at high density, feed low in the food chain, either as column feeders or benthic omnivores and can be grown using many by-products. The offspring can be produced all the year round in some countries. They have mild-tasting good quality white flesh attractive to consumers and are popular in different forms (such as whole or fillet, fresh or frozen, salted or smoked). The fillet yield of Nile tilapia varies from 25.4% (Clement and Lovell, 1994) to 35.7% (Rutten et al., 2004), processing yield (total fish weight minus weight of head, skin and viscera) is 51.0%, fat content of fillet is low (only 5.7 g/100 g), protein content of fillet is high (20.3 g/100 g), caloric value of fillet is 139 kcal/100 g, monounsaturated fatty acid comprise an average of 54.6 g/100 g of total fatty acids, n-3 highly unsaturated fatty acids comprises an average of 2.2 g/100 g of total fatty acids and the cholesterol content of fillet is only 31.3 mg/100 g (Clement and Lovell, 1994). All of these qualities explain much of their appeal to become a unique food fish to be cultured in a wide range of aquaculture ventures. Their culinary versatility and flexibility in preparation, reasonable price and classic flavour have established a market in almost every country in the world. Tilapias are now the second most farmed group of fish (behind carps) with annual global production exceeding 2.5 million metric tons in 2007 (FAO, 2009).

The production trend of tilapia is very positive. The dramatic annual increase in tilapia production from the top countries is given in Table 1. For example, tilapia production in China has increased from 44,832 metric tons in 1980 to over 1.2 million metric tons in 2007. In Indonesia, this figure is from 14,901 metric tons to 248,305 metric tons. The global production of tilapia in 2007 is 25 times higher than that in 1980.

Table1.1 Tilapia production (t) in the top ranked countries from 1980 to 2007

Country	**1980**	**1985**	**1990**	**1995**	**2000**	**2005**	**2007**
China (including Taiwan)	44,832	76,542	159,313	361,346	598,109	928,163	1,210,167
Egypt	9,000	22,346	24,916	21,969	157,425	217,019	265,862
Indonesia	14,901	29,302	53,768	74,125	85,179	189,570	248,305
Philippines	13,214	42,640	76,142	81,954	92,575	163,004	241,183
Thailand	8,419	16,542	22,895	76,383	82,581	203,911	190,258
Brazil	nr	nr	nr	12,014	32,459	67,851	95,091
Malaysia	366	314	1145	8,866	18,471	28,635	32,258
Honduras	6	35	120	172	927	28,376	28,356
Columbia	93	300	2040	16,057	22,870	27,953	27,960
Global	107,459	211,594	379,184	703,712	1,190,016	1,980,450	2,505,465

Source: FAO–Fisheries and Aquaculture Information and Statistics Service (2009). nr, not recorded.

In the 1960s and 1970s tilapia culture was aimed at the production of food for local consumption, utilizing primarily extensive or semi-intensive culture methods with minimal inputs of fertilizer or feeds (Watanabe et al., 2002). Advances in technology associated with the intensification of culture practices (for example, development of new strains and hybrids, monosex male culture, formulated diets and the utilization of greenhouses,

geothermal, or industrial waste heat and advanced water treatment methods) have resulted in the expansion of tilapia culture rapidly.

The culture practices of tilapia can be extensive, semi-intensive or intensive (Tsadik and Bart, 2007). There has been a gradual shift in tilapia culture from traditional semi-intensive to non-traditional intensive farm systems (El-Sayed and Kawanna, 2008). The diversification of culture systems as well as marketing programmes has also nurtured a growing demand for tilapia in domestic and international markets. But deciding the optimal culture method for tilapia farming can be quite complex (Graaf et al., 2005). Traditionally tilapia is often cultured in earthen ponds without supplemental feeding (Liti et al., 2005). Intensive monoculture in the concrete tanks is carried out in a few countries. Although practiced in some countries, cage culture of tilapia is yet to be commercialised on a wide scale basis. Pen culture of tilapia in open waters is practiced in the Philippines on an appreciable scale (Pillay, 1993).

Extensive culture systems in pond and reservoir ranching are widely practiced in developing countries and production ranges from 300-700 kg/ha/crop. In most developing countries, this type of producer has a low social, cultural, and economic status and limited access to technology, markets, and credit (Alceste, 2000). While its impact is hard to measure, extensive tilapia culture has also helped the rural poor to improve their household nutrition and raise the standard of living in some areas of the Americas (Fitzsimmons, 2000). Semi-intensive pond culture of tilapia is typically integrated with agricultural or animal husbandry activities because pond fertilization with organic fertilizers can promote natural pond productivity. In Brazil, for example, 0.1-ha ponds stocked with sex-reversed

male tilapia in monoculture or in polyculture with common carp and Chinese carp yield 3000 to 4000 kg/ha/crop when ponds are integrated with pig husbandry (Lovshin, 2000).

Intensive culture of tilapia is done in ponds, raceways, cages and tanks. This approach is useful for producers who use public or communal waters, including reservoirs, lakes, bays, irrigation systems, or village ponds. In the U.S., tilapia are being cultured in cages placed in abandoned phosphate mining pits in Florida and in watershed ponds in Alabama (Popma and Rodriquez, 2000). In Mexico, cage culture systems include floating cages, net pens that use staked sides and rest on the bottom, and wooden corrals that enclose portions of a lagoon (Fitzsimmons, 2000). Cage volumes and stocking densities range from $4m^3$ units stocked at 200 to 300 fish/m^3 to cages of 100 m^3 or larger, stocked at 25 to 50 fish/m^3. Yields range from 150 kg/m^3/crop in 4 m^3 cages to 50 kg/m^3/crop in 100 m^3. In Colombia, cage culture is practiced in large reservoirs constructed for hydroelectric generation (Popma and Rodriquez, 2000). Cages range from 2.7 to 45 m^3 in volume, with total volume of the reservoir exceeding 13,000 m^3. Sex-reversed males produced in land-based hatcheries are stocked into grow out cages at 30 g and are raised to 150 to 300 g in 6 to 8 months. Fish are fed extruded feeds with 24 to 34% crude protein. Annual yields at final densities of 160 to 350 fish/m^3 are 67 to 116 kg/m^3 (Popma and Rodriquez, 2000).

Intensive ponds of 1 ha or less and raceways or tanks supplied with large amounts of flowing water from rivers or streams are preferred for large-scale commercial production. In countries like the Caribbean and Central and South America, companies that grow tilapia using intensive systems target a high quality product for export markets. Such companies have access to monetary institutions to finance production, add value, and ship

to distant markets (Alceste, 2000). An example of a progression of tilapia culture from semi-intensive to intensive and highly intensive practices can be Jamaica, where the lowest level is the small farmer with 1 to 4 ha (75% of farms) that usually owns their land. Final yield averages 9000 kg/ha/y. The second level is the medium size operator with 5 to 20 ha (19% of farms). Fish are sold pond side, but are also sold to distributors or to a contract farming system. Final yield averages 16,000 kg/ha/y. The third level is the large operator with 21 to 45 ha (6% of farms). Large farms are typically partnerships or subsidiaries of larger firms engaged in other businesses. These farms stock at higher densities, use greater water exchange and aeration, and manage integrated systems for maintaining broodstock, producing fingerlings, processing, and marketing. Input costs are higher and feed efficiencies slightly lower, but larger fish are produced, and much greater yields of 45,000/kg/ha/y are generated. The production cost for a medium farm is $1.62/kg, while that of a large farm is estimated at $1.25 to 1.55/kg. This compares with a selling price of $2.65/kg (reviewed by Watanabe et al., 2002). Although definitive data on the economics of tilapia aquaculture production systems are lacking, diversified culture systems and some unique characters of this species has already established its market demand all over the world.

However tilapia present some big challenges to the fish culturist when significant proportion of yields become unmarketable due to their biological features. Swingle (1960) found that in 169-196 day culture cycles of mixed sex Mozambique tilapia, *Oreochromis mossambicus*, production exceeded 3,000 kg/ha but >90% of the harvest was composed of fish <100g. Verani et al. (1983) produced 4,944 kg/ha of mixed sex Nile tilapia (*O. niloticus*) in 11 months but the average weight harvested was <100g. High yields and efficient nutrient utilizations are meaningless unless a significant portion of the production

is marketable. Market sizes may vary but top prices are guaranteed for larger fish in exports and supermarkets.

1.2 Significance of Monosex production in Nile tilapia

Tilapia mature early and can be easily bred in captivity all the year round. They attain sexual maturity in 4 to 6 months while still being smaller than marketable size. They also spawn frequently (females every two to four weeks during the spawning season). Therefore, culture areas can become saturated with vast numbers of smaller and uneven sized fish, which are often unwanted by fish producers or hatchery operators (Pechsiri and Yakupitiyage, 2005). Females grow slower in this species. Males, and especially females, divert energy which could be utilised for somatic growth, into gamete production and behavioural interactions. In addition, competition with recruits in confined environments further suppresses the growth of stocked fish and can result in 30-50% of harvested biomass consisting of largely unmarketable recruits (Mair et al., 1995). Therefore in semi-intensive or intensive aquaculture, male tilapia are preferred for culture and harvest to reduce the possibility of unwanted reproduction and stunting in grow-out ponds, to obtain fast growing, more uniform sized fish and to gain more profit.

1.3 Possible ways of all male production in Nile (and other) tilapia

All (or mostly) male populations can be obtained in a number of ways in Nile tilapia. The first method is manual separation of males from females by visual observation on the basis of the genital papilla (the male genital papilla is elongated while the female papilla is rounded), which is labour intensive, time consuming and wasteful of discarded females. Monosex culture by either manually or mechanically selecting (grading by size) males

results in half of the fish being rejected. Fingerlings need to be 20-30 g for sexing, producing the equivalent of 4,000 kg/ha of females, which need to be discarded (Popma et al., 1984).

Inter-specific hybridization has also been used to result in male biased population in tilapia. Many *Oreochromis* hybrids are characterized by a majority of males, the occurrence of all-male broods is relatively common and this is where the major interest in hybridisation lies (Beardmore et al., 2001). Early attempts to control the sex of fingerlings by interspecific hybridization began half a century ago, by Hickling (1960). The report of monosex hybrids by Pruginin et al. (1975) created significant interest where they obtained 98-100% of males by crossing *Oreochromis niloticus* (♀) and *O. variabilis* (♂), *O. nigra* (♀) and *O. urolepis hornorum* (♂), *O. vulcani* (♀) and *O. u. hornorum* (♂), and, *O. vulcani* (♀) and *O. aureus* (♂) crosses. Hybridization between Nile tilapia (*O. niloticus*) and the blue tilapia (*O. aureus*) results in the production of predominantly male offspring and reduces unwanted natural reproduction in grow out ponds (Rosenstein and Hulata, 1993). In some commercial fish farms in Israel and Taiwan, the *O. niloticus* and *O. aureus* cross is utilized (Liao and Chen, 1983). This cross produces predominately males because of different sex-determining mechanisms in the two species: Nile tilapia has the XX/XY system with the male being heterogametic, whereas blue tilapia has ZZ/ZW with the heterogametic genotype being female (Lahav and Lahav, 1990; Wohlfarth, 1994). Other tilapia crosses producing predominately male offspring are Nile tilapia (♀) with Wami tilapia, *O. hornorum* or with the longfin tilapia, *O. macrochir* (♂) and the Mozambique tilapia, *O. mossambicus* (♀) crossed with the Wami tilapia (♂) (Wohlfarth, 1994).

However, the problem of consistency of producing all males by interspecific hybridization is apparent. Mass spawns between *O. niloticus* females and *O. aureus* males resulted in varied percentage of males, between 59 and 81% (Wohlfarth, 1994). It seems that although the inter-specific hybridization is a means of monosex tilapia production, it often leads to accidental introgression between the species, affecting the sex ratio (Beardmore et al., 2001). Attempts to commercialise monosex hybrids using *O. niloticus* female x *O. aureus* male cross or using male *O. u. hornorum* with females from *O. niloticus* or *O. mossambicus* have been disappointing in some cases (for example, in Israeli industry where tolerance to low temperature is also desirable character) with females usually occurring in putatively all-male broods. Failure to sustain production of all-male tilapia hybrids is most likely due to insufficient care in keeping broodstock segregated by sex and species, and in preventing introduction of hybrids into the broodstock ponds. However, with *O. niloticus* accepted as the best commercial species for the majority of freshwater aquaculture environments, dilution of the *O. niloticus* genome with genes from other species tends to reduce the performance potential in aquaculture, compared to pure *O. niloticus* (Beardmore et al., 2001). Table 1.2 summarises the hybrid combinations in tilapia with their possible effects.

Table 1.2 Hybridizations in tilapia with their effects on traits of interest

Species of tilapia hybridised (female parent x male parent)	**Effect/advantage**	**Reference**
Nile tilapia x blue tilapia (*O. niloticus x O. aureus*)	Hybrids are fertile with increased cold and salinity tolerance compared to Nile tilapia. Crosses of some strains yield all-male offspring; hybrid males have superior growth to blue tilapia. Applied commercially but results inconsistent. Some strains produce red colouration.	Lahav and Lahav, 1990; Wohlfarth, 1994; Bartley et al., 2001; Beardmore et al., 2001
Nile tilapia x Wami tilapia (*O. niloticus x O.urolepis hornorum*)	Cross yields predominantly male offspring with some strains producing red-skinned fish with salt tolerance. Some commercial applications.	Wohlfarth, 1994; Beardmore et al., 2001
Nile tilapia x long-finned tilapia (*O. niloticus x O. macrochir*)	Cross yields predominately male offspring, but strain of Nile tilapia important for good fry production.	Wohlfarth, 1994
O. niloticus x O. variabilis	All progenies monosex (male)	Beardmore et al., 2001
Mozambique tilapia x blue tilapia (*O. mossambicus x O. aureus*)	All progenies monosex (male)	Beardmore et al., 2001
Mozambique tilapia x Wami tilapia/blue tilapia (*O. mossambicus x O. urolepis hornorum*)	Cross yields predominately male offspring. Hybrids are fertile, however often with slow growth and dark colour. Certain strains produce the Florida red tilapia with good growth and salinity tolerance.	Krasnai, 1987; Bartley et al., 2001; Head et al., 1994; Wohlfarth, 1994
Mozambique tilapia x Nile tilapia (*Oreochromis mossambicus x O. niloticus*)	Hybridization of some strains produced tilapia with salinity tolerance. Also known as the Taiwan red tilapias, progeny of these hybrids display a variety of different skin colours.	Lim et al., 1993
O. spilurus niger x *O. macrochir/ O. u. hornorum*	All progenies monosex (male)	Beardmore et al., 2001
O. aureus x *O. u. hornorum*	All progenies monosex (male)	Beardmore et al., 2001
T. zilli x O. andersonii	All progenies monosex (male)	Beardmore et al., 2001

Chromosome set manipulation can provide alternative mechanisms for monosex production by single parent genome contribution. In contrast to the production of monosex females (e.g., Pongthana et al., 1999) by gynogenesis (one of the chromosome set manipulation techniques), this and other techniques (e.g., androgenesis) can be used as routes to monosex male production. Production of all-male triploid tilapia Androgenesis should result in offspring of equal sex ratio; females would be XX and males would be YY. Progeny testing will be required during experimental development to confirm that the males are fertile and that only male offspring (XY) will result when spawned with normal females (XX). Sex ratio of progeny from crosses of androgenetic females with normal males should be 1:1, and presumptive YY-male androgenetics crossed with normal females would be expected to produce only male progeny. The YY-males would then be a basis for developing a unique broodstock that would produce all-male progeny and add insight into the stability and fidelity of the sex-determining system in tilapia. However, autosomal modifier genes may alter the theoretical 1:1 progeny sex ratio (Shelton et al., 1983; Wohlfarth and Wedekind, 1991; Mair, 1993). In addition, thermal (cold or hot) or pressure shock treatment during the process must be timed to coincide with a cytological event, such as disruption of the spindle fibers during metaphase to prevent karyokinesis, or interference with the cell duplication during cytokinesis (Shelton, 2002). Thus, shock type and intensity, duration, and time of application need to be carefully combined and controlled for maximum yield of diploid progeny. Poor survival of androgenetic individuals of tilapia is attributed to these factors. The low probability of producing mature YY-males and the increasing evidence of complications with reference to sex determination for tilapias have become the major concerns of successful ploidy manipulation technique in tilapia.

Hormonal sex reversal is another option for the production of monosex male tilapia. The treatment of sexually undifferentiated fry by administration of androgen hormones has been shown to work well under carefully controlled conditions. Hormone treatment does not alter the genotype of the fish but directs the expression of the phenotype. A treated population of fish may be phenotypically mono-sex but genetically will be the same as determined at the moment of fertilization. As a result of hormone treatment, it is possible to have phenotypically male fish which are genetically female following androgen treatment or phenotypically female fish that are genetically male following estrogen treatment. The use of hormones to alter the sex ratios of fish was first demonstrated in medaka by Yamamato (1953) who concluded that the sex hormones, in addition to modification of secondary sex characters, also affect the gonads. He produced 100% female medaka (*Oryzias latipes*) with an estrogen and a nearly all male population with an androgen (Yamamoto, 1953, 1955). The technique of sex reversal has been used in over 25 species including rainbow trout *Oncorhynchus mykiss*, goldfish *Carassius auratus* (Yamazaki, 1976), grass carps, *Ctenopharyngodon idella* (Stanley et al., 1978) and tilapias (Clemens and Inslee, 1968; Nakamura and Takahashi, 1973).

Production of all male populations through administration of the androgen 17-α-methyltestosterone (MT) is considered to be the most effective and economically feasible method for obtaining all male tilapia populations (Guerrero and Guerrero, 1988). Such efficiency and simplicity in production techniques has resulted in hormone sex reversal becoming the commercial procedure of choice to produce male tilapia fingerlings and has been a significant factor in the rapid growth of the tilapia industry. However, failure to optimise the necessary parameters such as age and size of fry and feeding frequency can result in lower than expected rates of sex reversal (Mair et al., 1995). Concerns persist over

the safety of commercial sex reversal treatments both with regard to the safety of the farmer and of the consumer together with the possible environmental (hatchery, local environment) impacts. Hormone residues are likely to have negative impacts on environmental safety and could cause adverse developmental, neurobiological, genotoxic and carcinogenic effects (Al-Dobaib and Mousa, 2009); this procedure needs high level of control in use and dispersion of the products. In many countries, therefore, hormonal sex reversal is unacceptable.

The other major alternative way to produce (nearly) all males is the production of genetically male tilapia (GMT). Here supermales (YY) are created (Figure 1.1, usually the initial step is hormonal feminisation of XY males and crossing to untreated XY males), grown up as broodstock and crossed with females (XX) to obtain all normal males (XY).

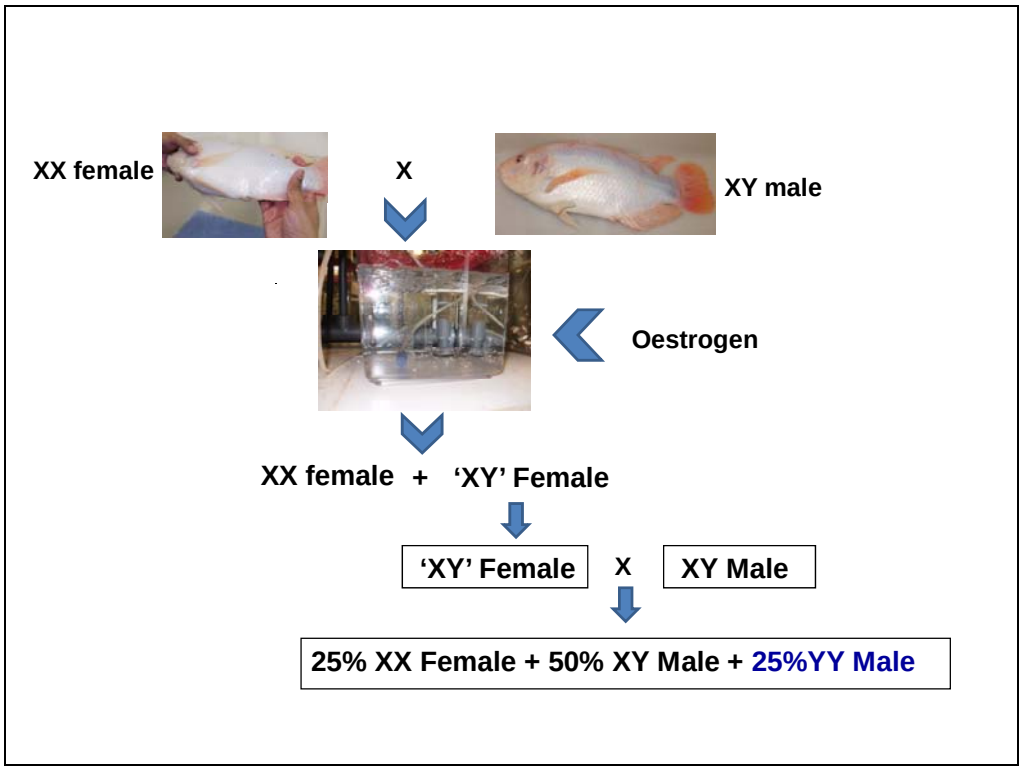


Figure 1.1 Conventional production technique of putative YY males

The success of this technology depends on the production of 'true' YY brood males with the hypothesis that the ultimate control of the sexual phenotype is by an XX/XY (male heterogametic) sex-determination system. The production of YY broods involves a number of crucial steps and once a pure YY line is established, it can be used for producing XY male progeny on a sustainable basis. The production of GMT from YY broodstock is an accepted, legal (environment-friendly) method of producing "all male" Nile tilapia.

However, the wide variation in progeny sex ratios resulting from putative YY questions the actual sex determination mechanism in this species. Putative YY-males have been found to produce 67-100% male populations (Mair et al., 1993). This wide variation indicates that the sex determining mechanism in tilapia is complex, and is a big barrier to the feasibility of application of this technology on a commercial scale. Therefore, a general understanding of sex determination mechanism in this species along with a comparison of that with other animals is needed before undertaking any large scale monosex production scheme.

1.4 Sex determination system in different animals

Sex determination is the genetic and/or environmental process by which sexual identity is established in an organism. It begins with the initial commitment by embryonic cells to a particular sexual fate and ends with sex-specific terminal differentiation. Sex determination results in the development of individuals with characteristics that allow them to be identified as males, females, or in some cases, hermaphrodites. In certain species, like the soil nematode *Caenorhabditis elegans*, differences in sexual characteristics can be very small (in *C. elegans*, the only distinguishing sexual characteristic is the presence of a testis

versus an ovotestis). In other species, the phenotypic differences between the sexes can be quite significant (Hake and O'Connor, 2008).

The first major breakthrough in understanding sex determination was the discovery of sex chromosomes in the early 1900s (Wenrich, 1946). From meticulous analyses of male and female insect chromosomes, scientists discovered that, although most chromosomes were present in equal numbers in both males and females, there were one or two additional chromosomes that were unequally represented in the two sexes. Analyses of additional species over the years have revealed that such chromosomal differences are associated with sex determination in many animals. However, this is not certain in all animals, and a subject of investigation whether single locus (XY or WZ) plays primary role (rather than chromosomal differentiation) in sex determining mechanism.

The mechanisms of sex determination can be classified into two broad categories: genetic sex determination (GSD) and environmental sex determination (ESD). The GSD system is principally based on the evidence of genetic factors and their variations between sexes, for example, presence or absence of heteromorphic sex chromosomes, allelic variation etc (Sarre et al., 2004). Sex determination by environmental factors such as temperature, pH, social environment etc defines ESD.

A brief description of sex determination systems (GSD and/or ESD) in other vertebrates, followed by a detailed discussion on the sex determination in some cultured fish species including tilapia is given in the next section.

1.4.1 Sex determination in humans and other mammals

The most defining moment in our lives is fertilization when we inherit either an X or a Y chromosome from our father- "the profoundly different journeys of male and female life are thus decided by a genetic coin toss" (Wilhelm et al., 2007).

In placental mammals, the presence of a Y chromosome determines sex. Normally, cells from females contain two X chromosomes, and cells from males contain an X and a Y chromosome. Occasionally, individuals are born with sex chromosome aneuploidies, and the sex of these individuals is always determined by the absence or presence of a Y chromosome (Hake and O'Connor, 2008).

Although the role of the Y chromosome in mammalian sex determination has been known since the early twentieth century, it was not until 1959 that scientists were able to identify the region of the Y chromosome that controlled this process (Jacobs and Strong, 1959). Later, researcher David C. Page analyzed the chromosomes of sex-reversed XX men (individuals who look like men but have two X chromosomes instead of one X chromosome and one Y chromosome). Using DNA hybridization with probes corresponding to different regions of the Y chromosome, he discovered that sex-reversed males carried genes from a 140-kilobase region on the short arm of the Y chromosome. Presumably, this region had been transferred to the X chromosome during a translocation (Page et al., 1985). Subsequent experiments narrowed down this region and found that the Y chromosome showed a reduced number of functional genes, containing between 70 and 200 genes along its 50-million base pair length (Noordam and Repping, 2006) and one gene, the sex-determining region of the Y, or *SRY*, was the master regulator of sex determination (Noordam and Repping, 2006). The Y chromosome induces testis formation

and thus male sexual development. Subsequent male sexual differentiation is largely a consequence of hormonal secretion from the testis. In the absence of a Y chromosome, gonads differentiate into ovaries and female development ensues. Molecular genetic studies have identified the Y-located testis determining gene SRY as well as autosomal and X-linked genes necessary for gonadal development. The phenotypes resulting from mutation of these genes, together with their patterns of expression, provide the basis for establishing a hierarchy of genes and their interactions in the mammalian sex determination pathway (Schafer and Goodfellow, 1996).

SRY encodes a protein containing a high mobility group (HMG) motif, which confers the ability to bind and to bend DNA. Genetic evidence supporting *SRY* as *TDF* (Testes determining factor) came from the observation of a male phenotype in XX mice transgenic for a small genomic fragment containing *Sry*, and from the study of XY sex-reversed individuals who harbor de novo mutations in the *SRY* coding sequence. Other non-Y-linked genes involved in sex determination were subsequently found by genetic analysis of XY sex-reversed patients not explained by mutations in *SRY*. These genes are *WT1* (Wilm tumor), *SF1* (Steroidogenic factor 1), *DAX1* (Divergent Antennapedia class homeobox gene 1) and *SOX9* (*SRY* related HMG-box 9). A regulatory cascade hypothesis for mammalian sex determination, proposing that SRY represses a negative regulator of male development, was supported by observation of mice that expressed a *DAX1* transgene and developed as XY sex-reversed females (Vilain and McCabe, 1998). The role of some sex-determining genes, such as *DAX1* and *SF1*, in the development of the entire reproductive axis, a functionally integrated endocrine axis, leads to a new concept. Normal sexual development may result from the functional and developmental integration of a number of different genes involved in sex determination, sexual differentiation, and sexual behavior.

In the absence of *SRY*, three genes may convert the undifferentiated gonad to become female sex organ. If the embryo has at least one X and lacks a Y chromosome, two genes work together to give the embryo the female phenotype. The first gene, called *DAX1* (Mizusaki et al., 2003), is found on the X chromosome. The second gene, *WNT4* is found on chromosome 1. Together, these genes stimulate the development of ovary tissue. However, despite their indubitable importance, the only other candidate that has emerged for this role to date is *FoxL2*, a member of the large family of forkhead/winged helix transcription factors, known to play important roles during vertebrate development (Kaufmann and Knochel, 1996). It is expressed in mesenchymal pregranulosa cells and later in granulosa cells (Schmidt et al., 2004). The ovary tissue excretes the hormone estrogen, which turns on other genes that control the development of the remaining female reproductive structures. Once testis or ovary differentiation has occurred, our sexual fate is further sealed through the action of sex-specific gonadal hormones.

Supporting evidence for *SRY* also derived from work in the mouse. First, *Sry* (the mouse ortholog) is deleted in a line of XY female mice (Gubbay et al., 1990). Second, *Sry* is expressed in the somatic component of the genital ridge at exactly the predicted time for testis determination, i.e., just before the appearance of testis cords (Koubova et al., 2006). Finally, transgenic XX mice carrying a genomic fragment containing the *Sry* gene develop as males (Koopman et al., 1991), which are sterile due to the adverse effect of two X chromosomes in spermatogenesis.

The early development of gamete appears to be controlled more by the environment than the genotype of the germ cells. Female primordial germ cells (PGCs) introduced in the testis will begin to differentiate into sperm, and male PGCs introduced into the ovary will

begin to differentiate into oocytes (Strachen and Read, 2004). The regulation of the cell cycle may be reflected by this since PGCs entering the testis arrest prior to meiosis while those entering the ovary commence meiosis immediately (Ross and Capel, 2005). Therefore, PGCs of either sex that constitute or colonise somatic tissue outside the gonad begin to differentiate into oocytes since there is no signal to arrest the cell cycle. In all of these unusual situations, however, functional gametes are not produced. Differentiation aborts at a relatively late stage, presumably because the genotype of the germ cells themselves also plays a critical role in gonad development (Strachen and Read, 2004).

Unlike the situation with primary sex characteristics, it appears that female secondary sex characteristics are the default state (Wilhelm et al., 2007). One of the genes regulated by *SRY* is *NR5A1*, which encodes another transcription factor steroidogenic factor. This activates genes required for the production of male sex hormones, including *HSD17B3* (encoding hydroxy steroid-17-β-dehydrogenase 3, which is required for testosterone synthesis) and *AMH* (the gene encoding anti-Mullerian hormone). Both hormones play important roles in the differentiation of male urogenital system (Nef and Parada, 2000). *AMH*, for example causes the Mullerian ducts (which become the fallopian tubes and uterus in females) to break down. Feminised XY individuals may be produced by the mutations inhibiting the production, distribution, elimination or perceptions of such hormones (Strachen and Read, 2004).

So, genes are not enough to make a male or female. To produce a human male requires not only the XY chromosome pair but also an adequate level of testosterone exposure during fetal development. If testosterone or the cellular receptors for it are lacking, as in androgen-insensitivity syndrome (AIS), an XY human may be born with female genitalia

and misidentified as a baby girl. AIS results from defects in the testosterone receptor, which prevents the body responding to the hormone even if it is produced at normal levels (Strachen and Read, 2004). XY individuals with this disease appear outwardly as normal females, but due to the effects of *SRY* and *AMH*, they possess undescended testes instead of ovaries, and they lack a uterus and fallopian tubes.

Conversely, if an XX fetus is exposed to a testosterone excess (from the adrenal glands), the labia may fuse into a scrotumlike sac, the clitoris may grow to resemble a penis, and the baby may be misidentified as a boy; this is called adrenogenital syndrome (AGS). The mistaken identity often comes to light only at puberty, when the individual fails to develop as he or she normally would for the mistakenly assumed sex. Such belated discovery of the child's genetic sex creates some difficult issues of gender identity (Villinski and Mattox, 2003).

Although testis development is brought about through the action of the sex determining region located on the short arm of the Y chromosome, correct doses of other genes on autosomes as well as the X chromosome, are also required. Sry appears to be widely expressed in human fetuses, suggesting the possibility that its influence on development is not confined to the testes (Mittwoch, 1997). There is additional evidence of a difference in developmental rates between XY and XX cleaving embryos, in which Sry and another gene in the sex-determining region named Zfy, for the zinc finger protein it encodes, are already expressed. These findings are consistent with the possibility that Y-chromosomal genes affect somatic sex differences prior to the formation of steroid hormones (Mittwoch, 1997).

1.4.2 Sex determination in birds

Birds are well known for their striking sexual dimorphisms, often characterised by gaudy plumage or ornamental feathering in males versus the more drab or cryptic colouration of females (Smith, 2010). Birds lack *SRY* and have a non-homologous set of sex chromosomes, designated ZZ male and ZW female (Griffiths, 1991; McBride et al., 1997; Stiglec et al., 2007). Research has been performed to reveal if it is the female-specific W chromosome of birds that causes the avian embryo to develop a female phenotype, analogous to the dominance mode of genetic sex differentiation seen in mammals, or it is the number of Z chromosomes that triggers male development, similar to the balance mode of differentiation seen in *Drosophila* and *C. elegans*. Although definite answers to these questions have not been given, some data have provided support for the latter hypothesis (Ellegren, 2000). The discovery that the Z-linked *DMRT1* gene, which is conserved across phyla as a gene involved in sexual differentiation, is expressed early in male development, which suggests that it might be the number of Z chromosomes that regulate sex in birds (Smith, 2010). On the other hand, the recent identification of the first protein unique to female birds, encoded by the W-linked *PKCIW* gene, and the observation that it is expressed early in female gonads, suggests that the W chromosome plays a role in avian sexual differentiation (Ellegren, 2001; Smith, 2010). Moreover, despite the potentially common features of sex determination in mammals and birds, comparative mapping shows that the avian sex chromosomes have a different autosomal origin than the mammalian X and Y chromosomes (Ellegren, 2000).

1.4.3 Sex determination in insects

When geneticists first began studying chromosomes in the early 1900s, insects were the organisms of choice. They are the most diverse class of organisms on the planet, so it is not too surprising that they show considerable diversity in their mechanisms of sex determination (Saccone et al., 2002). The majority of insects have dimorphic sex chromosomes that can be distinguished cytologically.

Clarence Erwin McClung (American geneticist and paleontologist) discovered the role of chromosomes in sex determination in a species of grasshopper (http://www.todayinsci.com). In 1901, he determined that female grasshoppers had two X chromosomes, but males had one. This arrangement, now known as XX-XO, with the O representing a lack of a chromosome, occurs in many insects. For these organisms, the number of X chromosomes in relation to the autosomal chromosomes (X: A) determines maleness or femaleness. A similar situation occurs in fruit flies. Male fruit flies are XY, but the Y does not have any sex-determining genes on it. Instead, sex is determined by the number of X chromosomes compared to the number of sets of autosomes (Suzuki, 2010). An XX fly with two sets of autosomes would be female. An XY fly with two sets of autosomes would be male.

Bees and wasps have no sex chromosomes at all (Robinson, 2005). Instead, they reproduce by haplodiploidy. How gender is determined under haplodiploidy in the absence of heteromorphic sex chromosomes is still an unanswered question although much progress has been made in recent years. Under haplodiploidy, males and females differ in ploidy level; females are diploid and develop from fertilized eggs, whereas males are haploid and develop parthenogenetically from unfertilized eggs. This mode of reproduction occurs in several invertebrate groups including pinworms, mites, thrips, and beetles, but occurs

ubiquitously only in the hymenopteran insects (ants, bees, wasps and sawflies). Almost all knowledge about the genetics of sex determination in haplodiploid systems has been obtained from this group of insects, of which the honeybee, *Apis mellifera*, and the jewel wasp *Nasonia vitripennis*, have been investigated most intensively (Beukeboom and Van de Zande, 2010). Recently, the complete genome sequences of these two species have been published (The Honeybee Genome Sequencing Consortium 2006; Werren et al., 2010), increasing their worth as hymenopteran model organisms. For a long time it has been known that multiple different sex-determining mechanisms exist within the Hymenoptera. Whiting (1933) was the first to show that sex determination in the wasp *Bracon* depends on the allelic state of a single locus. This mode of sex determination is called complementary sex determination (CSD). According to this model, if an individual is heterozygous for a certain locus, it develops into a female, whereas hemizygous and homozygous individuals develop into males. Since hymenopteran mother and sons share the same genes they are especially sensitive to inbreeding which may cause reduction in the number of different sex alleles present in a population, hence increasing the occurrence of diploid males (but infertile). This model of sex determination has now been reported for over 60 hymenopteran species (VanWilgenburg et al., 2006), including the honeybee.

The species of the insect order Lepidoptera (moths and butterflies) and their closest relatives, Trichoptera (caddis flies), share a female-heterogametic sex chromosome system. Originally a Z/ZZ (female/male) system, it evolved by chromosome rearrangement to a WZ/ZZ (female/male) system in the most species-rich branch of Lepidoptera. Further sporadic rearrangements created multi-sex chromosome systems; sporadic losses of the W changed the system formally back to Z/ZZ in some species. Primary sex determination

depends on a Z-counting mechanism in Z/ZZ species, but on a female-determining gene, *Fem*, in the W chromosome of the silkworm (Traut et al., 2007).

Study of the sex chromosomes of the *Drosophila melanogaster* (fruit fly) has played an important role in our understanding of heredity. Sex is primarily determined by the X: A ratio in this species (Cline and Meyer, 1996) and is independent of the Y chromosome. Conventionally, it is thought that the ratio of the number of X chromosomes to autosomes (X: A) constitutes the signal to determine the sex because XX: AA are females, X: AA are males and triploid flies bearing two X chromosomes and three sets of autosomes (XX: AAA) are intersexual. Under this model, the X: A signal is defined as the balance between a set of X-linked "numerator" proteins that promote female development and autosomally encoded "denominator" proteins that counteract the numerator elements. Erickson and Quintero (2007) claimed that although the X: A signal is a textbook standard, only one strong denominator element exists, and it cannot account for the effects of altered chromosome number (ploidy) on sex. To understand how X and autosome doses influence sex, they examined haploids (1X; 1A) and triploids during the brief embryonic period when sex is determined and found that ploidy affects sex indirectly by increasing in haploids, or decreasing in triploids, the number of embryonic cell cycles in which chromosomal sex is assessed. Their findings indicate that the fly sex-determination signal is more accurately viewed as a function of the number of X chromosomes rather than as a value of the X: A ratio.

Though the primary signal for sex determination seems quite variable among different insects, lower down the cascade there are some conserved genes or functions. These include the *transformer* (*tra*) and *doublesex* (*dsx*) genes (Dafa'alla et al., 2010). Both of

these genes were first identified in *Drosophila melanogaster* in classical genetic screens, loss-of-function mutants showing abnormalities in sexual differentiation (Baker and Ridge, 1980). The sex-specific alternative splicing of *tra* is regulated by *Sex-lethal* (*Sxl*). *tra* in turn regulates the sex specific alternative splicing of *dsx*. *Sxl* has therefore been described as the 'master switch' for sex determination in *Drosophila*. Homologues of *Sxl* have been identified in different groups of insects including other dipterans (Saccone et al., 1998; Lagos et al., 2005) and lepidopterans (Niimi et al., 2006), hymenopterans and coleopterans (Traut et al., 2006). However, the sex determination role of *Sxl* does not seem to be conserved or ancestral, rather it appears to be a recently acquired novel function restricted to a rather small group of insects (Dafa'alla et al., 2010).

1.4.4 Sex determination in fish with particular emphasis on tilapia

Reproductive mechanisms in fish are highly variable (Devlin and Nagahama, 2002). Most fish reproduce sexually. However a few have an asexual method of reproduction, for example, sailfin molly (*Poecilia formosa*), consists of all-female gynogens and to initiate embryogenesis, mating with a male (who does not contribute genetic material) from a related species (*P. latipinna*) is necessary (Schlupp and Ryan, 1996). Some fish are protandrous hermaphrodites, which change sex from male to female, for example, Asian sea bass, *Lates calcarifer* (Guiguen et al., 1993) and some are protogynous which change sex from female to male, for example, bluehead wrasse *Thalassoma bifasciatum* (Kramer and Imbriano, 1997).

Among gonochoristic fish species, both genetic and environmental mechanisms of sex determination are at work. Genetic sex is determined on the basis of inheritance of major sex factors (including chromosomal systems) and/or minor sex factors (polyfactorial with

no differentiated sex chromosomes). Chromosomal sex determination systems are classified into XX-XY (male heterogamety, for example in *Oncorhynchus mykiss*, Thorgaard, 1977; *Cyprinus carpio*, Nagy et al., 1981), WZ-ZZ (female heterogamety, for example in *Aulopus japonicas* with highly differentiated sex chromosomes: Ota et al., 2003; *Oreochromis aureus*: Guerrero, 1975, Campos-Ramos et al., 2001; two unlinked loci predicted by major WZ-ZZ and secondary XX-XY and have also been postulated to work in this species, Lee et al., 2004), multiple sex chromosomes ($X_1X_1X_2X_2$-X_1X_2Y in *Eigenmannia* sp and XX-X_1X_2Y in *Hoplias* sp for examples; Almeida Toledo and Foresti, 2001) and multifactorial systems (e.g., in *Xiphophorus maculatus*, male is XY or YY and female is XX, WX or WY; Kallman, 1984). In addition to major sex determination loci, other sex determining genes may influence the sex determination in some fish species such as blue poecilia (Kosswig, 1964) and *X. helleri* (Kallman, 1984).

Direct chromosome staining can be useful for detecting morphologically differentiated sex chromosomes in a few species (e.g., lake trout, *Salvelinus* sp: Reed et al., 1995). In *Leporinus* and *Clarias* sp, they have been used to identify female heterogamety (WZ-ZZ system; Pandey and Lakra, 1997). Most often the chromosomes are not morphologically differentiated and cannot be identified by chromosome staining techniques. In such cases, it requires other techniques (or combination of techniques) to analyse the system. For example, in salmonids and channel catfish, the evidence of female homogamety was revealed by hormonal sex reversal and progeny testing (Johnstone et al., 1978; Davis et al., 1990). Other genome manipulation techniques, for example chromosome set manipulations, have also been used (e.g., in *Cyprinus carpio*, Komen et al., 1991). Difficulty in sex chromosome identification has also led researchers to put considerable

effort to produce Y-specific probes, e. g., for several species of *Oncorhynchus* (Devlin et al., 1994; Donaldson and Devlin, 1996; Nakayama et al., 1998).

Environmental sex determination mechanisms, principally involving temperature, have been suggested for a range of fish. The first evidence of temperature dependent sex determination (TSD) was found in the atherinid Atlantic silverside, *Menidia menidia* (Conover and Kynard, 1981), where higher temperatures during a thermosensitive period during larval development result in a higher incidence of males. Sea lampreys, *Petromyzon marinus* (Beamish, 1993), American eels, *Anguilla rostrara* (Krueger and Oliveira, 1999), *Odontesthes bonariensis* (Strussman et al., 1997), sockeye salmon, *Oncorhynchus nerka* (Craig et al., 1996), channel catfish, *Ictalurus punctatus* (Patino et al., 1996) and seabass, *Dicentrarchus labrax* (Blazquez et al., 1998) are some more examples (out of around 60 species) of fish with TSD. Three major types of reaction norms have been postulated concerning the response of sex ratios to temperature (Reviewed by Penman and Piferrer, 2008): I) the number of males increases with temperature, II) the number of males decreases with temperature, and III) the number of males is higher at extreme (high and low) temperatures. The temperature or other environmental factors can affect (overlay or modify) sex ratios depending on the genetic background (Conover et al., 1992; Strüssman et al., 1997; Abucay et al., 1999). But measurement of the interactions between genotypic and environment factors is a complex trait since different populations and strains may exhibit different degrees of interactions depending on the genetic constitution and the relative strength of the sex factors (Mylonas et al., 2005). Other environmental factors, for example, pH can affect the sex ratios in the genus *Apistogramma* (Römer and Beisenherz, 1996) that may use changes in water pH brought by periodic precipitation as an environmental cue to determine sex.

The use of genetic markers to identify sex is relatively new but is now a widely used approach. Various methods, e.g., RAPD (Random Amplification of Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), VNTR (Variable Number Tandem Repeats) can be applied to identify sex-linked or sex-specific molecular markers in species with well established genetic sex determination such as in salmonids (Devlin et al., 2001), in three spine stickleback (Griffiths et al., 2000), in platyfish (Coughlan et al., 1999; Nanda et al., 2000) and also in some catfishes (Galbusera et al., 2000). The use of molecular markers is discussed in more detail (section 1.5) following discussion on tilapia sex determination systems.

Tilapia have been the focus of considerable biological research with genetics receiving much emphasis (Mair et al., 1997). The success of producing genetically monosex tilapia (as opposed to sex reversed tilapia production, SRT) progeny for commercial ventures depends on an understanding of the sex determining mechanism and application of this knowledge to precisely manipulate the genetics. The knowledge of the precise action of genetic influences (as well as other factors) on sex determination is likely to facilitate genetic manipulation in a proper and a predictable way that would allow monosex production of this species.

The system of sex determination has been analyzed using a variety of procedures for many species without differentiated sex chromosomes and where no appropriate markers existed. Analysing phenotypic sex ratios among fish produced by hybridisation, chromosome set manipulations and progeny testing of sex-reversed individuals has allowed identification of the basic genetic sex determining systems, XX-XY or WZ-ZZ (Reviewed by Penman and Piferrer, 2008). However, the precise mechanism of determining the sex in tilapia,

particularly in Nile tilapia, is not well understood. Early hypotheses were based on the sex ratios observed in hybrid crosses of different species (Chen, 1969). Later, Avtalion and Hammerman, (1978) developed a theory of autosomal influence to explain sex ratios in hybrids. Most researchers suggest that Nile tilapia has a predominantly monofactorial mechanism of sex determination with heterogametic XY males and homogametic XX females (Calhoun and Shelton, 1983; Mair et al., 1991a). However, this theory fails to explain some deviations from predicted sex ratios based on studies involving sex reversal and chromosome set manipulation (Mair et al., 1997). The existence of an autosomal sex modifying locus (with alleles *SR* and *sr*) epistatic to the gonosomal locus was hypothesized by Hussain et al. (1994). This hypothesis was developed to explain the occurrence of varying proportions of males in heterozygous and homozygous meiotic and mitotic gynogenetic progeny (Mair et al., 1991a; Hussain et al., 1994); the autosomal gene induces female to male sex reversal when sr is homozygous. However, this hypothesis still fails to explain some of the aberrant sex ratios observed in crosses of hormonally sex reversed fish. Additional autosomal influences and/or environmental influences have been suggested (Mair et al. 1990; Trombka and Avtalion 1993).

Environmental effects on sex differentiation of tilapia have also been demonstrated. Baroiller et al. (1995) presented evidence for a temperature effect on sex differentiation. They observed significantly higher proportions of males in progeny reared at high temperatures (36 °C) in putative all-female progeny from sex reversed neomales (XX) crossed with normal females. Abucay et al. (1999) reported the effects of environmental conditions during the period of sex differentiation on the sex ratio of the Nile tilapia (*Oreochromis niloticus*). Different sex genotypes were exposed to varying temperatures (putative all-female, all-male and all-YY males) and salinities (putative all-female progeny

only) for a minimum period of 21 days after first feeding and were on grown prior to sexing by gonad squash. The majority of the putative all-female progeny exposed to high temperature (36.54±0.39 °C) produced significantly higher percentages of males (up to 44.23%) compared to controls reared at ambient temperature (27.87±1.40 °C). Similarly, at high temperature, some of the all-male and YY male progenies had significantly lower percentage of males (up to 73.39%) compared to controls. Sex differentiation in YY males appears to be more labile than in normal XY males although this could possibly be attributable to different levels of inbreeding. Low temperature (25.78±0.24 °C) and varying levels of salinity (11.30 to 26.65 ppt) did not significantly affect sex ratios. An overall theory on how this environmental factor acts is therefore difficult to develop. Thus, currently available data indicate that sex determination in *O. niloticus*, while influenced by several factors, is best described as "predominantly monofactorial", with an underlying mechanism of male heterogamety playing the major role (Mair et al., 1997).

Cytogenetics (the branch of biology that deals with heredity and the cellular components, particularly chromosomes, associated with heredity) can give some hints to identify the sex determining mechanism in some fish. In tilapias, the karyotype analyses (organised profile of chromosomes) do not distinguish heterogametic sex chromosomes. When homologous chromosomes were observed with electron microscopy and were tightly paired, two unpaired regions were observed in the nuclei of female (heterogametic) *O. aureus* (Campos-Ramos et al. 2001). The two regions were on different chromosomes. There were no unpaired regions in the male. One of the unpaired regions corresponded to the unpaired region in bivalent 1 of male (heterogametic) *O. niloticus*. However, male Nile tilapia had only one such region whereas blue tilapia had two. It appears that *O. aureus* may have two

pairs of sex chromosomes which may contribute to the observed sex ratios, which is also supported by the works of Mair et al. (1991b) and Lee et al. (2004).

It seems from the discussion, there are a number of proposed theories on the mechanism of tilapia sex determination. However, none describes the mechanism specifically enough to account for all the variation observed. The uncertainty in understanding the exact process of sex determination mechanism in tilapia resulting from unpredictable sex ratios and other variable factors (especially unspecific minor-sex determining factors) raises questions as to whether sex inheritance in tilapia is predictable enough for a YY breeding program to be practical on the basis of simple Mendelian inheritance.

1.5. Molecular markers in fish sex determination mechanisms

DNA markers are expressed regions of DNA (genes) or more often some segment of DNA with no known coding function but whose pattern of inheritance can be determined (Avise, 1994). For aquaculture species, three types of polymorphic markers are predominant: AFLPs (Amplified Fragment Length Polymorphism), microsatellites and SNPs (Single Nucleotide Polymorphism). The value of each type of marker depends on the application for which they are intended, their ease and cost of genotyping and their level of information content i.e. how many different alleles are present in the population at the marker locus and the frequency of these alleles.

AFLPs are the result of PCR amplification of a subset of genomic fragments after the DNA has been cut with restriction enzymes and PCR primer binding sites have been ligated to the ends. These amplified fragments are then analysed by gel electrophoresis which

generates bands to be compared for polymorphisms. As they are dominant markers, it is not possible to distinguish between the heterozygote and dominant homozygote. As a result their information content is low. Microsatellite markers are generally dinucleotide repeats, and the alleles are scored according to the size, in base pairs, of the amplified DNA fragments containing the repeated DNA motifs (generally AT or CG). They are often highly polymorphic i.e., many alleles in a population and have high information content. However, detection of microsatellites is more difficult than detecting AFLPs (locus-specific primers are required) and they are more expensive as well to genotype. They may also not be present in sufficient density in the genome to be closely linked to genes and can affect mapping of genes responsible for important traits in aquaculture production. SNPs occur very frequently throughout the genome, approximately 1 every 1000 bases. They are not as informative as microsatellites are because they have a maximum of two alleles. Generally five SNPs are required to give the same amount of information as a single microsatellite (Glaubitz et al., 2003).

Molecular markers can play a vital role to locate master switch genes for sex determination. Unlike humans and other mammals where the male-inducing master sex-determining gene is SRY, no master sex-determination gene has yet been unambiguously identified in fish or other non-mammalian vertebrates. However, Matsuda et al. (2002) have described an outstanding candidate and subsequent research (for example, transgenic researches) has strengthened the evidence for the first master sex-determining gene in fish, from medaka, *Oryzias latipes*. Sex determination in medaka involves male heterogamety. Unlike human sex chromosomes, there is no visible cytogenetic difference between X and Y in medaka, and X-Y pairing occurs along almost the complete chromosome length. This suggests that the male-determining region on the Y chromosome should be relatively small

(Matsuda et al., 2002). They restricted this region to 530 kb by positional cloning and identified 52 putative genes by sequencing of about 422 kb from this region. Deletion analysis of the Y chromosome of an XY congenic female further shortened the region to 250 kb, containing 27 candidate genes. Only three of these were expressed in embryos; and only one gene, called *DMY*, was expressed exclusively in XY embryos and was present on the Y but not on the X chromosome. *DMY* has also been found in *O. curvinotus*, the most closely related species to medaka (Kondo et al., 2003). Functional evidence of the male sex-determining role of *DMY* in these species has been obtained with loss-of-function and gain-of-function studies. Thus, knockdown of *DMY* results in the initiation of female development in genetically male medaka (Paul-Prasanth et al., 2006) whereas transgenic *DMY* induced male development in genetically female medaka (Matsuda et al., 2007). However, the presence of some spontaneous XX males that may contain autosomal modifiers for sex determination (Nanda et al., 2003) suggests that *DMY* may not always be essential for male development and may be in the process of replacement by another master gene derived from one of such autosomal modifiers (Takehana et al., 2007). Such modifiers are normally rare, and caused by alleles of autosomal loci which can influence or override the master (sex chromosomal) gene(s) (Penman and Piferrer, 2008). In zebra fish or in puffer fish, no master gene(s) or sex-linked markers have been identified although these are well-studied fish species (Volff, 2005; Charlesworth and Mank, 2010).

Appropriate sex linked and sex specific markers are useful to reduce the time and effort involved in progeny testing. For example, identification of XX neomales by the absence of a Y-specific DNA marker (Devlin et al., 1994) allows separation of these fish from XY males as soon as they are large enough to be tagged. However, it is likely that in most cases only a very small proportion of the genome will be sex-specific or tightly sex-linked.

Simply screening male and female fish taken at random from species with an unknown or poorly characterized sex determination system has a relatively low probability of success (e.g., several sturgeon species), which could be due to the size of the genome, the number of markers screened and the proportion of the genome (if any) that is sex-specific in the species studied (Penman and Piferrer, 2008). Bulked segregant analysis (BSA; separate pooling of DNA from males and females) is therefore likely to be more useful (Ezaz et al., 2004b; Felip et al., 2005) for detecting sex-linked as well as sex-specific markers.

1.6 Genetic mapping in Tilapia

Since most performance and production traits are controlled by multiple genes and therefore inherited as quantitative traits, analysis of their associated quantitative trait loci (QTL) is emerging as a very important part of aquaculture genetics/genomics. QTL are largely unidentified genes that affect performance traits (such as growth rate and disease resistance) that are important to breeders (Liu and Cordes, 2004). Linkage markers for unknown single gene traits (e.g., sex) can also be useful for marker-assisted selection and positional cloning.

Relative chromosomal positions of QTL in a species genome can be identified in a two-step process that begins by constructing a genetic linkage map. Genetic linkage maps are constructed by assigning (mapping out) polymorphic DNA markers (such as microsatellites, SNP, or AFLPs) to chromosome configurations based on their segregation relationships. This requires two elements: polymorphic DNA markers and families in which these markers segregate. Once a linkage map has been constructed for a given species, it can be used in combination with studies of breeding and assessment of

quantitative traits to identify markers that are closely associated (linked) to QTL of interest, thus allowing the QTL to be positioned on the linkage map. This information can then be used in selection within a population to maximize growth, disease resistance, or some other desirable trait through marker-assisted selection (MAS). Typically, evenly spaced markers covering the entire genome are selected for screening of trait-linked markers, and this process is known as a genome scan for QTL. Once the QTL are mapped to a chromosomal region, fine mapping can be conducted using polymorphic markers near the chromosomal regions containing the QTL.

One of the problems faced by aquaculture is that some of the resources required to locate QTL accurately, such as dense linkage maps, are not yet available for many species. Thus the limitations for marker assisted selection are the number of genetic markers and QTL maps as well as suitable population for QTL detection. Recently, however, information from expressed sequence tag (EST) databases has been used to develop molecular markers such as microsatellites and single nucleotide polymorphisms (SNPs) (Martinez, 2007). Genetic maps are available for some cultured species, for example, tilapia, catfish, tiger shrimp, kuruma prawn, Japanese flounder, rainbow trout, carp, sea bass, sea bream and Atlantic salmon. However, the density of these genetic maps is very low with the map for rainbow trout having the highest density (1359 genetic markers and a sex phenotype: Nichols et al., 2003). A second generation map was also developed in rainbow trout ordering 1124 microsatellite loci spanning a sex-averaged distance of 2927.10 cM (Kosambi) and having 2.6 cM resolution was constructed (Rexroad III et al., 2008). In tilapia, linkage maps have also been produced, using different mapping approaches. The microsatellite DNA markers that were developed by Lee and Kocher (1996) were used in these studies.

First generation maps in tilapia

The first comprehensive attempt by Kocher et al. (1998) to map the tilapia genome documented the segregation of 62 microsatellite and 112 AFLP markers in 41 haploid embryos derived from a single *O. niloticus* female. The map consisted of 30 linkage groups spanning 704 cM and an estimated total map length of approximately 1,200 cM. McConnell et al. (2000) used 49 offspring of a backcross (male *O. niloticus* × *O. aureus* that was crossed with *O. niloticus* female). A partial genetic linkage map was constructed. The *O. aureus* male linkage map comprised 28 markers and 10 linkage groups, covering 213 cM, and a smaller genetic linkage map of the *O. niloticus* female comprised nine markers and four linkage groups, covering 41 cM.

Second generation map in tilapia

A second-generation linkage map of tilapia from the F_2 progeny of an interspecific cross between *O. aureus* and *O. niloticus* was constructed following the development of many more microsatellite markers. This map contains 525 microsatellite and 21 gene-based markers. It spans 1,311 cM, for an average marker spacing of 2.4 cM. The markers are linked in 24 linkage groups, 22 large and two small ones (now 22 LGs, Shirak et al., 2006; J.F.Baroiller, pers. comn). The linkages and order of markers in this map are largely congruent with the previous linkage maps of tilapia (Kocher et al., 1998; Agresti et al., 2000; McConnell et al., 2000).

This more comprehensive linkage map of the tilapia genome is one of the most extensive linkage maps available for fishes. This map was based largely on sequenced microsatellite markers that are supposed to be highly polymorphic and therefore informative in most crosses. The distribution of recombination along linkage groups can be very different

between males and females, suggesting that tilapia have a sex-specific pattern of recombination. Although the overall levels of recombination are nearly identical, internal linkages are often larger in females, while terminal linkages are larger in males (Lee et al., 2005). This map provides the infrastructure for systematic genome scans for detection of quantitative trait loci (QTL) in tilapias, as already demonstrated in several studies (Lee et al., 2003, 2004, 2005; Cnaani et al., 2004, 2008).

Sex-linked markers have been identified based upon the linkage map, in *O. niloticus* and *O. aureus* (Lee et al., 2003, 2004; Shirak et al., 2006; Cnaani et al., 2008, Baroiller et al., 2009). Lee et al. (2003) demonstrated that markers in LG1 were tightly linked to sex in 95% of the individuals from two out of three crosses studied in Nile tilapia (XX/XY) but in the third family, markers did not segregate for the same Y-haplotype. Additional sex-determining factors were hypothesized but BSA did not show any association of markers and sex. In *O. aureus* two unlinked loci were found to interact to determine sex (Lee et al., 2004). Analysis of epistatic interactions among the loci suggests the action of a dominant male repressor (the W haplotype on LG 3) and a dominant male determiner (the Y haplotype on LG1). One third of the individuals with the ZZXX genotype under this model were female, in contrast to none of the fish with the ZZXY genotype. Two distinct QTL for sex determination in tilapias were reported on LG23 in a hybrid cross between *O. aureus* and *O.mossambicus* (Cnaani et al., 2003, 2004). A variety of sex-determining systems was demonstrated for these closely related species. The pattern of epistasis among the alleles of the different systems identified on LG1 and LG3 is complex. Cnaani et al. (2008) confirmed the complexity of sex determination by DNA marker segregation patterns showing variations in both the strain and the species amongst the tilapia group. Segregation of sex-linked DNA markers (Lee et al., 2003, 2004; Shirak et al., 2006; Cnaani et al.,

2008) complies with what has been postulated previously on the existence of a single or a multi-allelic major sex determinant as well as an additional epistatic locus (or perhaps several loci) presumably autosomal (Hammerman and Avtalion, 1979; Mair et al., 1991a; Baroiller et al., 1995, 1996; Mair et al., 1997; Baroiller and D'Cotta, 2001).

1.7 Marker Assisted Selection

Traits such as growth, disease resistance, fillet quality, feed efficiency and maturation are under selection in many aquaculture species. If DNA markers are close to a variable locus or QTL, in which one allele has a more favourable effect on a quantitative trait then this information can be used more accurately to select broodstock which are genetically superior for the quantitative trait. Marker-assisted selection (MAS) or genome-wide marker-assisted selection (G-MAS) using linkage disequilibrium within families or across populations is not widely used in aquaculture (Martinez, 2007) but their application in actual breeding programmes is expected to be a fertile area of research.

To implement marker-assisted selection, phenotypic, pedigree and marker information is combined to estimate the breeding values (Fernando and Grossman, 1988). If accuracy of predicting breeding values is already high without marker information, the DNA marker data will not add much extra accuracy (Goddard and Hayes, 2002). Simulation studies investigating the advantage of marker-assisted selection with a trait that is measured on all animals prior to selection, growth rate for example, found only marginal gains from additional marker data (Lande and Thompson, 1990) whereas for traits such as disease resistance or meat quality (e.g., fillet colour in salmon), the gains are much larger. The

breeding candidate should be genotyped prior to selection to see if the QTL have large effect and to increase the potential breeding value.

Information from flanking markers on each side of the QTL is more accurate unless a single marker is very closely linked to the QTL. In practice, using markers which are not closely linked to the QTL is difficult because large families are needed to estimate allele effects accurately and marker-QTL phase information i.e., which marker allele is associated with the favourable QTL allele in each family should be re-estimated (Hayes and Andersen, 2005). By contrast, very tightly linked markers are easy to utilize because reliability on estimates of the QTL allele effects obtained is higher.

The great advantage of marker-assisted selection is that it is possible to identify breeding candidates of high genetic value at an early age. Marker-assisted selection also reduces the time and labour effort in progeny testing. In salmon breeding programmes, markers linked to the QTL explaining large proportion of genetic variation for fillet colour and disease resistance could be available. IPN (infectious pancreatic necrosis) QTL has already been used in commercial breeding programmes for salmon in Scotland and Norway (Houston et al., 2008). In tilapia, marker-assisted selection (MAS) is quite new. MAS can be well applied to sort out potential Nile tilapia broodstock by early selection before maturity and help reduce the time and effort in breeding programmes. However, high-resolution genetic map saturated by markers in the vicinity of a target locus (gene) is one of the limitations of MAS in this species. The successful implementation of MAS in Nile tilapia would be useful to supplement conventional methods of monosex production approach by hormonal sex reversal and progeny testing.

1.8 Aims and objectives of the present research

The present work on inheritance of sex and sex-linked markers in Nile tilapia is focused on obtaining better understanding of the complex dynamics of sex determination system in this species. Segregations of DNA microsatellite markers from across the genome were carefully observed in a variety of crosses to obtain informative sex-linked markers and to apply these markers in selecting broodstock for genetically male tilapia production. The main objectives of this PhD were:

(1) To validate a clonal line of females of Nile tilapia to be used for sex determination studies using molecular markers (mostly microsatellite DNA markers)

(2) To analyse sex determination system in the Stirling population of Nile tilapia using molecular markers (mostly microsatellite DNA markers), including attempting to identify markers linked to unknown genes (by studying inheritance of markers from genome wide selection) that cause departures from the sex ratios predicted by an XX/XY system

(3) To demonstrate marker-assisted selection to produce progeny with close to 100% genetically male offspring

1.9 Structure of the present thesis

This research is structured as follows, starting with a general introduction (this Chapter) and materials and methods (Chapter 2), followed by three major experimental chapters, each of which contains a brief introduction and methodology with details on experimental results and discussion. The first experiment, validation of a clonal line as a reference line for sex determination studies is described in Chapter 3. Chapter 4 describes sex linkage study in three groups/types of families (based on the progeny sex ratios) in the Stirling Nile tilapia (*Oreochromis niloticus*) population involving the validated clonal females crossed with putative XY males (type 'A'), putative YY males (type 'B') and 'unknown' (type 'C') groups of males. Screened markers (mostly microsatellite DNA) from LG1 were used to observe and confirm the LG1-associated pattern of inheritance of phenotypic sex in type 'A' families. Screened markers from LG1, LG3 and LG23 were used (based on some works suggesting the presence of sex determining genes in different tilapia sp.) to investigate inheritance of DNA markers in type 'B' and type 'C' families for association with sex using bulked segregant analysis (BSA) of female and male DNA pools, followed by further analysis (on DNA from individuals) using informative markers in BSA. This Chapter also deals with genome wide scan of selected markers (except markers from LG1, LG3 and LG23), approximately evenly spaced from the rest 21 LGs, and study them in type 'B' families in BSA for any association with sex. Chapter 5 deals with marker-assisted selection for genetically male tilapia (GMT) production. Finally, a general discussion on the experimental outcomes with future implications of this research work is given in Chapter 6, followed by literature references and appendices.

Chapter 2. General Materials and Methods

2.1 Experimental fish and basic maintenance

The fish used throughout the study were the Nile tilapia, *Oreochromis niloticus* (Linnaeus), introduced to the University of Stirling in 1979 from Lake Manzala, Egypt. The basic maintenance of the experimental stock rigorously followed working procedures under ASPA (Animals Scientific Procedures Act, 1986) and monitored by the Home Office in the United Kingdom. An accredited training for personnel working under ASPA had to be followed and a Personal Licence obtained before carrying out experimental work with fish approved by ASPA.

All experimental fish were maintained in the Tropical Aquarium Facilities (TAF) of the Institute of Aquaculture, University of Stirling. Selection of broodstock was done initially from the TAF's broodstock in larger tanks and selected fish were transferred into separate tanks with labelling which included project and personal licence number, project holder's name, name of species, tank number, number of fish kept, the category of procedures (to be applied), starting date of the experiment and notes on any special requirements for the experimental fish in question.

Individual brood fish were held in the square (with rounded corners) fibreglass tanks (generally the males) or in glass aquaria (generally the females). Continuous water flow and aeration was in operation in each tank. All tanks were in water recirculating systems (Figure 2.1) within a controllable rearing environment and with proper facilities for filtering and cleaning the water before recycling back through the fish rearing tanks. New water was added to the systems to replace the water used to flush out fish waste materials as well as to make up water loss due to evaporation. The water quality parameters,

particularly dissolved oxygen, ammonia and nitrate and nitrite contents were checked periodically. The standard temperature of the water in the TAF was maintained at 27.5±0.5 ^{o}C.

Figure 2.1 Water Recirculatory System in Tropical Aquarium Facilities, with spawning circular tanks and glass aquaria (L) and circular and rectangular fibreglass tanks (R).

The main experimental brood stock kept in individual rearing tanks consisted of different genotypes:

i. Clonal line females (XX) (details are discussed in Chapter 3)
ii. Outbred XX females
iii. Putative YY females
iv. Clonal neomales (XX neomales)
v. Outbred XY males
vi. Putative YY males

2.2 Fish handling and breeding

Handling of fish was performed with care. Nets of proper mesh sizes were used to take the fish out of tanks and these were held in plastic buckets filled with water from storage tanks

(temperature 27.5 ^{o}C) before being transferred to the wet laboratory for experimental procedures. Nets were soaked in disinfectants (Total Farm Iodophor, Downland marketing Ltd, Carlisle; major components phosphoric acid, iodine and non-ionic surfactant) before and after use. A solution of 100 ml of Iodophor was used in 40 liters of warm water for this purpose. Fish were anesthetised to avoid excessive handling stress prior to tagging, breeding or fin biopsy sampling. For this purpose, benzocaine (ethyl-4-aminobenzoate, Sigma-Aldrich, UK) solution at a final concentration of 1:10,000 was used. A stock solution was first prepared by dissolving benzocaine powder at 10% (w/v) in ethanol. This concentration took about 5 minutes to anesthetise the fish.

Females were kept in glass aquaria so that they could be easily observed for signs of readiness to spawn. A swollen reddish genital papilla was the main sign of this. Females also showed nest building behaviour during the onset of spawning. Handling of such fish to breed them always followed sedation as described above. The anesthetic agent is absorbed across the gills (and referred to as inhalation anesthesia) resulting in gill and fin immobilization and loss of balance.

Stripping was carried out in the wet lab of the TAF. Male fish that were ready to give milt were kept in another bucket with aeration and anesthetised if the eggs of the female were of good quality (e.g., not under ripe or over ripe, and not whitish in external observation) and of sufficient quantity. The anesthetised fish was taken out of the bucket and placed on the bench on wet tissue paper. The eggs were released by slight ventral pressure (stripping) and kept in a Petri dish partially filled with clean water (temperature 27.5 °C) directly from the incubator system where the eggs were to be hatched. The spent fish was immediately

transferred to another bucket filled with oxygenated water for recovery. The male fish was then placed in anesthetic solution. Meanwhile, the collected eggs were washed several times with water to remove any faeces, mucus and scales. The washed eggs were left with enough water just to cover the eggs. The milt was collected from anaesthetised selected males in glass capillaries (1 mm diameter, Drummond Scientific Co. USA) directly from the urogenital pore of the male once the urine was drained, by applying ventral pressure. This avoided activation of the sperm. Milt was added to the eggs in a proportion of approximately 100µl of milt per 500 eggs and gently stirred for about 1 minute, then left standing for another 2-3 minutes to ensure maximum fertilization rate before they were transferred into a plastic downwelling incubator in a recirculation system. The spent fish were transferred to an aerated bucket filled with water for about 10 minutes for recovery and then returned to their original tanks.

Fertilized eggs were washed with clean water and transferred to a series of 750 ml round bottomed plastic jars (soft drink bottles) for incubation. These jars were connected to a recirculating system where warm water was fed from a 125 litre (L) overhead tank to the jars by gravity. The water from the overhead tank first passed through a 30 W UV sterilisation unit (flow rate 20 L/min, UV dosage 62,000 µW sec/cm^2), then through 20 mm PVC pipe to the jars. They received water from the PVC pipe flow via Perspex tubing connection with the flow in the jars being controlled by small airline taps in such a way that the eggs in the jars were kept in gentle motion at all times. UV treatment of the water system was done to prevent bacterial infection of the eggs. The wastewater was discharged into the bottom settling tank (180 L capacity) via two filters filled with shell unit positioned just above the settling tank. The shell filters helped to maintain the pH of the

system and act as a surface for bacteria. Fertilized eggs were kept in hatching incubators for a total period of 11 days before they were ready to transfer to circular plastic tanks. The initial number of eggs was recorded and dead eggs and embryos were removed by siphoning during this period.

Fry were transferred into 25 L circular plastic tanks in a recirculation system (except those used for sex reversal) immediately after the yolk sac was absorbed (day 12) and reared for 3 months. The fry density was 60 per tank. Fry for sex reversal experiments (section 2.4) were transferred into 10 L rectangular static tanks and kept for three weeks before transferring them in 25 L circular plastic tanks. The fish were immersed in an overdose of anesthesia (0.05% benzocaine) for about 10 minutes before destruction of the brain and being sexed at this age by gonad dissection and examination of a squash preparation (Guerrero and Guerrero, 1988). Fish that were kept for further crosses were kept either in similar 25 L tanks with densities of 20-25 fish/tank, a range suggested to minimize aggression, or in large tanks (2 m diameter).

Every brood fish was tagged by a TROVAN Passive Integrated Transponder (PIT) tag that had a unique 10 digit code. The routine technique of anesthesia was followed before introducing the tag with the aid of a special wide tip syringe (previously disinfected in 70% ethanol) on the lateral-abdominal side of the fish, lifting a scale and making an incision under it. The incision was sealed with the same scale and fish was immediately placed into clean aerated water until full recovery before return to the original tank or an aquarium to rear as broodstock.

2.3 Feeding regimes

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; manufacturer Skretting, Preston, UK; Product code 470405) twice a day. Hatchlings were fed with powdered food (0.25-0.50 mm in diameter), prepared by grinding feed size 5 (4 mm diameter) and fed *ad libitum* for the first four to six weeks followed by feeding a mixture of ground food and no. 3 feed size as the fish grew. Advanced fry and fingerlings weighing between 5 g to 40 g received no. 3 size feed twice a day at a rate of approximately 5% of their body weight. Fish weighing 40 g to 80 g and >80 g were fed with no. 4 and no. 5 sized feed respectively, at a rate of approximately 2% of their body weight per day.

Composition of feed ingredients:

Wheat, Soybean meal, maize gluten (60%), fish meal, sunflower meal, fish oil, minerals, vitamins

Nutrient compositions:

Trout food no.3: 8% oil, 5.7% ash, 0.9% phosphorus, 38% protein, 4% fibre, +12000 iu/kg Vit.A, +2000 iu/kg Vit.D3, 100 iu/kg Vit.E as alpha tocopherol acetate, antioxidants (BHT, butylated hydroxytoluene; BHA, Butylated hydroxyanisole)

Trout food no.4: 18% oil, 9% ash, 1% phosphorus, 54% protein, 1% fibre, +12000 iu/kg Vit.A, +2000 iu/kg Vit.D3, 250 iu/kg Vit.E as alpha tocopherol acetate, antioxidants (BHT, BHA)

Trout Food no.5: 8% oil, 8% ash, 1.2% phosphorus, 40% protein, 2% fibre, 12000 iu/kg Vit.A, 2000 iu/kg Vit.D3, 100 iu/kg Vit.E as alpha tocopherol acetate, antioxidants (BHT, BHA)

2.4 Steroid food preparation

Steroid hormone treatment for masculinisation was performed in clonal female (inbred line) to change their sex to 'neomale' (phenotypically male, genetically female).To prepare 100 g of food for masculinisation, the procedure according to Abucay and Mair (1997) was carried out with some modifications, as follows: a 3 mg/ml stock solution of 17α-methyltestosterone (MT) hormone (Cat. No. M7252, SIGMA, UK) was prepared in absolute ethanol, then 1 ml of stock solution was diluted in 30 ml absolute ethanol. In a fume hood, this diluted solution (the whole 30 ml) was mixed with 100 g ground and sieved fish food then dried. The final concentration of the hormone was 30 mg/kg.

Steroid hormone treatment for feminisation was performed in XY and YY males to change their sex to 'neofemale' (phenotypically female, genetically male). To prepare 100 g of food for feminisation, 100 mg of diethylstilbestrol (DES) hormone (Cat. No. D4628, SIGMA, UK) was dissolved in 30 ml absolute ethanol. This stock solution was mixed with 100 g ground and sieved fish food by gently pouring the stock solution onto the food and stirring with a spatula to homogenise the sample. The final concentration of hormone in the food was 1g/kg.

The treated food was allowed to dry before being packed into individual sealed plastic bags, stored at 4°C and protected from direct light. Treated food was kept for a maximum of three months.

2.5 DNA extraction and quantification

2.5.1 Collection of tissue

Tissues from dorsal or anal fin were collected from live (broodstock) or dead (gonad-squashed offspring) fish and preserved in 95% ethanol in 1.5 ml Eppendorf tubes. The date of collection of tissue, PIT tag number, cross details and sample number were recorded. The same information as a laser printed label were also kept in every Eppendorf, immersed in ethanol with the finclips.

2.5.2 DNA extraction

Three extraction techniques were used:

i. Phenol-chloroform protocol (Taggart et al., 1992)

ii. REAL DNA extraction protocol (Purification kit by REAL laboratories, Spain)

iii. HotSHOT genomic DNA preparation

The first method is the standard and preferred way to extract high quality and reliable quantity of DNA. However, as the protocol was time-consuming and dealt with highly toxic phenol, this method was replaced with the other two as the number of samples increased.

The second technique of DNA extraction was easier compared to the phenol-chloroform method and could be used in tube or plate extraction method. The quality of DNA was good and the quantity was sufficient enough for the PCR analyses required, giving consistent results and allowing an efficient extraction of more samples per unit time (200 to 250 samples with one kit). This method was used extensively throughout the research work.

The third method of extraction was the quickest method of DNA extraction but the quality of DNA was not always good and too much starting tissue inhibited PCR reactions. This protocol yields relatively short DNA fragments but can work for PCR. This method was used during the later stages when time was a more limiting factor. These three methods of DNA extraction are described in detail below:

2.5.2.1 Phenol-chloroform extraction method

The tissue samples (approximately 50 mg) were placed in individual nucleic acid free 1.5 ml microcentrifuge tubes containing 340 µl of 0.2M EDTA (Ethylenediaminetetraacetic acid) solution (pH 8.0) with 0.5% SDS (Sodium lauroylsarcosine, Sigma). Ten µl of 20 mg/ml proteinase K (ABgene) was added to each tube, mixed briefly and the tube was incubated overnight at 55 ^{o}C in a hybridisation oven (Techne Hybridizer HB-1). During incubation, the tubes were tumbled to ensure constant and homogenous mixing. Following this step 10 µl of 20 mg/ml DNAse free RNAse (ABgene) was added to each tube, which was then shaken vigorously and incubated for 60 min at 37 ^{o}C in a hybridization oven. About 350-400 µl of buffered phenol (Fisher Scientific) was added to each tube and vortexed for 10 seconds. About 350-400 µl of chloroform (Fisher Scientific) was added to each tube and shaken vigorously for 10 sec. The tubes were centrifuged at 14,000 rcf for 5 min to separate the organic and aqueous phases. About 300 µl of the top aqueous layer was removed to a clean tube, carefully avoiding proteins at the aqueous: organic interface. About 900 µl of chilled 92% ethanol was added to the aqueous solution and mixed by vigorous inversion of the tubes 5-6 times to precipitate the DNA. After allowing the precipitate to stand for 2-3 min, most of the ethanol was carefully decanted off. One ml of 70% ethanol was added to wash the DNA pellet, centrifuged at 14,000 rcf for 2 min and

again decanted off. Finally the tubes were left in a rotary evaporator (Stuart Scientific) for around an hour to allow the ethanol to evaporate completely. The DNA was allowed to partially dry for 5-10 min at room temperature before resuspending in 50 µl TE buffer (10 mM Tris.Cl, 1 mM EDTA; pH 8.0). The DNA was stored at -20 ^{o}C in a freezer.

2.5.2.2 REAL DNA extraction protocol

The reagents were supplied with REAL DNA extraction kit (REAL laboratory, Spain). The tissue samples were placed in individual nucleic acid free 1.5 ml microcentrifuge tubes and 600 µl of lysis solution was added. Three µl of proteinase K was added, mixed with gentle vortexing for 1 min and kept in a hybridization oven overnight at 55 ^{o}C. Three µl of RNAse was added to each tube and gently vortexed for 1-2 min followed by incubation at 37 ^{o}C for 60 min. Samples were brought to room temperature and 360 µl of protein precipitation solution was added. Mixing was done by vortexing at high speed for 30 sec and then centrifuged at 14,000 rcf for 5-10 minutes. Precipitated protein formed a pellet. The supernatant containing the DNA was poured into a fresh microtube containing 300 µl of isopropanol and mixed a few times. The DNA pellet should be visible at this stage. The tubes were centrifuged at 14,000 rcf for 3 min and supernatant was removed by decanting off the tube or by pipetting. 600 µl of 70% ethanol was added, centrifuged at 14,000 rcf for 2 min to wash the pellet. Ethanol was removed carefully avoiding touching the pellet and the tubes were air-dried by keeping upside down on absorbent paper. Finally 100-250 µl hydration solution was added and incubated at 65 ^{o}C for 1 hr or keeping at room temperature overnight for proper dilution of DNA. DNA was stored at -20 ^{o}C (in freezer) for longer term storage.

2.5.2.3 HotSHOT (hot sodium hydroxide and tris) genomic DNA preparation

Extraction of genomic DNA using HotSHOT (Truett et al., 2000) method is one of the quickest methods of DNA extraction. Fin tissue (less than 10 mg) was placed in a 0.65 mL tube. 75 µl alkaline lysis reagent (Table 2.1) was added and the sample was heated to 95 ºC for 10 minutes to an hour (30 minutes is optimal) in a thermocycler. The solution was cooled to 4 ºC (optional) and 75 µl neutralization buffer (Table 2.2) was added. DNA was used immediately.

Table 2.1 Preparation of Alkaline Lysis Reagent

Reagent	**Final Conc.**	**Amount for 200 mL**
NaOH	25 mM	200 mg
EDTA	0.2 mM	14.88 mg
Note: Distilled H_2O was added to make a final volume of 200 mL. pH of Alkaline Lysis Reagent would be 12. There was no need to adjust the pH of this solution.		

Table 2.2 Preparation of Neutralization Buffer

Reagent	**Final Conc.**	**Amount for 200 mL**
Tris-HCl	40 mM	1.3 g
Note: Distilled H_2O was added to make a final volume of 200 mL. pH of Neutralization Buffer will be 5. There was no need to adjust the pH of this solution.		

2.5.3 Genomic DNA quantification

Genomic DNA was quantified using a Nanodrop (ND-1000) spectrophotometer. DNA concentration was standardised to 100 ng/µl by adjusting with molecular grade water. The

quality of DNA was measured by OD parameter, OD260/OD280 = 1.8-2.0 being good enough for running PCR.

2.6 Polymerase chain reaction

Amplification of DNA was performed by Polymerase Chain Reaction (PCR). PCR was routinely performed in 0.2 ml well DNAse free plates.

Both ABgene™ Taq and KBioscience Taq DNA polymerase kit (UK) were used which included 10× Buffer IV (750 mMTris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$ and 0.1% (v/v) Tween20), 25 mM $MgCl_2$ and 5 U/µl Taq polymerase. Primer sequences were retrieved from NCBI database and ordered from MWG (Germany) or Sigma (UK). The stock concentration of primers was adjusted to 100 pmol/µl (100 µM) by resuspending in molecular grade water. PCR reactions were performed using a fluorescent labeled tailed primer method. This approach reduced the cost of purchasing individual fluorescent tagged primers for each locus under investigation. The principles of the tailed primer method (Raposo, 2001) are briefly described below:

In order for the DNA fragments to be detected by the Beckman-Coulter sequencer, they must be labeled with Beckman-Coulter dyes. The simplest way to label the DNA fragments is to incorporate the dye into a PCR product using a labeled primer. A less expensive way to label the DNA fragments is using the tailed primer method.

This method employs a two-part primer in which a standard primer sequence or "tail" is added to the 5'-end of the primer sequence. The tail sequence usually corresponds to a readily available standard primer such as an M13 universal primer. Only one primer used

in the PCR reaction needs to have the tail. There are three M13 universal primers labeled with the three different Beckman-Coulter dyes (5'Dye-CACGACGTTGTAAAACGAC-3'). So the tail on the forward or reverse primers should have the same sequence as the M13 labeled primers. In the example below, the forward primer is the one that has the M13 universal tail.

The amplification is performed with three primers; the forward primer with an M13 universal tail, a reverse primer (non-tailed, not to be confused with the M13 forward or reverse primers) and a labeled primer (with a Beckman-Coulter dye).

Initial synthesis of newly formed DNA is primed from M13-tailed primer and the reverse primer on the original template DNA. DNA generated in the first round of synthesis becomes the template for further amplification with either the forward or reverse primers. By the second round of amplification, the product being amplified will have the M13-tail sequence incorporated into the PCR product. Hence by the third round, being in 10 fold excess, the M13 labeled primer will take the place of the forward primer (the M13-tailed primer) to create an amplified product containing the fluorescence dye that can be detected by the Beckman-Coulter sequencer (Figure 2.2).

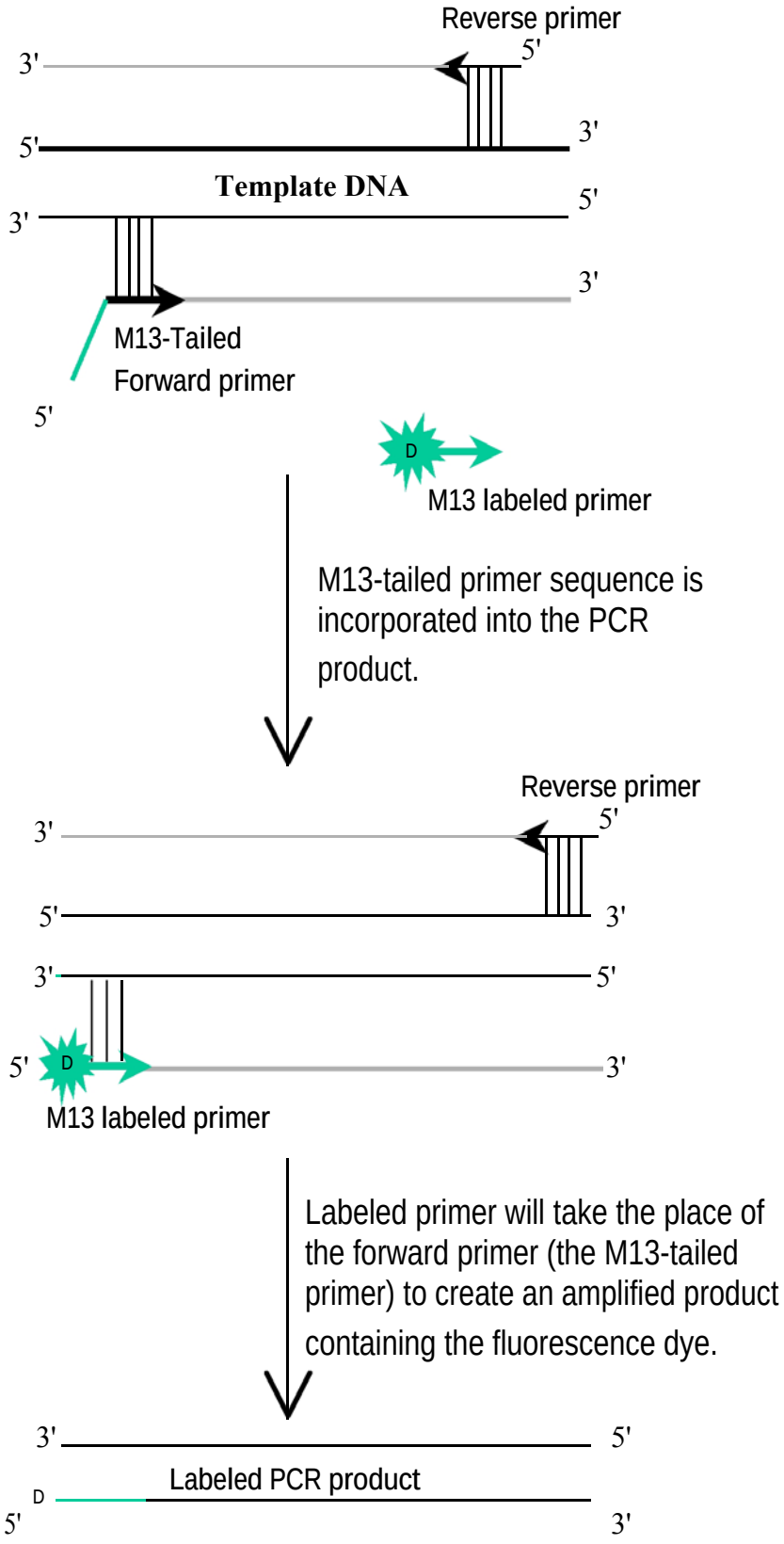


Fig. 2.2 Principles of 'tail' primer with Beckman-Coulter dyes (after Raposo, 2001)

Standard PCR reaction mixtures for amplification of DNA are given in Table 2.3.

Table 2.3 Calculated PCR recipe for 20 µl and 15 µl reaction mixture

Stock solution and components	**Per reaction**	**For 20 µl Mastermix**	**For 15 µl Mastermix**
10X Reaction buffer	1X	2 µl	1.5 µl
25 mM $MgCl_2$	1.5 mM	1.2 µl	0.9 µl
2 mM stock dNTPs	0.2 mM	2.0 µl	1.5 µl
10µM (10 pmol/µl) Labeled primer	0.3 µM	0.6 µl	0.45 µl
10µM (10 pmol/ µl) FW/RV primer	0.3 µM	0.6 µl	0.45 µl
5 µM Tailed primer	0.02µM	0.08 µl	0.06 µl
5 U/ µl Taq polymerase	0.05U/µl	0.2 µl	0.15 µl
H_20	-	12.32 µl	8.99 µl
DNA	0.05µg to 1 µg	1 µl	1 µl

The thermocylcer conditions varied for different fluorescent primers in polymerase chain reaction. A general amplification condition in tail primer method was:

Table 2.4 Thermocycler conditions for PCR

Step	**Temperature**	**Duration**	**Cycles**
Initial activation (with KBioscience Taq)	95 ºC	14 min	-
Denaturation	95 ºC	1 min	40
Annealing	57 ºC/58 ºC/60 ºC	1 min	
Extension	72 ºC	1 min	
Final extension	72 ºC	30 min	-

2.7 Genotyping

The labeled PCR fragments were genotyped using the CEQ 8800 capillary sequencer to observe the specific allele makeup of the individuals with reference to a specific character (i.e., sex) under consideration. For each capillary run, 0.9 µl product of single PCR reaction was added into a 96 well sequencer plate (Beckman Coulter®,USA) containing 30 µl SLS (Sample Loading Solution) and 0.25 µl DNA Size Standard kit-400 (SS400, Beckman Coulter®,USA) containing fragments labeled with D1-red dye. One drop of mineral oil was added on the top of each sample. An electrophoresis buffer tray, 96 well plate with flat bottom (Beckman Coulter®, USA), was prepared. Each row of 8 samples ran for 45 min using Beckman Frag-3 genotyping method.

2.8 Statistical analyses

Genotype data were generated automatically by the sequencer, and analysed later with the same Beckman-Coulter software (Fragment analysis module) which identified and quantified the detected allelic fragment. All genotype results were transferred to Excel spreadsheets and linkage analysis (wherever necessary) was performed using CRI-MAP (version 2.4). The significance of linkage between markers was hypothesized from LOD scores (significant LOD value >3.00) from two-point analyses in CRI-MAP. QTL Express was used to locate quantitative trait locus. Test of association (between markers and QTL) was done mainly by chi-square at its 0.05 level. Correlation studies (e.g., sex ratios from clonal and outbred females) were done using SPSS version 17.

Chapter 3

Validation of a clonal line of females as a reference line for sex determination studies

3.1 Introduction

Clonal individuals play a significant and expanding role in many scientific disciplines, including biomedicine, genomics, toxicology, immunology and evolutionary biology (Trevarrow and Robinson, 2004). They are central to much basic research and have promising applications in medicine, industry and conservation. Reliable cloning can be used to make farming more productive by replicating the best animals. It can make medical testing more accurate by providing test subjects that all react the same way to the same drug. It can allow mass production of genetically altered animals, plants and bacteria. It may replicate exceptional animals like rescue dogs. It may settle once and for all what part of personality is dependent on genetics and what part on environment. In short, it can be beneficial to almost every area of biological science.

The term "clones" refers to identical copies of genetic material, cells or whole organisms. Mitotically produced cells are genetically identical, and thus all the somatic cells of an individual are technically a clone. Monozygotic twins are clones, because they have identical DNA. Protozoa and bacteria can reproduce asexually by binary fission, a process in which a single-celled organism undergoes cell division (Nill, 2002) and results in two cells with identical genetic composition. Many plants reproduce asexually via a process known as apomixis. Some multicellular animals can also reproduce asexually by budding or binary fission (e.g., polyp separates into two halves in some species of sea anemone *Anthopleura elegantissima*) resulting in exact clones (Geller et al., 2005). However, the ability to intentionally create a clone in the animal kingdom involving complex laboratory

techniques on the cellular level is a relatively recent scientific advancement that is at the forefront of modern biology.

The first deliberate cloned animal was created by Hans Dreisch in the 1894 (Gould, 2007). Dreich's original goal was to prove that genetic material is not lost during cell division. Dreich's experiments involved sea urchins, which he picked because they have large embryo cells, and grow independently of their mothers. Dreich took a 2-celled embryo of a sea urchin and shook it in a beaker full of sea water until the two cells separated. Each grew independently, and formed a separate, whole sea urchin. In 1902, Hans Spemann, an embryologist, used a hair from his infant son as a knife to separate a 2-celled embryo of a salamander, which also grows externally. He later separated a single cell from a 16-celled embryo. In these experiments, both the large and the small embryos developed into identical adult salamanders (Gould, 2007). Major advances in cloning came in November of 1951, when a team of scientists in Philadelphia cloned a frog embryo by taking the nucleus out of a frog embryo cell and used it to replace the nucleus of an unfertilized frog egg cell. Once the egg cell detected that it had a full set of chromosomes, it began to divide and grow (Briggs and King, 1952). This nuclear transfer technology was later used to produce clone of the sheep, Dolly in 1996 followed by other mammals, e.g., cows, goats, horses, mules, pigs, rabbits, rats, mice, cats, and dogs (King et al., 2006), and essentially are outbred clonal individuals.

The recent scientific developments of cloning broadly cover three main methods: i) artificial embryo twinning, ii) somatic cell nuclear transfer (and microinjection), and iii) induced parthenogenesis.

Artificial embryo twinning mimics the natural process of creating identical twins. In nature, twins occur just after fertilization of an egg cell by a sperm cell. In rare cases, when the resulting fertilized egg, called a zygote, tries to divide into a two-celled embryo, the two cells separate. Each cell continues dividing on its own, ultimately developing into a separate individual within the mother. Since the two cells came from the same zygote, the resulting individuals are genetically identical. Artificial embryo twinning uses the same approach, but it occurs in a Petri dish (or any external media) instead of in the mother's body (http://learn.genetics.utah.edu). This is accomplished by manually separating a very early embryo into individual cells, and then allowing each cell to divide and develop on its own. The resulting embryos are placed into a surrogate mother, where they are carried to term and delivered. Again, since all the embryos came from the same zygote, they are genetically identical. The born animal is a clone of its brothers and sisters.

Somatic cell nuclear transfer (SCNT) uses a different approach than artificial embryo twinning, but it produces the same result: an exact clone, or genetic copy, of an individual. This was the method used to create Dolly the Sheep (Campbell et al., 1996). To make Dolly, researchers isolated a somatic cell from an adult female sheep. Next, they transferred the nucleus from that cell to an egg cell from which the nucleus had been removed. After a couple of chemical tweaks, the egg cell, with its new nucleus, was behaving just like a freshly fertilized zygote. It developed into an embryo, which was implanted into a surrogate mother and carried to term. The microinjection technique, also said to be a subset of nuclear transfer (fusion), uses only the adult cell nucleus to fuse with an egg cell rather than fusing entire adult animal cell. In nuclear transfer, the born animal is a clone of its parent.

Parthenogenesis (from the Greek, *parthenos*, "virgin", *genesis*, "birth": Liddel and Robert, 1940) is a form of mode of reproduction by which offspring arise from a single parent and does not involve meiosis, ploidy reduction, or fertilization. Parthenogenesis occurs naturally in certain invertebrates (e.g., water fleas, aphids, nematodes, some bees, some scorpion species, and parasitic wasps) and in some vertebrates, particularly in some reptiles and fish (Watts et al., 2006), and also has been recorded occasionally in mammals, including humans (Kono et al., 2004). The 'virgin birth' is also a religious tenet of Christianity and Islam (for the believers), which hold that Mary miraculously conceived Jesus (pbuh) while remaining a virgin (Gospels of Matthew and Luke; Quran, 3:47, 3:59, 66:12).

Induced parthenogenesis is artificially performed 'natural parthenogenesis' where offspring inherit the genes of a single parent only. Normal egg cells form after meiosis and are haploid, with half as many chromosomes as their mother's body cells. Haploid individuals, however, are usually non-viable, and parthenogenetic offspring usually have the diploid chromosome number. If the chromosome number of the haploid egg cell is doubled during development, the offspring is "half a clone" of its mother. If the egg cell was formed without meiosis, it is a full clone of its mother.

Fish species that have external fertilization can be reproduced (cloned) by induced parthenogenesis, e.g., gynogenesis or androgenesis (techniques reviewed by Dunham 2004; Komen and Thorgaard, 2007) that may result in clonal individuals or novel genotypes (such as YY males or WW females). The nuclear content of either the sperm or egg is destroyed by UV or gamma irradiation, and the treated gamete then is fused with an untreated egg or sperm to form a haploid embryo. The haploid embryo, which is a good

resource for gene-mapping (being equivalent to large single gametes that contain enough DNA for gene-mapping purposes; Kocher et al., 1998), will continue to develop but will normally die before hatching. However, it is possible to make the embryo diploid by inhibition of the second meiotic division (in gynogenesis) or first mitotic division; such individuals retain a duplicated set of chromosomes from the untreated gamete. Eggs fertilised by UV treated milt can become meiotic gynogenetic offspring by shocks that interfere with the second meiotic division, causing the retention of the second polar body. In terms of homozygosity, meiotic gynogenetic offspring are on average homozygous at 50% of the loci that were heterozygous in the mother because they retain a pair of sister chromatids, which undergo recombination (Purdom, 1969; Nace et al., 1970; Allendorf and Leary, 1984; Hussain et al., 1994). If the shock is delayed to suppress the first mitotic division (in mitotic gynogenesis or androgenesis) then two haploid copies of the maternal or paternal chromosomes respectively are retained to produce a mitotic gynogenetic offspring (also known as double haploid or dihaploid offspring). These offspring carry only the duplicated set of chromosomes and are, by definition, fully homozygous individuals.

Several authors have pointed out that the timing of the temperature or pressure shock should coincide with the prophase of the first mitotic division (Nagoya et al., 1990; Komen et al., 1991). However, embryonic development is probably highly asynchronous, as evidenced by the fact that the optimal "window" for heat or pressure shocks can be several minutes (Komen and Thorgaard, 2007). In common carp and zebrafish, a comparison has been made between window length and yields obtained when embryos were shocked immediately after fertilisation (when all eggs are in prophase of the second meiotic division) and those of embryos shocked to inhibit the first mitosis. In the first case, the

window was only 1-2 min and the yields could be as high as 50%. In the second case, the optimal window was 6-8 min, and the yield was correspondingly lower (Komen et al., 1991; Hörstgen-Schwark, 1993). However, there is no reduction in the length of the optimum window when comparing genetically uniform F_1 hybrids with outbred female common carp.

The dihaploids (mitotic gynogenetics or androgenetics) are homozygous at all loci but different individuals in the same family will be fixed for different alleles at any given locus depending on the recombinant event that generated that gamete. These dihaploid individuals from either a gynogenetic or androgenetic background can be used to generate clonal or isogenic lines as all gametes produced by such an individual will be identical, even after recombination. When these gametes are used in a second round of induced parthenogenesis, all of the offspring will be identical and clonal. Mitotic or meiotic gynogenesis, or androgenesis may be used in this second round (Hussain et al., 1998) to advance the line. It is therefore possible to generate clonal lines in as little as two generations for any particular species or strain of fish. However, it may be easier to hormonally sex reverse a proportion of the fish within a line to obtain both sexes (second generation or later), which then allows the line to be propagated by simple crosses. Additionally, it is possible to produce genetically uniform but non-inbred groups of fish by crossing between clonal lines which are referred to as outbred clones (Sarder et al., 1999).
Gynogenetic and androgenetic double haploids have been produced in at least 24 fish species (12 species of Cyprinidae, 3 species of Salmonidae, 3 marine species and 6 other fresh water species, reviewed by Komen and Thorgaard, 2007). Fully inbred clonal lines have been successfully produced in nine of these species by gynogenesis and four by

androgenesis. Table 3.1 presents the species for which homozygous clones and/or F_1 hybrids have been produced using gynogenesis or androgenesis.

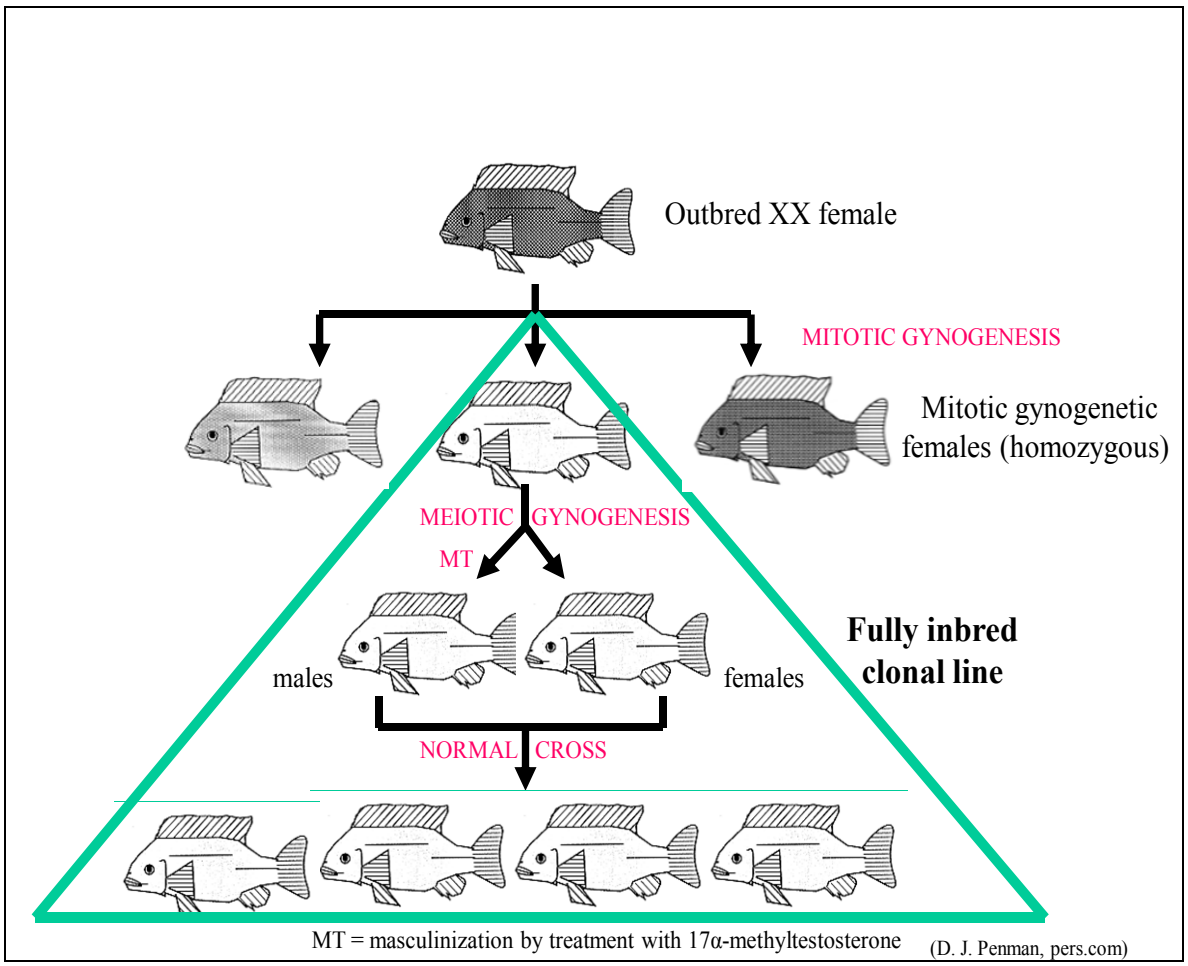
Table 3.1 Species for which homozygous clones have been produced using gynogenesis (G) and androgenesis (A) (after Komen and Thorgaard, 2007)

Common name	**Species name**	**G/A**	**Clone**	**F_1 hybrid**	**Reference**
Zebrafish	*Brachydanio rerio*	G	+	+	Streisinger et al. (1981)
Medaka	*Oryzias latipes*	G	+	+	Naruse et al. (1985)
Common carp	*Cyprinus carpio*	G	+	+	Komen et al. (1991)
		A	+	+	Bongers et al. (1997)
		G	+	+	Ben-Dom et al. (2001)
Nile tilapia	*Oreochromis niloticus*	G	+	+	Müller-Belecke and Hörstgen-Schwark (1995)
		G	+	+	Hussain et al. (1998)
		A	+	+	Sarder et al. (1999)
Amago salmon	*Oncorhynchus rhodorus*	G	+	+	Kobayashi et al. (1994)
		A	+	-	Nagoya et al. (1996)
Ayu	*Plecoglossus altivelis*	G	+	+	Han et al. (1991)
		G	+	+	Takagi et al. (1995)
Rainbow trout	*Oncorhynchus mykiss*	G	+	+	Quillet (1994)
		A	+	+	Young et al. (1995)
Hirame	*Paralichthys olivaceus*	G	+	+	Hara et al. (1993)
Red seabream	*Pagrus major*	G	+	+	Kato et al. (2002)

In Nile tilapia, completely homozygous gynogenetic individuals have been produced by using irradiated sperm and interfering with the first mitotic division using late heat shock (at temperature 42- 42.5 ^{o}C) starting at 27-29 minutes after fertilization. Müller-Belecke and Hörstgen-Schwark (1995) produced first-generation clonal fish from mitotic gynogenetic females in *O. niloticus*. Hussain et al. (1998) produced first-generation clonal *O. niloticus* and an outbred clonal group by crossing between a mitotic gynogenetic female and a mitotic gynogenetic male in the Stirling Nile tilapia population. Since then, clonal lines of females have been produced by i) either mitotic gynogenesis or meiotic gynogenesis from mitogyne individuals (clone founders homozygous females) and by ii) hormonally sex reversing a proportion of the fish within a line to obtain both sexes, and

crossing them to produce and advance homozygous line at the Tropical Aquarium Facilities (TAF), Institute of Aquaculture, Stirling. A schematic diagram of production of gynogenetic clonal line is presented in Figure 3.1. The clonal line used in the present experiments is one of the lines produced by Ezaz et al. (2004b).

Figure 3.1 Production of gynogenetic clonal lines in *Oreochromis niloticus*



In fisheries and aquaculture research, such clonal fish present tremendous potential. Genetic uniformity allows for comparisons of the same genotype over time and under different ambient conditions. This allows estimation of genetic correlations and detection of genotype-by-environment interactions and phenotypic plasticity for complex traits such

as sex and gonadal differentiation, stress response, and disease resistance (Bongers et al., 1998). Production of uniform, homozygous experimental material is particularly advantageous for many genetic mapping and genome sequencing studies in which interpretations are facilitated by homozygosity. Large-scale BAC (bacterial artificial chromosome) fingerprinting and sequencing can benefit when variation due to allelic heterozygosity is eliminated by using homozygous clonal material (Wayne and McIntyre, 2002). The current sequencing of the Nile tilapia and Atlantic salmon genomes is based on the clonal line and mitogynes respectively (Liu, 2007). Another promising avenue of research lies in the development of lines for studying physiological effects of mitochondrial variation. When androgenesis is utilized, there is the potential to produce individuals with identical nuclear genotypes, but which vary in their mitochondrial genotype (Brown and Thorgaard, 2002). They exploited this approach to produce lines of rainbow trout which are identical or near-identical in their nuclear genome but which differ in their mitochondrial haplotype. Bercsenyi et al. (1998) produced androgenetic dihaploid goldfish from irradiated common carp eggs. The hybrid progeny had inherited the nuclear genotype from the goldfish and the mitochondria from the carp. These hybrids will be useful for dissecting the significance of mitochondrial haplotype variation for development, physiological functioning and evolution of species. Brown et al. (2006) found that differences in development rate among rainbow trout from one clonal line potentially could be related to variations in mitochondrial type following androgenesis. Dihaploids and clones can also be used for the analysis of epistatic interactions and estimation of genetic correlations, as well as for the detection of QTL. Markers for QTL related to “difficult traits”, such as meat quality and disease resistance, are needed to execute marker-assisted selection (MAS), marker-assisted introgression (MAI) and

marker-assisted differentiation (MAD) breeding programmes (Komen and Thorgaard, 2007). In sex ratio studies, clonal lines play a highly advantageous role for examples as reference strains and constant factors in crosses to analyse sex-determination mechanism. Any genetic variation acquired by the offspring is attributable to the individuals with whom the clonal animals are mated. This knowledge of sex determination is useful in monosex production approach in many species.

There are some limitations of gynogenesis or androgenesis approaches as well. Reduced reproductive capacity, survival and fertility, and extremely low yields of dihaploids are obstacles. Heat and pressure shocks are easy to apply, but have wide ranging, undesirable side-effects on embryo development (Yamaha et al., 2002). In addition, maintenance of clonal lines and quality control of them for sustainable use are fundamental challenges.

3.1.1. Verification of gynogenetic or androgenetic inheritance

Once putative gynogenetics or androgenetics have been obtained, it is important to determine the success of the procedure to avoid contamination in setting up homozygous lines. Phenotypic (morphological) 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 common carp (Nagy et al., 1978) and tilapia (Don and Avtalion, 1988; Varadaraj 1990; Myers et al., 1995). However, such morphological markers are rare (Ezaz et al., 2004a), and, confirmation using biochemical or molecular markers is required to allow the unambiguous identification of inheritance. The screening for homozygosity at greater number of loci with large number of genetic markers should allow more accurate verification of gynogenetic clonal females.

Genetic markers can provide evidence on whether diploid gynogenesis results from a blockage of the first mitotic division, or from a blockage of polar body extrusion. Biochemical markers such as allozyme loci have been used to evaluate the success of gynogenesis in tilapia (Hussain et al., 1998; Sarder et al., 1999). Multilocus DNA fingerprinting has been used in several fish species including tilapia (Carter et al., 1991; Muller-Belecke and Horstgen-Schwark, 1995), African catfish (Volckaert et al., 1994) and sea bass (Felip et al., 2000). RFLP (Restriction Fragment Length Polymorphism), RAPD (Random amplified polymorphic DNA) and simple sequence repeat-anchored PCR (SSRa-PCR) have also been useful to study the clonal status of clone founders and to examine the uniformity of the gynogenetic offspring of founders and the genetic differences among the clones (Jenneckens et al., 1999; Galbusera et al., 2000; Peruzzi and Chatain, 2000).

Microsatellite DNA markers could be a proper tool to investigate the homozygous nature of clonal line fish. They have a number of desirable properties, including high polymorphism and consequently high information content, and ease of amplification. Microsatellites are generally found in the non-coding part of the genome and so have no effect on gene expression. Single nucleotide polymorphism (SNP), on the other hand can either be in non-coding DNA or in a specific gene. SNP detection involves comparing sequences from multiple animals for base substitutions and high throughput technology such as mass arrays allows a low cost for genotyping large numbers of animals for large number of markers (Vignal et al., 2002). However in terms of polymorphism, SNPs are not as informative (since they deal a maximum of two alleles) as microsatellites are. Polymorphic microsatellite markers are therefore considered to be very useful in identifying the minor genetic variation in population. They can detect and compare

polymorphism between clonal and outbred animals, identify loci with high gene-centromere recombination rates in meiotic gynogenetics which can be used to discriminate meiotic from mitotic gynogenetics (Ezaz et al., 2004b), and can serve as unique 'identifiers' of clonal lines.

3.1.2 Objectives of the study

This study was performed to verify fully inbred females of a clonal line (previously developed by gynogenesis) using DNA markers (mostly microsatellite DNA) from across tilapia genome, and to validate the line as a reference line for studies on sex determination of Nile tilapia. The specific objectives were to:

i. Screen for marker homozygosity at loci across the genome
ii. Observe the progeny sex ratio clonal line females x clonal neomales
iii. Correlate sex ratios from crosses between a range of males of different genotypes with clonal and outbred females

3.2 Materials and methods

3.2.1 Fish Stock

A number of fully inbred clonal lines of *O. niloticus* were produced previously by gynogenesis (Sarder et al., 1999; Ezaz et al., 2004b). Many of these showed low fertility. One XX line that showed good fertility was maintained in the TAF, Institute of Aquaculture, Stirling and the line advanced through breeding of clonal females and clonal neomales (the latter produced by hormonal masculinisation). This line was verified to evaluate them as a standard reference line for sex determination studies.

Six sexually mature females from this inbred line were selected for the study. Each of the females was tagged with a passive integrated transponder (PIT) tag and kept in a glass tank, table 3.2 shows the tag numbers and the tank numbers of aquaria they were reared in. Six outbred females were also reared (to be used for comparison of sex ratios with clonal females for a range of males, Section 3.2.7, this chapter) and the same information on these females is also given in Table 3.2. The general maintenance followed the methods described in Chapter 2 (sections 2.1 to 2.3).

Table 3.2 PIT tag numbers and tank numbers of the clonal females in TAF

Clonal line females		**Outbred females**	
PIT tag number	**Tank number**	**PIT tag number**	**Tank number**
00 068C F2E0	N1a	00 0633 2FB5	N3a
00 068C D9B1	H35	00 0638 E8DD	N3b
00 068D 0073	N1b	00 064E 1D98	H35b
00 0633 EA38	N2a	00 068D 0559	H36b
00 064E 4714	N2b	00 068D 0EEC	H37b
00 068C D6BE	H36	00 064C EFB4	H38b

3.2.2 DNA extraction and quantification

DNA was extracted from fin clips using the phenol-chloroform and REAL kit method (details described in Chapter 2 sections 2.5.2.1 and 2.5.2.2) and quantified with a Nanodrop spectrophotometer (Chapter 2 section 2.3).

3.2.3 Selection of DNA markers

DNA markers were selected from the recent tilapia linkage map (Lee et al., 2005). The criteria of this selection were to pick up markers at intervals from each linkage group and to cover the whole genome. A total of 97 markers (93 microsatellites and four gene-based markers) covering all 24 LGs were selected for this study (Table 3.3). Markers from LG1, LG3 and LG23 were given more emphasis in selection criteria (n=29) because sex determining genes have been mapped on these LGs in different species of tilapia (Lee et al., 2003, 2004; Shirak et al., 2006; Appendix II). The oligo sequences of screened markers are given in Appendix III (LG1 markers), Appendix IV (LG3 markers), Appendix V (LG23 markers), Appendix VI (modified SNP Wt1b marker), Appendix VII (fluorescent label primers) and Appendix VIII (Markers from other LGs except 1, 3 and 23).

Table 3.3 Screening of DNA markers for verification of clonal line of gynogenetic females

Linkage group	Name of markers	No. in each LG
1	GM633, UNH985, UNH931, UNH213, GM201, UNH148, UNH995, UNH104, GM258, UNH719, UNH846, modified WT1b_short	12
2	GM420, ***GM096,*** UNH860, ***UNH854,*** UNH159	5
3	GM354, GM271, UNH971, GM150, GM128, GM526, UNH982, ***ClCn5, dmo***	9
4	***GM470, UNH124,*** UNH170, GM553	4
5	***UNH817,*** UNH884, UNH309, UNH980	4
6	UNH948, UNH908, UNH968, GM440	4
7	GM205, UNH899	2
8	GM027	1
9	UNH843, UNH886, GM343, UNH132, GM062	5
10	UNH994, UNH960, GM080, GM472	4
11	UNH990, UNH192, GM215, GM399, UNH878, UNH979	6
12	GM377, UNH874, UNH1009, Rasgrf	4
13	GM373, UNH954	2
14	GM070, GM665, UNH885	3
15	GM664, UNH880, GM129	3
16	GM056, GM168, ***UNH176***	3
17	UNH103, UNH974, UNH440	3
18	UNH904, UNH888, GM285	3
19	UNH419, UNH943, UNH844,	3
20	UNH174, UNH866, GM363	3
21	UNH957, GM221	2
22	GM531, UNH905, UNH840	3
23	GM557, UNH848, UNH197, GM597, UNH898, UNH879, GM576, UNH907	8
24	GM173	1

Markers with italic bold: Failed in either PCR or genotype

3.2.4 DNA amplification

Polymerase chain reaction of the quantified DNA from clonal females was carried out in 20 µl reaction mixtures. The components used for a single reaction mixture (of 20 µl) are given in Chapter 2 Table 2.3. The thermocycler conditions varied for different fluorescent primers; three such primers were used: M13 blue (ggataacaatttcacacagg), CAG tag green (cagtcgggcgtcatca) and Godde black (catcgctgattcgcacat). The forward and reverse sequences of the primers were retrieved from the NCBI databank and any one of the three fluorescent sequences (blue or green or black) was added at 5' end of either the forward or of the reverse primer, thus that primer was the 'tailed' primer. Three fluorescently labeled primer sequences were added randomly for all the microsatellites tailing. The annealing temperatures for markers from LG1, LG3 and LG23 were determined from salt-adjusted and base stacking melting temperatures (Rychlik and Rhoads, 1989; Mueller et al., 1994; Santalucia, 1998) and used in PCR (Table 3.4 and 3.5) with some modifications and standardization from thermal gradient PCR. The formulae are given below and are available online (www.promega.com/biomath) to calculate T_a.

Salt-Adjusted T_m Calculations

$T_m = 81.5°C + 16.6°C \times (\log_{10}[Na^+] + [K^+]) + 0.41°C \times (\%GC) - 675/N$; N is the number of nucleotides in the oligo.

Base-Stacking T_m Calculations

The most sophisticated T_m calculations take into account the exact sequence and base stacking parameters, not just the base composition. The equation used is:

$$T_m = \frac{\Delta H \frac{kcal}{°C*Mol}}{\Delta S + R \ln([primer]/2)} - 273.15°C$$

Where:

ΔH is the enthalpy of base stacking interactions adjusted for helix initiation factors

ΔS is the entropy of base stacking adjusted for helix initiation factors and for the contributions of salts to the entropy of the system

R is the universal gas constant (1.987Cal/°C*Mol)

For the rest of the markers from genome wide selection, annealing temperatures of 57 ^{o}C, 58 ^{o}C and 60 ^{o}C were used for primers having M13 tail, CAG tag green and Godde black, respectively. The other steps in thermocycler conditions were kept same as described in Chapter 2 (section 2.6, Table 2.4).

Table 3.4 Annealing temperatures for markers from LG1 used in PCR

LG	Marker	Annealing temperatures used in PCR cycles
1	GM633_L1_CAG_F	2 cycles at 65, 38 cycles at 60
	GM633_L1_R	
	UNH985_L1_F	2 cycles at 64, 38 cycles at 60
	UNH985_L1_God_R	
	UNH931_L1_M13_F	2 cycles at 64, 38 cycles at 57
	UNH931_L1_R	
	UNH213_L1_M13_F	2 cycles at 65, 38 cycles at 58
	UNH213_L1_R	
	GM201_L1_CAG_F	2 cycles at 65, 38 cycles at 60
	GM201_L1_R	
	UNH148_L1_M13_F	2 cycles at 65, 38 cycles at 57
	UNH148_L1_R	
	UNH995_L1_God_F	2 cycles at 64, 38 cycles at 60
	UNH995_L1_R	
	UNH104_L1_CAG_F	2 cycles at 65, 2 cycles at 56, 36 cycles at 60
	UNH104_L1_R	
	GM258_L1_M13_F	2 cycles at 65, 2 cycles at 60, 36 cycles at 57
	GM258_L1_R	
	UNH719_L1_God_F	2 cycles at 64, 38 cycles at 60
	UNH719_L1_R	
	UNH846_L1_CAG_F	2 cycles at 65, 38 cycles at 61
	UNH846_L1_R	

Table 3.5 Annealing temperatures for markers from LG3 and LG23 used in PCR

LG	Marker	Annealing temperatures used in PCR cycles
3	GM354_L3_M13_F	2 cycles at 65, 2 cycles at 60, 36 cycles at 57
	GM354_L3_R	
	GM271_L3_F	2 cycles at 62, 38 cycles at 60
	GM271_L3_God_R	
	UNH971_L3_F	2 cycles at 66, 2 cycles at 63, 36 cycles at 60
	UNH971_L3_CAG_R	
	GM150_L3_F	2 cycles at 63, 38 cycles at 58
	GM150_L3_M13_R	
	GM128_L3_God_F	2 cycles at 64, 38 cycles at 61
	GM128_L3_R	
	GM526_L3_M13_F	2 cycles at 65, 2 cycles at 63, 36 cycles at 60
	GM526_L3_R	
	UNH982_L3_CAG_F	2 cycles at 65, 2 cycles at 62, 36 cycles at 60
	UNH982_L3_R	
23	GM557_M13_F	2 cycles at 65, 2 cycles at 60, 36 cycles at 57
	GM557_R	
	UNH848_CAG_F	2 cycles at 65, 38 cycles at 61
	UNH848_R	
	UNH197_Godde_F	2 cycles at 65, 2 cycles at 56, 36 cycles at 60
	UNH197_R	
	GM597_F	2 cycles at 64, 2 cycles at 60, 36 cycles at 57
	GM597_M13_R	
	UNH898_F	2 cycles at 65, 38 cycles at 61
	UNH898_CAG_R	
	UNH879_F	2 cycles at 64, 38 cycles at 61
	UNH879_Godde_R	
	GM576_M13_F	2 cycles at 64, 2 cycles at 62, 36 cycles at 57
	GM576_R	
	UNH907_CAG_F	2 cycles at 66, 38 cycles at 60
	UNH907_R	

3.2.5 Genotyping and fragment analyses

CEQ^{TM} 8800 Series, a specialized and efficient tool for genetic analysis system with robust software and chemistries, was used for analyses of the fragments after PCR gel electrophoresis (described in chapter 2). An array of eight capillaries takes full advantage of the 96-well plate format, while reducing the cost and complexity associated with larger arrays. The components required for genotyping and the procedure have been explained in Chapter 2 section 2.7.

3.2.6 Evaluation of sex ratios between clonal females and sex-reversed neomales

Once the clonal nature of the females was determined, sex ratios were observed in crosses between females and neomales (hormonally masculinized XX individuals) within this line. A total of 8 crosses were performed, four neomales each crossed to two females, with six different females involved. The survival rates in clonal line progeny was also compared with those in outbred groups on day 11, after the completion of yolk sac absorption period.

3.2.7 Evaluation of sex ratios involving clonal and outbred females with a range of males

Progeny sex ratios were also observed using clonal and outbred females. Each type was crossed with XY males, putative YY males and clonal neomales (XX males) to determine the correlation of the sex ratios produced by both types of females. Three XY males, three putative YY males and two neomales were used in this study.

3.2.8 Statistical analysis

The correlation coefficient of sex ratios between clonal female and outbred female crossed with different males was analyzed using SPSS (version 17.0) following arcsine transformation of data.

3.3 Results

3.3.1 Proof of clonal nature

Out of 97 DNA markers, 89 (87 microsatellites and two gene-based markers) successfully amplified the DNA. Analyses of the fragments after genotyping revealed that the clonal individuals (six broodstock with markers from LG1, 3 and 23; and three broodstock from the rest of the LGs) were homozygous at all of the loci concerned. The name of all the markers, number of the clonal females used and the genotypes with these markers are given in Table 3.6. Numbers of alleles from outbred individuals are also shown for predicting marker polymorphisms. Sixty seven markers were found to be polymorphic in outbred individuals (Table 3.6).

Table 3.6 Inheritance of alleles in clonal line of females (XX) with DNA markers from all linkage groups of tilapia

Linkage group and no. of markers screened	Name of markers	No. of clonal females used for genotyping	Genotypes of clonal females	No. of alleles found in outbred animals	Presence of mono-(M) or polymorphism (P)
LG1 (12)	GM633	6	206/206	3	P
	UNH985	6	144/144	2	P
	UNH931	6	227/227	3	P
	UNH213	6	211/211	2	P
	GM201	6	164/164	2	P
	UNH148	6	172/172	3	P
	UNH995	6	184/184	3	P
	UNH104	6	147/147	3	P
	GM258	6	144/144	3	P
	UNH719	6	127/127	3	P
	UNH846	6	190/190	3	P
	WT1short	6	64/64	2	P
LG3 (7)	GM354	6	142/142	2	P
	GM271	6	134/134	2	P
	UNH971	6	215/215	3	P
	GM150	6	217/217	2	P
	GM128	6	157/157	2	P
	GM526	6	260/260	2	P
	UNH982	6	124/124	1	M

Table 3.6 (Cont'd) Inheritance of alleles in clonal line of females (XX) with DNA markers from all linkage groups of tilapia

Linkage group and no. of markers screened	Name of markers	No. of clonal females used for genotyping	Genotypes of clonal females	No. of alleles found in outbred animals	Presence of mono-(M) or polymorphism (P)
LG23 (8)	GM557	6	268/268	2	P
	UNH848	6	208/208	2	P
	UNH197	6	205/205	2	P
	GM597	6	151/151	2	P
	UNH898	6	286/286	2	P
	UNH879	6	238/238	2	P
	GM576	6	242/242	2	P
	UNH907	6	134/134	2	P
LG2 (3)	GM420	3	137/137	1	M
	UNH860	3	216/216	2	P
	UNH159	3	251/251	2	P
LG4 (2)	UNH170	3	162/162	1	M
	GM553	3	257/257	2	P
LG5 (3)	UNH884	3	160/160	3	P
	UNH309	3	199/199	2	P
	UNH980	3	233/233	2	P
LG6 (3)	UNH948	3	197/197	2	P
	UNH908	3	124/124	1	M
	UNH968	3	226/226	2	P
	GM440	3	275/275	1	M
LG7 (3)	GM205	3	127/127	1	M
	UNH899	3	158/158	1	M
LG8 (1)	GM027	3	176/176	2	P
LG9 (5)	UNH843	3	125/125	1	M
	UNH886	3	185/185	1	M
	GM343	3	191/191	2	P
	UNH132	3	131/131	1	M
	GM062	3	286/286	2	P
LG10 (4)	UNH994	3	235/235	2	P
	UNH960	3	182/182	2	P
	GM080	3	245/245	2	P
	GM472	3	355/355	2	P
LG11 (6)	UNH990	3	168/168	3	P
	UNH192	3	156/156	1	M
	GM215	3	223/223	3	P
	GM399	3	273/273	2	P
	UNH878	3	120/120	1	M
	UNH979	3	271/271	1	M
LG12 (4)	GM377	3	312/312	2	P
	UNH874	3	214/214	2	P
	UNH1009	3	173/173	3	P
	Rasgrf	3	119/119	2	P

Table 3.6 (Cont'd) Inheritance of alleles in clonal line of females (XX) with DNA markers from all linkage groups of tilapia

Linkage group and no. of markers screened	Name of markers	No. of clonal females used for genotyping	Genotypes of clonal females	No. of alleles found in outbred animals	Presence of mono-(M) or polymorphism (P)
LG13 (2)	GM373	3	318/318	2	P
	UNH954	3	178/178	2	P
LG14 (3)	GM070	3	144/144	1	M
	GM665	3	238/238	1	M
	UNH865	3	235/235	1	M
LG15 (3)	GM664	3	245/245	1	M
	UNH880	3	204/204	1	M
	GM129	3	120/120	1	M
LG16 (2)	GM056	3	243/243	2	P
	GM168	3	336/336	3	P
LG17 (3)	UNH103	3	232/232	2	P
	UNH974	3	210/210	3	P
	UNH440	3	198/198	2	P
LG18 (3)	UNH904	3	184/184	2	P
	UNH888	3	226/226	1	M
	GM285	3	162/162	2	P
LG19 (3)	UNH419	3	202/202	3	P
	UNH943	3	155/155	2	P
	UNH844	3	133/133	1	M
LG20 (3)	UNH174	3	190/190	3	P
	UNH866	3	167/167	2	P
	GM363	3	210/210	1	M
LG21 (2)	UNH957	3	192/192	4	P
	GM221	3	197/197	3	P
LG22 (3)	GM531	3	231/231	2	P
	UNH905	3	168/168	3	P
	UNH840	3	153/153	2	P
LG24 (1)	GM173	3	285/285	2	P

3.3.2 Sex ratios and viability in clonal lines

The overall sex ratio result (at three months) obtained from 8 crosses involving all 6 clonal females showed that 105 of 106 progeny sexed were females. However, in almost all the crosses the number of offspring was small (ranging from 2 to 26). Table 3.7 presents the sex ratios from these crosses between clonal line females and neomales.

Table 3.7 Sex ratios in crosses between clonal line females and neomales

Neo♂♂	Clonal♀♀	No. male (M)	No. female (F)	Sex ratios (M: F)
00 068C FD8F	00 068C F2E0	0	22	0:100
	00 068C D9B1	0	17	0:100
00 0633 E607	00 068D 0073	0	12	0:100
	00 0633 EA38	0	2	0:100
00 064D 1843	00 064E 4714	1	13	7:93
	00 068C D6BE	0	4	0:100
00 064C EBEE	00 068C D6BE	0	26	0:100
	00 064E 4714	0	9	0:100
Total		**1**	**105**	**0.9: 99.1**

The viability of the offspring from clonal group is shown in Table 3.8 which shows mean survival rate of only 5.16% on day 11 (after yolk sac absorption period) significantly different ($P<0.05$) from that in outbred males crossed with outbred females (68.46%).

Table 3.8 Viability studies in clonal line compared to outbred crosses

Cross groups		**No. of eggs**	**No. live on day 4 (hatched fry)**	**No. live on day 11 (YSR fry stocked in tanks)**
Clonal ♂♂ x Clonal ♀♀				
00 068C FD8F	00 068C F2E0	200	40 (20%)	35 (17.5%)
	00 068C D9B1	200	20 (10%)	17 (8.5%)
00 0633 E607	00 068D 0073	380	26 (6.8%)	12 (3%)
	00 0633 EA38	100	2 (2%)	2 (2%)
00 064D 1843	00 064E 4714	200	18 (9%)	14 (7%)
	00 068C D6BE	150	4 (2.6%)	4 (2.6%)
00 064C	00 068C D6BE	250	29 (11.6%)	26 (10.4%)
	00 064E 4714	150	14 (9.3%)	10 (6.67%)
00 068C E132	00 068C F2E0	350	0 (0%)	0 (0%)
00 064D 0E7A	00 068C DD59*	150	0 (0%)	0 (0%)
			Mean %=7.13±6.24	Mean %=5.76±5.45[a]
Clonal ♂♂ x Outbred ♀♀				
00 068C FD8F	00 0638 E8DD	300	35 (11.67%)	32 (10.67%)
00 0633 E607	00 0633 2FB5	250	55 (22%)	49 (19.6%)
			Mean %=16.84±7.30	Mean %=15.14±6.31[b]
Outbred ♂♂ x outbred ♀♀				
00 068C FBE2	00 0633 2FB5	350	NR	240 (68.57%)
00 064C FEED	00 0638 E8DD	300	NR	205 (68.33%)
			-	Mean %=68.45±0.17[c]

$P(\chi^2 a,b)>0.05$; $P(\chi^2 a,c)<0.05$, *this clonal female was not verified but was from same line

3.3.3 Sex ratios involving clonal and outbred females with a range of males

Table 3.9 shows and compares the sex ratios obtained from clonal females and outbred females when crossed with different types of male individuals. Correlation analysis of sex ratios obtained from paired (half-sib) crosses involving clonal and outbred females with a range of males (clonal line neomales, XY males and YY males) was conducted.

Table 3.9 Comparison of sex ratios of clonal and outbred females with males

		Sex ratios with clonal female		Sex ratios with outbred female	
		% Male (No.)	% female (No.)	% Male (No.)	% female (No.)
Clonal male	00 068C FD8F (J12)	0 (0)	100 (39)	0 (0)	100 (28)
	00 0633 E607 (H51)	0 (0)	100 (14)	0 (0)	100 (49)
	00 064C EBEE (H53)	0 (0)	100 (35)	-	-
XY male	00 068C D9E3 (G15)	49(71)	51(75)	47(189)	53(194)
	00 068C FBE2 (G16)	52 (61)	48(57)	57(34)	47(26)
	00 064E 46A8 (G26)	49(60)	51(62)	51(33)	49(32)
YY male	00 013E 315C (C3)	95(404)	5 (36)	88(112)	12(15)
	00 068C E167 (G35)	93(206)	7(15)	93(167)	9(13)
	00 068C F8BB (H60)	97(76)	3(4)	99(277)	1(1)

The correlation coefficient between the sex ratios obtained from the clonal and outbred females was 0.994 ($P<0.01$; $n=8$) (Figure 3.2). The chi-squared test statistics of the paired values of sex ratio did not differ significantly ($P>0.05$, d.f 1) between clonal and outbred female sets.

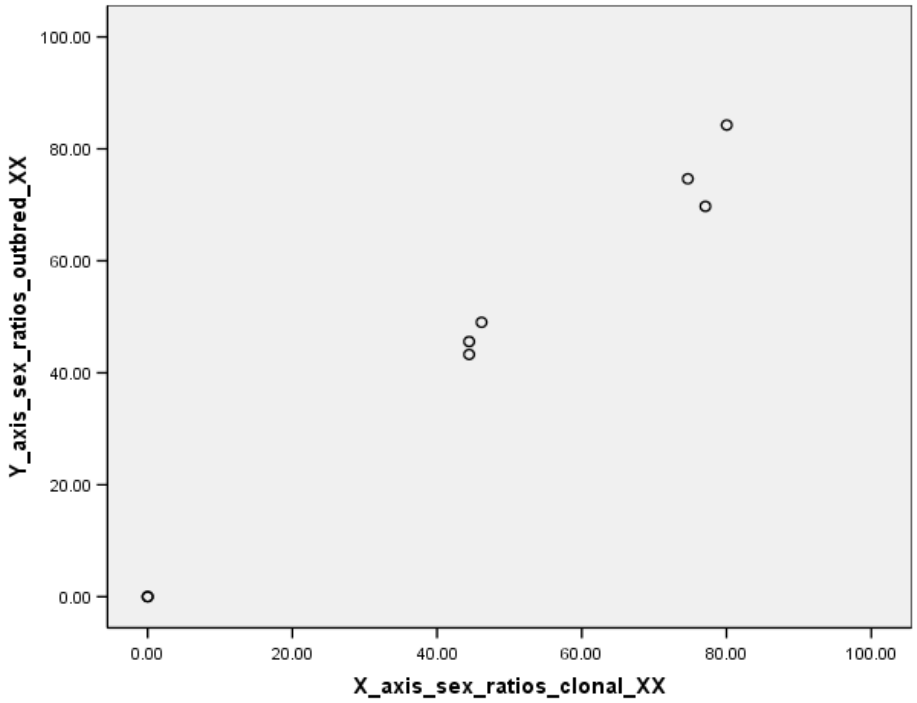


Figure 3.2 Correlation of sex ratios (%) between clonal and outbred females crossed with a range of males

3.4 Discussion

Some published works are available on the verification of the gynogenetic clonal females (founders) as well as clonal line individuals in Nile tilapia (*Oreochromis niloticus*). However, very few studies have been performed to validate the clonal lines by using polymorphic microsatellite markers. Some of these methods and the outcomes have been discussed below and the interpretation of the results from the present study is given.

The clonal status of the clone founders, the genetic uniformity of gynogenetic offspring from each clone founder and the genetic differences between clonal lines on DNA level in Nile tilapia other than the Stirling strain have been observed by using different DNA based

techniques (Jenneckens et al., 1999), e.g., multilocus DNA fingerprinting, random amplified polymorphic DNA (RAPD) and simple sequence repeat-anchored PCR (SSRa-PCR). Multilocus DNA fingerprinting and RAPD demonstrated that carryover of male chromosomal DNA by the use of UV-irradiated sperm for induction of gynogenesis did not occur and that the clonal lines could be accurately distinguished from each other. However, the primers used in SSRa-PCR could not determine the absence of paternal genomic transmission due to a lack of visible informative paternal bands. In such cases, microsatellite markers could be advantageous because of their high information content.

A study by Hussain et al. (1998) was aimed at producing heterozygous and homozygous clones of Nile tilapia using reproductively viable mitotic gynogenetic fish. Eggs were collected from a female heterozygous at the adenosine deaminase (*ADA**) locus for 135/113 alleles. Two batches of eggs were fertilized separately with UV irradiated sperm, initially collected from a normal diploid male and exposed, respectively, to optimal early pressure shock treatment (Hussain et al., 1991) for the production of meiotic gynogens and late pressure shock treatment (Hussain et al., 1993) for the production of mitotic gynogens. These mitotic gynogens were reared to sexual maturity as they were viable, and four of them used as broodstock (two female homozygous for the 113 *ADA** allele and two males homozygous for the 135 *ADA** allele). Eggs from each of the two females were divided into batches for the production of meiotic gynogens or inbred clones, ICL (by UV treatment followed by early pressure shock of eggs), and diploid controls or outbred clones, OCL (separately fertilized by each of the two males). The use of the *ADA** marker enabled both clones to be identified and confirmed that there was no paternal inheritance in the ICL produced by gynogenetic reproduction (retention of 2nd polar body). All of the

outbred clonal progeny (N=80) were heterozygous (113/135) and all of the inbred clonal progeny (N=7) were homozygous at *ADA** locus. Sarder et al. (1999) studied the same isozyme locus *ADA** genotypes of control and gynogenetic progeny from experiments in which *O. niloticus* females (genotype 113/135) and an *O. aureus* males (genotype 138/138) were used as parents. The absence of the male parent's genotype in gynogenetic offspring was an evidence of successful gynogenesis, Female parents had 113/135 or 135/135 genotypes. The *ADA** locus was useful to discriminate meiotic (113/135) and mitotic gynogenetic offspring (113/113 or 135/135) where the female parent was heterozygous (113/135) for this locus. The use of such allozyme markers to detect the variation, in the absence of marker genes or more reliable polymorphic markers (e.g., microsatellites or SNPs) is quite common for assessing parental contribution and the genetic status of the progeny in all forms of ploidy manipulations. The ability to study a large number of variable allozyme loci has also been particularly useful in estimating the frequency of heterozygotes in meiotic gynogenesis and thereby the level of recombination that has occurred at the first meiotic division (reviewed by Seeb and Miller, 1990). *ADA** used in the studies of Hussain et al. (1998) and Sarder et al. (1999) to determine the gynogenetic status was known polymorphic enzyme locus. This locus was found to give highest 'y' value (proportion of heterozygotes, y= 1- NR/Total, where NR is number of recombinants) of 1.0 (100% recombinants) in six different meiotic gynogenetic progeny groups, a total of 150 meiotic gynogenetic offspring from heterozygous females, in a study conducted by Hussain et al. (1994) on estimation of gene-centromere recombination frequencies in gynogenetic diploids of Nile tilapia. In contrast to the *EST-2** locus where there was no evidence of any recombinant genotypes (y=0.00) in any of the meiotic gynogenetic offspring (N=150) (and hence suggests the *EST-2** locus is very close to its respective

centromere) the results for the *ADA** locus suggested that it was placed distally on a chromosome arm.

Microsatellite markers are co-dominant and can express polymorphism in individuals. Therefore they are more appropriate than allozyme markers (codominant markers) or AFLPs (dominant markers) in analyzing the products of chromosome set manipulations, for example, clonal gynogenetic line of females. Polymorphic microsatellite loci were investigated in several gynogenetic families and clonal progeny of Nile tilapia by Ezaz et al. (2004b) to verify gynogenesis and clonal lines. They worked with 6 microsatellite markers in two meiotic gynogenetic families in selected loci with high gene-centromere recombination rates, which could be used to discriminate meiotic from mitotic gynogenetics. Microsatellite loci UNH189 and UNH211 showed 96.7% and 92.0% heterozygosity, respectively and thus indicated very low probability of an individual meiotic gynogenetic being homozygous for both loci. Polymorphic microsatellite loci were also used successfully to verify maternal inheritance in gynogenetic populations of African catfish and European sea bass by Galbusera et al. (2000) and Peruzzi and Chatain (2000). Genotyping of clonal line gynogenetic females with a moderately large number of microsatellite DNA primers was considered to be a reasonable way to verify the clonal nature and to validate them as 'pure inbred' and to help establish the line as a reference line for sex ratio studies in Nile tilapia. The present study showed that the representative sample of females from the clonal line were demonstrated to be homozygous at all of the 89 microsatellite loci, thereby strengthened the hypothesis of being fully inbred clonal females.

The viability of clonal female offspring (as shown in Table 3.7) was found to be low. One popular explanation behind such viability is that these low and variable survival rates are caused by expression of deleterious genes and inbreeding depression. In theory, double haploid clone founders produced by androgenesis or gynogenesis should suffer from inbreeding depression due to the expression of homozygous deleterious mutations (Purdom et al., 1985). Many of these mutations could act during early embryo development, thereby causing a significant reduction in survival of double haploid fry. Assuming an average genetic load of 1-2 harmful recessive genes for any female parent, the mortality in her doubled haploid progeny could be as high as 50-75% (Komen and Thorgaard, 2007). Müller-Belecke and Hörstgen-Schwark (2000) obtained survival of clonal line females at first-feeding varying between 1 and 20% in comparison to about 45% in controls. Sarder et al. (1999) obtained mean survival rates of 63.11 ± 6.41%, 30.89 ± 6.30%, and 4.34 ± 1.24% respectively at yolk sac resorption in diploid control, meiotic gynogenetic, and mitotic gynogenetic groups of *O. niloticus*. In zebrafish, gynogenesis was used to identify 14 recessive maternal effect mutations. Homozygosity for these mutations in adult females led to the inviability of their offspring (Pelegri et al., 2004). As homozygous animals show inbreeding depression, it is likely that the performance of homozygous clones in terms of viability and fertility usually follows that of the parent from which they were derived. The offspring derived from the clonal line of females (produced from mitogynes) in this study may have deleterious genes fixed which have caused poor fertility of germ cells.

Analysis of sex ratio results obtained from 8 crosses involving all 6 clonal females and 4 neomales in this study confirms sex ratios very close to all-female (99.1%). This result supports earlier results (Sarder et al., 1999; Ezaz et al., 2004b) in producing nearly all

female progeny in mitotic gynogenetic clonal lines. Analyses of sex ratios of gynogenetic *O. niloticus* by Penman et al. (1987) revealed all females among 89 meiogynes pooled from progeny of seven different mothers whereas Mair et al. (1991a) found eight males within a greater sample size of 187 meiogynes derived from 11 mothers. They suggested that the occurrence of males in gynogenetics may result from some form of "natural sex reversal" of females and this sex reversal mechanism develops from homozygosity of rare autosomal, recessive, sex-influencing genes. Sarder et al. (1999) observed presence of males in all of four mitogyne groups and in three out of four meiogyne groups. They also observed sex ratios in gynogenetic inbred clonal lines of Nile tilapia. With the exception of the clonal line founded from one female, all of the lines contained only female fish regardless of how the clonal groups were propagated.

The presence of fairly high proportions of males in both meiogyne and mitogyne groups has been observed in some studies although this is not predicted by the XX/XY sex determining system. Hussain et al. (1994) obtained 7.5% and 47.5% males in the meiotic and mitotic gynogenetic progenies of *O. niloticus* respectively and suggested that an epistatic locus (SDL-2, two alleles, SR and sr) causing sex reversal from female to male under homozygous condition. Thus a female that is heterozygous at this locus (XX SRsr) would be expected to produce non-recombinant homozygous males (XX srsr) and both non-recombinant and recombinant females (XX SRSR and XX SRsr respectively) in meiotic gynogenesis and only XX srsr males and XX SRSR females in mitotic gynogenesis. Komen et al. (1992, 1995) described a recessive mutation in *Cyprinus carpio* (mas) which in the homozygous state in XX animals (XX mas/mas) results in female to male sex reversal. Müller-Belecke and Hörstgen-Schwark (1995) reported 35.3% male

progeny in the mitogynes of *O. niloticus*. All female meiogynes were derived from two females which produced males among mitotic gynogenetic progenies. Gynogenetic reproduction of six mitogyne females led to all female-homozygous clones. Progenies of five mitogyne males mated with different females were all male, all female or predominantly female (Crosses to one of the control females produced only female offspring, while crosses to the mitotic mother of the clonal males produced high percentages of males). They suggested the occurrence of two or more minor sex determining factors, which are able to override the XX-XY mechanism when they act in combination and occur in homozygous state might account for the sex ratios observed. They also interpreted in an another way that the genotype exhibits greater instability in sex differentiation, perhaps through greater susceptibility to environmental effects. The presence of males was also evident in a study by Sarder et al. (1999) in a gynogenetic clonal line. Data from progeny testing of those males suggested that that line was homozygous for an allele or combination of alleles at an autosomal locus or loci which caused female to male sex reversal but with limited penetrance.

Following the discussion and based on the assumption of locus polymorphism (autosomal or sex-determining locus) in (many) outbred females for determination of sex in Nile tilapia, an interpretation on occurrence of progeny sex can be done on the basis of meiotic gynogenesis, mitotic gynogenesis and clonal line individuals produced from mitotic gynogens. As an example, if the female parent is XX but heterozygous for a hypothetical recessive autosomal sex reversal locus (SR/sr), meiotic gynogenesis will result in XX, SR/SR and XX, SR/sr genotypes consisting of females and XX, sr/sr consisting of male and/or females depending on the penetrance and position of the locus. Mitotic gynogenesis

will produce XX, SR/SR (only females) or XX, sr/sr (males and/or females depending on the penetrance) and occurrence of more males may be apparent. In producing clonal line of females, these mitotic males are eliminated (since they are not used as clone founders). A clonal line of nearly all females founded from mitotic female clone founders (e.g., the outcome of the present study) thereby could hypothetically account for a strong selection against the sex reversal allele (sr/sr) with strong penetrance.

The homozygous nature of the clonal line females in this study with good number of microsatellite markers, the yield of very close to 100% female progeny with sex-reversed neomales and the strong positive correlation with the outbred females in giving sex ratios with a range of males suggests that this line of clonal females could be used as standard, 'reference line' for sex determination studies in Nile tilapia.

Chapter 4
Sex linkage study in the Stirling Nile tilapia (*Oreochromis niloticus*) population

4.1 Introduction

Teleost fish display an amazing variety of sex determination systems. The primary sex determination in most species is genetic (Valenzuela et al. 2003); nevertheless, the sex differentiation of fishes is remarkably plastic and is determined by both genetic and environmental factors in many species (Baroiller and D'Cotta 2001). Although genetic factors probably regulate sex determination in most fishes, relatively few teleosts have karyotypically distinct sex chromosomes (Arkhipchuck, 1995) and sex chromosomes can display variable degrees of molecular differentiation (Volff, 2005).

In tilapia species in general, a variety of evidence suggests that sex determination is principally monofactorial (Wohlfarth and Wedekind 1991). There are no gross morphological differences between the sex chromosomes (Majumder and McAndrew, 1986) and the sex chromosomes of tilapia are thought to be still at an early stage of differentiation (Shirak et al., 2006). The hypothesised sex-chromosome systems suggest that some species have the XX: XY system (*Oreochromis mossambicus, O. niloticus*) whereas others have the WZ: ZZ system (*O. aureus, O. macrochir, O. urolepis hornorum*) (Lee et al., 2004). A wide variety of techniques have been used to analyse the sex determination system in these species, for example, sex reversal and progeny testing, ploidy manipulation, inter- and intra-specific hybridization. Several karyotype studies have also been carried out to identify the sex chromosomes in tilapia species (Foresti et al., 1993; Carrasco et al., 1999; Campos-Ramos et al., 2001; Harvey et al., 2002; Ocalewicz et al., 2009). Foresti et al. (1993) worked with XY type males to identify the difference between each type of chromosome by synaptonemal complex analysis of meiotic

chromosomes. Similar work was done by Carrasco et al. (1999) with three type of individuals-XX, XY, YY and they found an absence of pairing in the terminal portion of largest chromosome pair in 25% of the pachytene preparations obtained from XY males while normal pairing was observed in homogametic individuals from both XX and YY genotypes. The inhibition of pairing of this large chromosome was explained by the accumulation of heterochromatin corresponding to the sex determining region (Griffin et al., 2002).

Although the genetic sex of Nile tilapia is thought to be an XX/XY male heterogametic system (Jalabert et al., 1975; Penman et al., 1987; Mair et al., 1991a) controlled by a major gene, mass spawning and interspecies (or intraspecific) cross-breeding shows unexpected sex ratios based on a simple monofactorial sex determination model (Mair et al. 1987; Rosenstein and Hulata, 1994). It has been postulated that the departures from the sex ratios predicted by a chromosomal monofactorial model can be caused by other unidentified genetic factors, which appear to be autosomal (Majumdar and McAndrew, 1983; Mair et al., 1991a) and to have partial penetrance (Mair et al., 1991a; Hussain et al., 1994; Sarder et al., 1999). In Nile tilapia, the success of monosex (genetically male) production largely depends on a clear understanding of the genetic factors affecting sex where effects of temperature (or other environmental factors) are controlled in a regulated environment. Studies on sex linkage help us to understand the total phenomenon of the sex determination mechanism, in such species with genetic sex determination (GSD) systems.

The advent of DNA analyses techniques has opened up possibilities to identify sex-linked and sex-specific markers in different fish species. A variety of molecular approaches are

now used to find the sex determining region or to identify the sex determining gene. Some of these approaches, e.g., DNA profiling/fingerprinting, look directly for sequences which differ between the male and female genomes; "candidate gene" approaches, look for genes or sequences that are sex-determining or sex-linked in one species and assessed in a new target species; linkage mapping approaches, where phenotypic sex is scored with many segregating markers (mostly DNA-based) to place the sex determining gene(s) into one or more linkage groups, which may lead to positional cloning of such genes later; and molecular-cytogenetic approaches, e.g., to relate linkage and karyotypic data (Penman and Piferrer, 2008). The study of sex linkage in Nile tilapia is likely to give us an insight into the molecular mechanism of sex determination by identifying DNA markers linked to unknown genes (QTL) which can help to improve the production of genetically male tilapia.

DNA markers have been used in a number of studies to identify association between markers and the QTL (for sex) in different tilapia species and their hybrids (Shirak et al., 2002; Lee et al., 2003, 2004; Cnaani et al., 2004; Ezaz et al., 2004a; Karayucel et al., 2004; Cnaani et al., 2008). In Nile tilapia, notable works on sex-linkage using DNA markers are those of Lee et al. (2003) and Lee and Kocher (2007). Lee et al. (2003) found an association between microsatellite DNA markers in tilapia linkage group (LG) 1 with sex (tightly linked) in two out of three crosses studied in Nile tilapia (XX/XY) but no association with markers in any of the LGs in the third one. Genes that were known to be involved in the sex determination pathway of other vertebrates (e.g., *WT1b, CYP19A1*) were also mapped in Nile tilapia but they were excluded as being the major sex-determining genes after break point analyses (Lee and Kocher, 2007). Mapping of a

functional gene close to QTL for sex determination has also been performed but based on hybrid crosses (Shirak et al., 2006). Some work suggests there may be autosomal modifiers in establishing sex of tilapia (e.g., LG1 and LG3 markers contributed in some families of both Nile and blue tilapia: Cnaani et al. 2008). Another model of sex determination, in the absence of any interaction (or in inconsistent pattern of interaction) between two such sex chromosomes is the segregation of an allelic series (multiple alleles) at the sex determining locus (Wohlfarth and Wedekind, 1991) which was partially supported by Cnaani et al. (2008) because of the apparent differences among families of the same species. However, they did not have evidence for multiple alleles segregating at the same sex determining locus across crosses. Therefore, the current state of knowledge on the sex determination system of Nile tilapia is vague. A possible hypothesis suggests a 'predominant' monofactorial genetic system, and but we need a better understanding of the genetic factors behind the establishment of sex. Further linkage mapping studies using DNA markers (mostly microsatellites from the current linkage map of tilapia: Lee et al., 2005) in intra-specific Nile tilapia families are likely to give better explanation of how sex is genetically determined.

The present chapter describes an investigation of the association of DNA markers (mostly microsatellites) with sex in a variety of crosses involving XX clonal females and different types of males (designated on the basis of progeny sex ratios and pedigree information) to get a further understanding of the genetic sex determination mechanism in Stirling red Nile tilapia in a controlled environment with a regulated temperature (27.5±0.5 ^{o}C). The inheritance pattern of sex- linked markers in this species, screening for QTL for sex, presence of any allelic series at sex determination loci and segregation patterns of those

alleles (if present) and interaction of any autosomal locus/loci were of fundamental scientific curiosity in this current study.

In this study, three types of sires (crossed with clonal line females) would be identified based on progeny sex ratios: type 'A'- apparently normal XY males; type 'B'- putative YY males and type 'C'- not matching either of these presumed sex determination hypothesis. By using molecular markers from LG1 (thought to be the site of main sex determining gene in Nile tilapia), the inheritance of phenotypic sex by their allelic segregation will be observed to confirm LG1 associated pattern in type 'A' families. In the other two types of families, in addition to LG1 markers, LG3 and LG23 markers are used to investigate any association of markers from these LGs that can explain slight to moderate departure of sex ratios from prediction. LG3 is thought to be the site of main sex determining gene in blue tilapia (Lee et al., 2004), and in LG23, QTLs for sex determination have been found in hybrid tilapia (Shirak et al., 2006). In addition, markers from genome wide scan (n=62; except these three LGs) are applied to investigate any association between any of the markers and sex in type 'B' families only. So the objectives of this study were:

i. To study the inheritance of alleles/genotypes in clonal females x putative XY males (type 'A') by using screened markers from linkage group (LG)1 to confirm the LG1-associated pattern of inheritance of phenotypic sex and the structure of LG1

ii. To study the inheritance of alleles/genotypes in clonal females x putative YY males (type 'B') producing >90% male progeny by using screened markers from LG1, LG3 and LG23 for association with sex using bulked segregant analysis (BSA) of female and male

DNA pools, followed by further analysis (on DNA from individuals) using informative (sex-linked polymorphic) markers in BSA

iii. To study the inheritance of alleles/genotypes in clonal females x unknown group sires (type 'C') producing ~60-80% male progeny by using the same markers (as in objective ii) for association with sex using bulked segregant analysis (BSA) of female and male DNA pools, followed by further analysis (on DNA from individuals) using informative (sex-linked polymorphic) markers in BSA

iv. To undertake a genome wide scan of selected markers (apart from LG1, LG3 and LG23 markers), approximately evenly spaced from the remaining 21 LGs (Lee et al. 2005), and study them in type 'B' families in BSA for any association with sex, followed by further analysis (on DNA from individuals) using informative markers (if any, in BSA)

4.2 Materials and Methods

4.2.1 Screening of broodfish and PIT tagging

Five putative XY and 16 putative YY male fish were taken from stock tanks at Tropical Aquarium Facilities (TAF), tagged and kept at individual tanks. Putative YY males were selected from the pedigreed stock of putative YY fish produced in the TAF. The general maintenance followed the procedures described in Chapter 2 section 2.1.

4.2.2 Breeding of fish

Three putative XY and 14 putative YY fish (randomly chosen) were successfully bred (Table 4.1 in Results section) with the clonal line females. Details of breeding procedures were discussed in Chapter 2, section 2.2.

4.2.3 Selection of different types of families for sex linkage study

A total of three groups/types of crosses (each type consisting of three families) were selected from those described in section 4.2.2. A frequency distribution of the sex ratio from each male was observed.

The first type of cross was selected from the apparent normal males giving sex ratios not significantly different from 1:1 (and therefore should be XY). This normal XY group was considered as type 'A' for sex linkage study. Type 'B' included the families giving high frequencies of males (with >90 % male) and significantly different from 1:1 sex ratio. This type of crosses was repeated to try to get enough females (shown in Table 4.1) for results from subsequent marker association studies to be statistically meaningful. The sires of type 'B' group were designated as putative YY males. The third type consisted of families

giving intermediate sex ratios (~64-80% males), considering them as neither normal XY males nor putative YY males. This type was designated as group 'C'. Throughout this experimental chapter (along with other chapters, if mentioned), the designations of these types are used. The details of the selected families are given in Table 4.2.

4.2.4 Fin biopsy and DNA extraction

Fin samples were biopsied from the parents and offspring in each of the 9 families screened. DNA was extracted, quality checked and quantified by Nanodrop. Details of the DNA extraction methods are given in Chapter 2 (section 2.5).

4.2.5 PCR and Genotyping

The method of PCR has been described in Chapter 2 (section 2.6). PCR was performed for DNA samples of parents and offspring (n=48) of type 'A' families with LG1 markers (n=12), then for type 'B' and 'C' families, initially with LG1 markers and then with LG3 markers (n=7) and LG23 markers (n=8). The names of the markers are given in Chapter 3, section 3.3, and the oligo sequences are given in Appendix III to VII. Genotyping of amplified DNA was performed using CEQ Beckman-Coulter Sequencer 8800 as described in Chapter 2 section 2.7. Specific strategies were taken to reduce the amount of genotyping of large number of samples considering the cost. These approaches were:

i) Genotyping of parents DNA: Identifying polymorphic loci in sires and checking the clonal females were homozygous

ii) Bulk segregant analyses (BSA): Pooling of DNA samples from male and female progeny separately into two bulk samples and diluting to 50-100 ng/µl. Thus, only two (bulk) samples represented all male and female progeny DNA of a single

family. Polymorphic loci were run only (from LG1, LG3, LG23 and genome wide scan, depending on family type). While this is regarded as a 'dirty' method, a quick analysis of microsatellite loci can be done in order to identify sex-associated alleles.

iii) Individual genotyping approach: If BSA suggested an association with sex, individual samples were run to allow statistical analysis and build linkage maps.

4.2.6 Genome wide scan in families of type 'B'

The pooled DNA samples from male and female progeny along with their parents in type 'B' families were amplified with 62 additional markers from 21 other LGs (in addition to 27 markers from LG1, 3 and 23) and genotyped at those loci. The genotypes were carefully studied to investigate any interaction between alleles at different loci, particularly with LG1 marker alleles.

4.2.7 Fragment and statistical analyses

The analyses of fragments were performed using Beckman-Coulter software (Fragment analysis module) which identified and quantified the detected allelic fragments. All genotype results were transferred to Excel spreadsheets where data were used for further linkage mapping analysis. The CRI-MAP software v 2.4 (Green and Crooks, 1990) was used to construct linkage maps. These maps were created through Build and Flips options. The software is available online at http://www.ba.cnr.it/Embnetut/Crimap. The graphical representation of linkage maps was done by MapChart version 2.2 (Voorrips, 2002). Chi-square (goodness of fit) statistics were performed to test for significant difference of observed sex ratios in each cross from 1:1 ratios. The same statistics were used to test the allelic segregation at each locus for polymorphic informative markers.

4.3 Results

4.3.1 Sex ratios from breeding of clonal females with a range of males

The cross details and sex ratios from various crosses are presented in Table 4.1.

Table 4.1 Breeding of clonal females with different types of males

Put. XY males tag and tank no.	**Tag no. of clonal females**	**No. of male/ female**	**% male**	**χ^2 (against H_0=1:1)**	**Inferred sire genotype**
00 068C D9E3 (G15)	00 0633 EA38	71/75	48.6	0.11, $P(\chi^2_{[1]})>0.05$	**XY ♂**
00 068C FBE2 (G16)	00 0633 EA38	61/57	51.6	0.136, $P(\chi^2_{[1]})>0.05$	
00 064E 46A8 (G26)	00 068C F2E0	60/62	49.2	0.033, $P(\chi^2_{[1]})>0.05$	
Put. YY males tag and tank no.					
00 068D 0B95 (C8)	00 068C F2E0	45/62	42.1	0.033, $P(\chi^2_{[1]})>0.05$	
	00 068C D9B1	68/43	61.3	0.033, $P(\chi^2_{[1]})>0.05$	
	00 068C DD59	9/7	56.3	0.033, $P(\chi^2_{[1]})>0.05$	
	Overall/mean	**122/112**	**52.1**	**0.427 $p(\chi^2_{[1]})>0.05$**	**XY ♂?**
00 061E E4E0 (G34)	00 068C D6BE	26/13	66.7	4.33, $P(\chi^2_{[1]})<0.05$	**??**
00 013E 315C (C3)	00 068C F2E0	72/4	94.7	60.84, $P(\chi^2_{[1]})<0.001$	
	00 068C F2E0	110/2	98.2	104.14, $P(\chi^2_{[1]})<0.001$	
	00 064C FDF1	222/30	88.1	146.28, $P(\chi^2_{[1]})<0.001$	
	Overall/mean	**404/36**	**91.8**	**307.78, $P(\chi^2_{[1]})<0.001$**	**YY♂?**
00 064E 44F0 (G4)	00 068D 0073	80/46	63.5	9.12, 0.01 >P>0.001	
	00 068C D9B1	7/3	70.0	1.6, $P(\chi^2_{[1]})>0.05$	
	Overall/mean	**87/49**	**64.0**	10.62, $P(\chi^2_{[1]})<0.01$ but >0.001	**??**
00 064C F99B (G5)	00 068D 0073	107/41	72.3	29.43, P<0.001	
	00 068C D9B1	13/5	72.2	3.5, P>0.05	
	Overall/mean	**120/46**	**72.3**	**32.99, P<0.001**	**??**
00 068C E167 (G35)	00 068D 0073	197/14	93.4	158.72, $P(\chi^2_{[1]})<0.001$	
	00 064E 4714	9/1	90.0	6.4, $P(\chi^2_{[1]})<0.05$; >0.01	
	Overall/mean	**206/15**	**93.2**	165.07, $P(\chi^2_{[1]})<0.001$	**YY?**
00 064C FC4D (G24)	00 068C F2E0	10/170	5.5	142, $P(\chi^2_{[1]})<0.001$	**XX neo♂?**
00 064E 1559 (G7)	00 068C F2E0	32/81	28.3	21.24, $P(\chi^2_{[1]})<0.001$	**??**
00 064C F5FC (G14)	00 068C F2E0	50/13	79.4	21.73, $P(\chi^2_{[1]})<0.001$	
	00 068C F5C9	36/8	81.8	17.81, $P(\chi^2_{[1]})<0.001$	
	Overall/mean	**86/21**	**80.4**	39.48, $P(\chi^2_{[1]})<0.001$	**??**
00 068C F8BB (H60)	00 064E 4714	7/2	77.8	2.778, $P(\chi^2_{[1]})>0.05$	
	00 068C DD59	69/2	97.2	63.225, $P(\chi^2_{[1]})<0.001$	
	Overall/mean	**76/4**	**95.0**	**64.8, $P(\chi^2_{[1]})<0.001$**	**YY♂?**
00 064C FEED (H61)	00 064E 4714	18/2	90.0	12.8, $P(\chi^2_{[1]})<0.001$	
	00 064C FDF1	57/24	70.4	13.44, $P(\chi^2_{[1]})<0.001$	
	Overall/mean	**75/26**	**74.3**	**23.77, $P(\chi^2_{[1]})<0.001$**	**??**
00 068C D843 (H62)	00 068D 0B19	23/1	95.8	20.16, $P(\chi^2_{[1]})<0.001$	
	00 068C D9B1	27/3	90.0	19.2, $P(\chi^2_{[1]})<0.001$	
	00 068C F2E0	30/0	100	30, $P(\chi^2_{[1]})<0.001$	
	Overall/mean	**80/4**	**95.2**	**68.66, $P(\chi^2_{[1]})<0.001$**	**YY♂?**
00 068C E90E (H63)	00 068C D9B1	12/1	92.3	9.30, 0.01 >P>0.001	
	00 068C F2E0	36/7	83.7	19.56, $P(\chi^2_{[1]})<0.001$	
	Overall/mean	**48/8**	**85.7**	**28.57, $P(\chi^2_{[1]})<0.001$**	**??**
00 068C F923 (H64)	00 068C D9B1	4/2	66.7	-	-

4.3.2 Selection of three groups of families for sex linkage study

The first selected group (type 'A') of families for linkage study were sired by three XY males represented in the first three crosses in Table 4.1. The other two groups were selected from the observation of frequency distribution (Figure 4.1) of sex ratios sired by pedigreed putative YY sires in TAF. Thus second group (type 'B') consisted of families from highest frequency class (91-100). The third group with intermediate sex ratios (60-80% male progeny) also showed higher frequency. Table 4.2 presents all three types of crosses (consists of nine families) for sex linkage study.

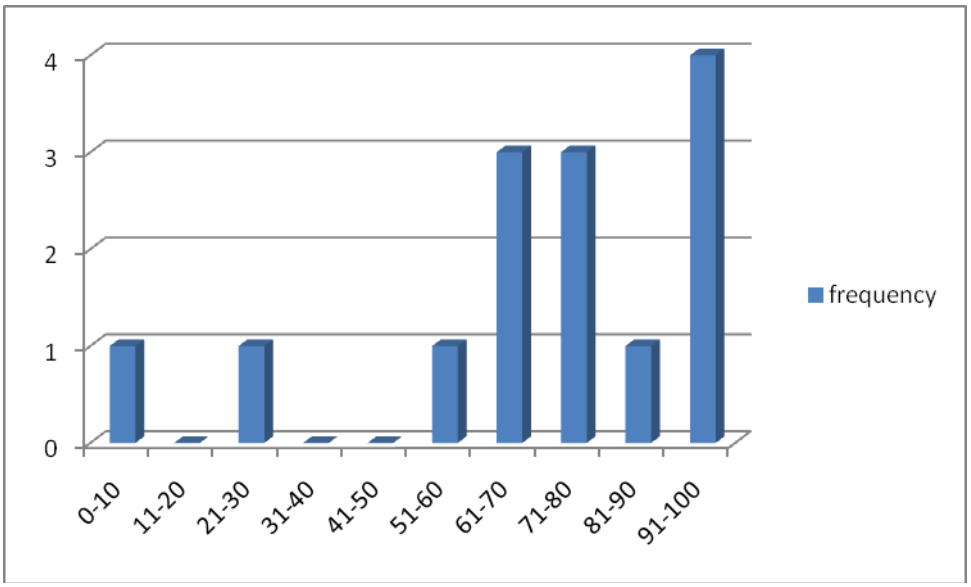


Figure 4.1 Frequency distribution of sex ratio means (% male offspring) of putative YY males x clonal XX females

Table 4.2 Selection of three types of crosses (consists of nine families) for sex linkage study

Designated group/type of cross for sex linkage study	Designated Family	Tag no. of Sire x Dam	No. of male/ female	% male	Inferred Sire genotype	Basis of selection
Type A	Fam 1	00 068C D9E3 x 00 0633 EA38	71/75	48.6	XY	Sex ratios do not significantly differ from 1:1 (Table 4.1), ($P(\chi^2_{[1]})>0.05$)
	Fam 2	00 068C FBE2 x 00 0633 EA38	61/57	51.6	XY	
	Fam 3	00 064E 46A8 x 00 068C F2E0	60/62	49.2	XY	
Type B	Fam 4	00 068C E167 x 00 068D 0073 and 00 064E 4714	206/15	93.2	Putative YY	Sex ratios differ significantly from 1:1 (Table 4.1), ($P(\chi^2_{[1]})<0.001$); High frequency of males (91-100%, Figure 4.2)
	Fam 5	00 013E 315C x 00 068C F2E0, 00 068D 0B19 and 00 064C FDF1	404/36	91.8	Putative YY	
	Fam 6	00 068C F8BB x 00 064E 4714 and 00 068C DD59	76/4	95.0	Putative YY	
Type C	Fam 7	00 064E 44F0 x 00 068D 0073 and 00 068C D9B1	87/49	64.0	??	Sex ratios differ significantly differ from 1:1 (Table 4.1); intermediate frequency of males (64-80%)
	Fam 8	00 064C F99B x 00 068D 0073 and 00 068C D9B1	120/46	72.3	??	
	Fam 9	00 064C F5FC x 00 068C F2E0 and 00 068C F5C9	86/21	80.4	??	

4.3.3 Analyses of marker genotypes in type 'A' families

Heterozygosity was detected in seven loci (out of 12) in LG1 in the sires of type 'A' families. The genotyping and subsequent analyses of pooled progeny DNA samples (BSA) from male (N=23) and female (N=23) showed polymorphisms at all of the seven markers. Individual progeny DNA were genotyped (after amplification of DNA with seven markers from LG1) along with parental DNA samples with those markers (N=48 in each family). The segregations of alleles with polymorphic markers are presented in Table 4.3. The details of the genotype profiles for each marker in BSA are given in Appendix IX.

4.3.4 Linkage and QTL analyses in type 'A' families

Six loci were found to be significantly linked (LOD>3.00) following linkage analyses using the genotype information (Table 4.3). UNH719A was not found to be linked with any of those six loci (LOD<1.00) in Crimap 'twopoint' analysis. Crimap 'Build' produced the order of the linked markers for the six loci concerned (Table 4.4). The positioning of QTL (sex) obtained by 'QTL Express' from the individual genotype results of three families of type 'A' cross is given in Table 4.5. The graphical representation of linked markers and the QTL for sex on LG1 is given in Figure 4.2.

Table 4.3 Segregation of LG1 markers in individuals of type 'A' families

Marker name	Sire tag code	Sire genotype	Dam genotype	Possible genotypes in progeny	No. of progeny with different genotypes	
					Male (N=23)	Female (N=23)
UNH985	D9E3	144/154	144/144	144/144 144/154	2 21	23 0
	46A8	144/154	144/144	144/144 144/154	1 22	22 1
	FBE2	144/154	144/144	144/144 144/154	0 23	23 0
UNH931	D9E3	227/261	227/227	227/227 227/261	1 22	22 1
	46A8	227/261	227/227	227/227 227/261	1 22	22 1
	FBE2	227/261	227/227	227/227 227/261	4 19	21 2
UNH213	D9E3	190/211	211/211	211/211 190/211	2 21	22 1
	46A8	190/211	211/211	211/211 190/211	1 22	22 1
	FBE2	190/211	211/211	211/211 190/211	6 17	22 1
UNH995	D9E3	184/236	184/184	184/184 184/236	4 19	21 2
	46A8	184/236	184/184	184/184 184/236	3 20	21 2
	FBE2	184/236	184/184	184/184 184/236	0 23	23 0

Table 4.3 (cont'd) Segregation of LG1 markers in individuals of type 'A' families

Marker name	Sire tag code	Sire genotype	Dam genotype	Possible genotypes in progeny	No. of progeny with different genotypes	
					Male (N=23)	Female (N=23)
UNH104	D9E3	147/197	147/147	147/147 147/197	4 19	21 2
	46A8	147/197	147/147	147/147 147/197	2 21	22 1
	FBE2	147/197	147/147	147/147 147/197	0 23	23 0
UNH719A	D9E3	121/127	121/127	121/121 121/127	2 21	1 22
	46A8	121/121	121/127	121/121 121/127	1 22	0 23
	FBE2	121/127	121/127	121/121 121/127	5 18	5 18
UNH719B	D9E3	141/null	Null/null	Null/null 141/null	7 16	17 6
	46A8	141/143	Null/null	141/null 143/null	18 5	9 14
	FBE2	141/null	141/null	Null/null 141/141	0 23	3 20

Table 4.4 Male-specific map of Nile tilapia LG1 produced from offspring genotypes of XY males x XX clonal females (type 'A' families)

#	1	2	3	4	5	6
Marker	UNH931	UNH213	UNH985	UNH995	UNH104	UNH719B
Distance cM	0.0	2.9	5.8	5.1	0.7	30.0
Position cM	0.0	2.9	8.7	13.8	14.5	44.5
# Sires Genotyped (%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)
# Dams Genotyped (%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)
# HS Genotyped (%)	138 (100%)	138 (100%)	138 (100%)	138 (100%)	138 (100%)	138 (100%)
# Het Sires	3	3	3	3	3	3
# alleles	2	2	2	2	2	3

Table 4.5 Mapping the QTL in linkage group 1 in cross type 'A'

Linkage group	Trait	QTL location	F value
1	sex	9 cM	355.1 ***

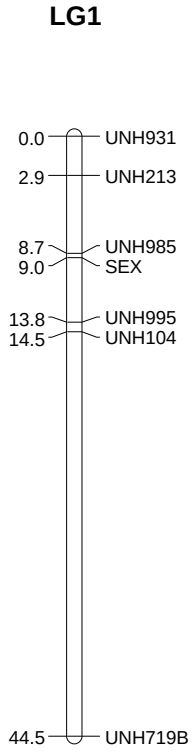


Figure 4.2 The location of sex on linkage group (LG) 1 in Nile tilapia (from XY males x clonal females XX)

4.3.5 Analyses of marker genotypes in type 'B' families

The sires of type 'B' families were found to be heterozygous at seven loci (out of 12 markers screened) in LG1, four loci in LG3 (out of seven markers screened) and seven loci in LG23 (out of 8 markers screened). The genotyping and subsequent analyses of pooled progeny DNA samples (BSA) from males (N=20 from each of the two families and N=28

from third family) and females (N=10 from each of the two families and N=4 from third family) showed that four markers from LG1 were polymorphic in sires of all three families. These were UNH995, GM258, UNH719 and UNH846. The other three markers showed polymorphism in at least one sire. Among those, only UNH995 seemed to show a strong association with sex during pooled sample analyses. The four markers from LG3 and seven markers from LG23, although showing polymorphism in all of the three families of type 'B', did not show any association with sex in the BSA. The details of genotype profiles in BSA for families of type 'B' are presented in Appendix X. Individual progeny DNA were genotyped with UNH995, the only marker seemed to be tightly linked to sex in families of type 'B' and the analysis of allelic segregations is given in Table 4.6.

Table 4.6 Association between UNH995 (LG1) and phenotypic sex in individual progenies of family type 'B' (putative YY males x clonal females)

Cross type	Sire tag code	Sire genotype	Dam genotype	Male progeny		Female progeny	
				Genotype	No.	Genotype	No.
Type 'B' YY x XX (producing > 90% male in progeny sex ratios)	Fam 4 00 068C E167	184/236	184/184	184/184 184/236	11/20 9/20 ns	184/184	10/10 *
	Fam 5 00 013E 315C	184/236	184/184	184/184 184/236	9/20 11/20 ns	184/184	10/10 *
	Fam 6 00068C F8BB	184/236	184/184	184/184 184/236	15/28 13/28 ns	184/184	4/4

* χ^2 (against expected ratio of 1:1 of each genotype in female progeny): $P<0.05$;
ns: Not significant: $P>0.05$

4.3.6 Analysis of marker genotypes in family 'C' families

The sires of type 'C' families were found to be heterozygous at nine loci (out of 12 screened) in LG1, six loci in LG3 (out of 7 markers screened) and all of the eight loci in LG23. The genotyping and subsequent analyses of pooled progeny DNA samples (BSA) from male (N=23) and female (N=23) showed five markers from LG1, four markers from LG3 and eight markers from LG23 to be polymorphic in all three families. Among these, only markers from LG1 showed association with sex in BSA. Three polymorphic LG1 markers in family 7, two in family 8 and three in family 9 seemed to be associated with sex. Individual progeny DNA were genotyped with these markers in each family of type 'C' to test the association between marker and sex. Table 4.7, 4.8 and 4.9 illustrate the genotypes for individual male and female progeny for these markers in families 7, 8 and 9 respectively. The details of genotype profiles in BSA for families of type 'C' are presented in Appendix XI.

Table 4.7 Segregation of LG1 polymorphic markers in family 7 (00 064E 44F0 male x clonal XX female) of cross type C (unknown sire genotype producing ~60-80% male progeny)

Cross type	Marker and LG	Unknown Sire (44F0) genotype	Dam (0073 clonal ♀) genotype	Male progeny		Female progeny	
				Genotype	No.	Genotype	No.
Type C Family 7	UNH931 (LG1)	227/240	227/227	227/227 227/240	10/23 13/23 ns	227/227 227/240	21/23 2/23 ***
	UNH995 (LG1)	236/252	184/184	184/236 184/252	12/23 11/23 ns	184/252	23/23 **
	UNH104 (LG1)	190/210	147/147	147/190 147/210	12/23 11/23 ns	147/210	23/23 **

*** χ^2 (against expected ratio of 1:1 of each genotype in female progeny): P<0.001; ** P<0.01; ns: Not significant: P>0.05

Table 4.8 Segregation of LG1 polymorphic markers in family 8 (00 064C F99B male x clonal XX female) of cross type C (unknown sire genotype producing ~60-80% male progeny)

Cross type	Marker and LG	Unknown Sire (F99B) genotype	Dam (0073 clonal ♀) genotype	Male progeny		Female progeny	
				Genotype	No.	Genotype	No.
Type C Family 8	UNH104 (LG1)	147/190	147/147	147/147 147/190	11/23 12/23 ns	147/147	23/23 ***
	UNH719 (LG1)	127/141	127/127	127/127 127/141	10/23 13/23 ns	127/127 127/141	19/23 4/23 **

*** χ^2 (against expected ratio of 1:1 of each genotype in female progeny): P<0.001
** χ^2 (against expected ratio of 1:1 of each genotype in female progeny): P<0.01
ns: Not significant: P>0.05

Table 4.9 Segregation of LG1 polymorphic markers in family 9 (00 064C F5FC male x clonal XX female) of cross type C (unknown sire genotype producing ~60-80% male progeny)

Cross type	Marker and LG	Unknown Sire (F5FC) genotype	Dam (F2E0/F5C9 clonal ♀) genotype	Male progeny		Female progeny	
				Genotype	No.	Genotype	No.
Type C Family 9	UNH931 (LG1)	227/245	227/227	227/227 227/245	6/18 12/18 ns	227/227 227/245	4/20 16/20 **
	UNH995 (LG1)	236/252	184/184	184/252 184/236	10/18 8/18 ns	184/252	20/20 ***
	UNH104 (LG1)	190/210	147/147	147/190 147/210	8/18 10/18 ns	147/210	20/20 ***

*** χ^2 (against expected ratio of 1:1 of each genotype in female progeny): P<0.001
** χ^2 (against expected ratio of 1:1 of each genotype in female progeny): P<0.01
ns: Not significant: P>0.05

4.3.7 QTL analyses using three families of cross type 'C' and using 6 families altogether from cross type 'A' and 'C'

Following the QTL analyses in three XY families in section 4.3.4, two additional maps were constructed and QTL was positioned by

i) using the genotype information from family 7, 8 and 9 of cross type 'C' with 4 markers (Table 4.10 and 4.11 and Figure 4.3a); and

ii) using the genotype information, collectively, from three families of type 'C' and from three families of type 'A', with 4 markers (Table 4.12 and Table 4.13 and Figure 4.3b).

Table 4.10 QTL analyses for type 'C' families

#	1	2	3	4
Marker	UNH931	UNH995	UNH104	UNH719
Distance cM	0.0	27.5	0.0	11.0
Position cM	0.0	27.5	27.5	38.5
# Sires Genotyped (%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)
# Dams Genotyped (%)	3 (100%)	3 (100%)	3 (100%)	3 (100%)
# HS Genotyped (%)	138 (100%)	138 (100%)	138 (100%)	138 (100%)
# Het Sires	2	2	3	1
# alleles	2	3	3	2

Table 4.11 Mapping the QTL in linkage group 1 for type 'C' families

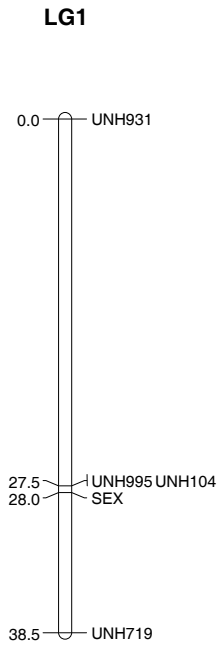
Linkage Group	**Trait**	**QTL Location**	**F value**
1	SEX	28 cM	23.87 ***

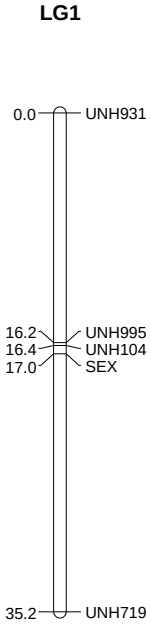
Table 4.12 QTL analyses for genotypes of 6 families, combining crosses of type 'A' and 'C'

#	1	2	3	4
Marker	UNH931	UNH995	UNH104	UNH719
Distance cM	0.0	16.2	0.4	18.6
Position cM	0.0	16.2	16.6	35.2
# Sires Genotyped (%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)
# Dams Genotyped (%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)
# HS Genotyped (%)	276 (100%)	276 (100%)	276 (100%)	276 (100%)
# Het Sires	5	5	6	4
# alleles	3	3	5	3

Table 4.13 Mapping the QTL in linkage group 1 from combined A+C crosses:

Linkage Group	Trait	QTL Location	F value
1	1 (sex)	17 cM	53.4 ***





(Figure 4.3a) (Figure 4.3b)

Figure 4.3 The location of sex on LG1 in Nile tilapia (using genotype data from type 'C' families, 4.3a; and combined data from type 'A' + 'C' families, 4.3b)

4.3.8 Genome wide scan (GWS)

An investigation of 62 DNA markers (mostly microsatellites) from all other LGs (i.e., except LG1, 3 and 23) to follow the segregation of alleles in pooled DNA samples of selected offspring in cross type 'B' (families of putative YY sires x clonal line females) showed a number of polymorphic markers from different LGs that seemed to be associated with sex from the BSA results. Genotyping of individual samples for these selected markers showed the segregation patterns illustrated in Tables (4.14, 4.15 and 4.16). The details of all the markers in the genome wide scan (GWS) and the fragment sizes from pooled DNA samples of male and female offspring along with the parental samples are given in Appendix XII.

Table 4.14 Inheritance of polymorphic markers putatively associated with sex in BSA (during GWS) and genotyping individual samples (♂N=20, ♀N=10) for family 4 of cross type 'B'

Cross type (fam)	Marker (LG)	Sire (E167 putative YY♂) genotype	Dam (0073 Cl XX ♀) genotype	Male progeny			Female progeny		
				BSA Genotype	Individual Genotype	N=20	BSA Genotype	Individual Genotype	N=10
'B' (4)	UNH159 (2)	251/257	251/251	251/257 (257 LP)	251/251 251/257	11 9 ns	251/257	251/251 251/257	5 5 ns
	UNH884 (5)	133/146	160/160	133/146/160 (133 P)	133/160 146/160	9 11 ns	133/146/160 (133 NP)	133/160 146/160	2 8 ns
	UNH309 (5)	199/203	199/199	199/203	199/199 199/203	9 11 ns	199/199	199/199 199/203	4 6 ns
	GM027 (8)	176/191	176/176	176/191	176/176 176/191	10 10 ns	176/191 (191 NP)	176/176 176/191	5 5 ns
	UNH960 (10)	153/182	182/182	153/182	182/182 153/182	8 12 ns	153/182 (153 LP)	182/182 153/182	6 4 ns

P= Prominent, LP= Less prominent, NP= Not prominent (fragment peak size relative to size standard); ns=Not significant, P>0.05 (against expected ratio of 1:1 of each genotype)

Table 4.15 Inheritance of polymorphic markers putatively associated with sex in BSA (during GWS) and genotyping individual samples (♂N=20, ♀N=10) for family 5 of cross type 'B'

Cross type (fam)	Marker (LG)	Sire (315C putative YY♂) genotype	Dam (0B19 Cl XX ♀) genotype	Male progeny			Female progeny		
				BSA Genotype	Individual Genotype	N=20	BSA Genotype	Individual Genotype	N=10
'B' (5)	UNH159 (2)	251/257	251/251	251/257 (257 LP)	251/251 251/257	9 11 ns	251/257	251/251 251/257	5 5 ns
	GM215 (11)	223/248	223/223	223/248	223/223 223/248	9 11 ns	223/223	223/223 223/248	6 4 ns
	GM377 (12)	280/310	310/310	280/310	280/310 310/310	11 9 ns	310/310	280/310 310/310	4 6 ns
	UNH974 (17)	184/232	210/210	184/210/232	210/232 184/210	11 9 ns	210/232	210/232 184/210	6 4 ns

LP= Less prominent (fragment peak size relative to size standard)
ns=Not significant, P>0.05 (against expected ratio of 1:1 of each genotype)

Table 4.16 Inheritance of polymorphic markers putatively associated with sex in BSA (during GWS) and genotyping individual samples (♂N=16, ♀N=4) for family 6 of cross type 'B'

Cross type (fam)	Marker (LG)	Sire (F8BB putative YY♂) genotype	Dam (0073 Cl XX ♀) genotype	Male progeny			Female progeny		
				BSA Genotype	Individual Genotype	N=16	BSA Genotype	Individual Genotype	N=4
'B'(6)	UNH159 (2)	247/257	251/251	247/251/257	247/251 251/257	8 8 ns	251/257	247/251 251/257	1 3 ns
	UNH440 (17)	176/198	198/198	176/198	176/198 198/198	7 9 ns	198/198	176/198 198/198	2 2 ns
	UNH904 (18)	155/174	184/184	155/174/184	155/184 174/184	9 7 ns	155/184	155/184 174/184	2 2 ns

ns =Not significant, P>0.05 (against expected ratio of 1:1 of each genotype)

The genotyping of individual samples (in Tables 4.14, 4.15 and 4.16) in genome wide scan did not show any significant association between markers and sex. However, a single marker from LG5, UNH884, showed marginal P value (0.057) for significant association.

Therefore, an attempt was made to observe if there was any autosomal interaction between marker(s) from main sex determining region (LG1) and that in LG5.

4.3.8.1 Inter-allelic interaction study between UNH995 (LG1) and UNH884 (LG5)

The potential interaction of UNH995, a tightly sex-linked LG1 marker (as observed by QTL analyses) with one of the LG5 markers (UNH884) selected from GWS, in family 4 of cross type 'B' was observed. The genotypes in individual progenies in type 'B' with UNH995 (from Table 4.6) and genotypes in individual progenies in family 4 of type 'B' (marginal P value was observed only in this family) with UNH884 (from Table 4.14) are given in Table 4.17.

Table 4.17 Interaction study of UNH995 (LG1) and UNH884 (LG5) loci in family 4, cross type 'B' (Putative YY males x clonal XX females)

Marker and Cross type	Family	Sire genotype	Dam genotype	Male progeny Genotype (n=20)	Female progeny Genotype (n=10)
UNH995 Type 'B'	Fam 4	184/236	184/184	184/236= 9 184/184=11 ns	184/236=0 184/184=10 *
UNH884 Type 'B'	Fam 4	133/146	160/160	133/160=9 146/160=11 ns	133/160=2 146/160=8 ns

* χ^2 (against expected ratio of 1:1 of each genotype in female progeny): $P<0.05$; ns= not significant

From Table 4.17, it can be seen that one of the two types of UNH995 genotypes (184/236) produced exclusively male progeny. The other genotype (184/184) produced both types of progeny. An interaction could be hypothesised as, in presence of one of the two UNH884 genotypes (133/160), the progenies with 184/184 (UNH995) could be male-biased, whereas the other (146/160) segregates approximately equally. There was no interaction

between these markers to explain female biased progeny. In addition, individual genotyping of progeny DNA from another family (family 5) of the same cross type 'B' was done with UNH884 (although, BSA did not show any association) and did not show any association with sex (Table 4.18).

Table 4.18 Individual genotypes for UNH884 (LG5) in family 5 of cross type 'B'

Cross type (Family)	Marker and LG	Sire genotype	Dam genotype	Male progeny		Female progeny	
				Individual Genotype	No.	Individual Genotype	No.
B (5)	UNH884 (5)	146/160	160/160	146/160 160/160	8/16 8/16	146/160 160/160	11/24 13/24

4.4 Discussion

4.4.1 Sex ratios in clonal females and different males

The sex ratios in the three families of cross type 'A' (Table 4.2) were not significantly different ($P>0.05$) from 1:1 and there was a strong association between polymorphic sex and LG1. Therefore it can be postulated that a monofactorial model of sex determination is at work in all three families with female homogamety (XX) and male heterogamety (XY). The sex ratios in the three families of cross type 'B' (Table 4.2) were >90% male and significantly different ($P<0.001$) from 1:1. The sex ratios in three families of cross type 'C' (Table 4.2) were significantly different from 1:1 ($P<0.01$or $P<0.001$) and in the range of 64-80% male. In contrast to the arbitrary designations of the families by Mair et al. (1997), where potential YY genotypes producing sex ratios significantly not different from 1:1 or only significant at the 5% level ($0.01<P<0.05$) were designated as XY and sex ratios significantly different at a probability level of 0.1% ($P<0.001$) were designated as YY, the current study assigned a criteria of $P<0.001$ with >90% male progeny in the families as putative YY (cross type B). The other type of families (type 'C' families) had no such specific criteria (one family with $P<0.01$ and two families $P<0.001$, against 1:1 in progeny sex) and selected from the frequency distribution with a criterion of up to ~80% male progeny. The sires of these families are neither XY nor YY based on the sex ratios obtained.

The basic sex determination mechanism of some species of *Oreochromis* (besides *niloticus*) has been proposed to be 'predominantly' monofactorial by several authors. Jalabert et al. (1971) studied the sex determination of *O. niloticus* and *O. macrochir* by evaluating the sex ratios of hybrid progeny and concluded that *O. niloticus* had a basic XX:

XY sex determination. However the sex ratios from the back cross of the male hybrid to a female *O. niloticus* did not conform to the expected ratios. Clemens and Inslee (1968) and Chen (1969) proposed that in *O. mossambicus* the female was the homogametic sex (XX) and the male heterogametic (XY) as in *O. niloticus*. There are some other studies where significant variations were observed from predicted sex ratio of 1:1 in this species. Shelton et al. (1983) reported sex ratios from mass spawnings of *O. niloticus* that ranged from 31 to 83% male. Progeny sex ratios from normal XY males and XX females varied from 34.2 to 70% with a mean close to 50%, with a slight but significant overall excess of males (Mair et al., 1997). Tuan et al. (1999) reported a range of 15 to 100% male among 95 families of *O. niloticus*. Calhoun and Shelton (1983) examined the sex ratios within individual spawns of Nile tilapia. Numerous spawns significantly deviated from 1:1 sex ratios. Some females produced as high as 90% male progeny and others as high as 70% female progeny. Calhoun and Shelton (1983) reported that the female component of variation was responsible for 13-fold more variation in progeny sex ratios than the male component of variation suggesting the possibility that the modifying loci may actually lie on the X chromosome rather than the autosomes (Dunham, 2004). This would result in variants of the X chromosome of varying strength, if there was genetic variation at these modifying loci or at the X allele itself. However, where an appropriate XX: XY pattern of inheritance is assumed, the male would produce either an X or Y sperm, and an X-containing egg would be fertilized. If no autosomal factors influence sex ratios, or no variation exists at the XY locus, the fertilization of the egg with a Y sperm should result in male progeny. This would be particularly evident in a highly inbred population such as one produced by androgenesis (Phelps and Warrington, 2001). The highly inbred clonal line of females used as dams in the present experiment may also account for such uniform sex ratios provided

that the male component of variation was only 'Y' in absence of such variants (autosomal or XY locus) in the three families concerned in the current study.

There are a number of studies where significant variations were observed from predicted sex ratios concerning the YY nature of the male parents, like the sex ratios in family type 'B' and 'C' in the current study (both types were selected from YY pedigree). The sex ratios produced by the males that were designated as YY genotypes in a study by Mair et al. (1997) were not quite in accordance with those predicted by the hypothesis of simple monofactorial sex determination, many of them being somewhat lower than the expected 100% male. Mair et al. (1991a) observed a single female in the progeny of one of four YY males progeny tested. YY male genotypes of Nile tilapia sired a mean of 95.6% males when mated with XX females (Beardmore et al., 2001) and reflected predominantly monofactorial genetic sex determining mechanism. However, Scott et al. (1989) observed no females in the sexing of 285 progeny of a single YY male crossed to 10 separate females.

The families of type 'C' in the current study showed sex ratios significantly different from 1:1, but approximately intermediate sex ratios (% male) between the other two groups (XY and putative YY). Significant overall excess of male progeny (as in type 'C' of the present study) has also been observed in a number of studies on Nile tilapia where an arbitrary designation of the sires was hypothesized. Mair et al. (1997) observed 70% male offspring in one family of Nile tilapia, but did not designate the sires as putative YY on the basis of an arbitrary designation ($P>0.01$ but <0.05 against 1:1 denoted as XY) for a range of sex ratios obtained from such families. Such criteria can be set to designate inferred genotypes

in the absence of genetic markers. The present experiment did not categorize such families in either YY or XY, and attempts to identify if families of such group (type 'C' crosses) possess any genetic variation (at single sex determining locus or any autosomal locus/loci) that can be identified by a sex linkage study.

The existence of autosomal influence or a polyfactorial mechanism are popular hypotheses for the departed sex ratios observed. Capili (1995) described highly variable sex ratios of progeny from two YY males of Nile tilapia, ranging from 36% to 100% male (Capili, 1995). This high level of heterogeneity was explained by polyfactorial sex determination in this species. The greater sample size and varieties of families may account for such aberrant sex ratios with significant percentage of females in progeny of arbitrary YY males. Mair et al. (1997) increased the overall proportions of males in the progeny of YY males derived from crosses of selected YY males (on the basis of the 100% male sex ratios in initial progeny tests) and indicated that the sex ratios could be some form of genetic response to the selection. The mean sex ratios of non-selected YY was lower than that from 'selected' YY and was explained by the erroneous inclusion of one or more sex ratios from XY males, incorrectly designated as YY males. It is therefore important to carefully define which statistical criterion should be set to designate the YY genotype. In the present study although two families were observed (in cross type 'C') with significant deviation from 1:1 at $P<0.001$ (and another at $P<0.01$), they were not designated as putative YY males (type 'B', also with $P<0.001$ against 1:1 sex ratio in progeny) considering a hypothesis that these families could be genetically different from normal (XY) or putative super (YY) males, and this difference may be due to the effect of autosomal loci or due to different variants at the XY locus.

Autosomal sex modifying loci have been postulated to explain the deviation from predicted sex ratios in some studies. Karayucel et al. (2004) postulated two unlinked 'sex reversal' loci in the Nile tilapia to explain the presence of males in a clonal line of females. Shirak et al. (2006) also observed effects of autosomal loci for sex determination in hybrid tilapia.

An autosomal sex reversal locus was also postulated to explain the sex ratio departure in blue tilapia, *O. aureus* (Mair et al., 1991b, Hussain et al., 1994). Two unlinked loci have been hypothesised to control sex in blue tilapia (Lee et al., 2004), the action of a dominant male repressor (the W haplotype on LG3) and a dominant male determiner (the Y haplotype on LG1). Cytogenetic studies on male (ZZ), female (WZ) and neomale (WZ) blue tilapia (with synaptonemal complex), revealed unpaired regions in two bivalents in heterogametic individuals, the largest one and a small bivalent (Campos-Ramos et al., 2001). This was intriguing given other evidence for a secondary sex-determining locus in this species (Mair et al., 1991b; Lee et al., 2004).

It seems therefore, the sex ratios obtained in different crosses are supported by factors such as additional genetic, polygene or multiple alleles playing role to positively skew the male percentage in a population in a hypothesized XY male parents (additive or dominant locus or multiple alleles suppressing the expression of female genotype) or negatively skewing the male percentage in crosses sired by hypothesized YY male parents (suppressing the expression of 'male' determining locus or alleles by other loci/alleles).

Environmental parameters are also thought to play a role (particularly temperature, Baroiller et al., 2009) in sex differentiation of this species. But in the present research the

environmental effects particularly of temperature is highly unlikely (because of controlled environmental conditions) and reinforces some other genetic sex determination system apart from single chromosomal effect which could be revealed by analysing the inheritance of markers in these families as well as genome wide scanning of the markers and studying the inheritance pattern (follows next).

4.4.2 QTL and association study of markers

Study on the seven loci from LG1 and segregation of alleles in individual male and female offspring in cross type 'A' and QTL analyses of the genotypes revealed six loci to be significantly linked (LOD> 3.00). UNH719A was not found to be linked with any of those six loci (LOD< 1.00) in CRI-MAP 'twopoint' analysis. The order of the markers produced by Crimap 'Build' showed that the genetic map length from the present study considering six loci was 44.5 cM (Table 4.4, Figure 4.2, results section). This was a sex-specific (male) map. No female recombination map could be constructed in the females due to their inbred clonal nature- homozygous for every locus (described in Chapter 3). Considering those six markers, the genetic map produced by Lee and Kocher (2007) gives a map length of 42.2 cM, very close and comparable with the male-specific map in current study. The extensive linkage map of tilapia genome constructed from F_2 progeny of an interspecific cross between *Oreochromis niloticus* and *O aureus* (Lee et al., 2005) shows significance linkage between SEX and six markers at the end of LG3 for a single family consisting of 70 individuals. In LG1, the map distance (Lee et al., 2005) between UNH931 and UNH719 was shorter (33 cM) compared to the LG1 map of Lee and Kocher (2007) and that in the current study. The linkage and order of markers in the present study are largely congruent except the double flip of UNH985 with previous maps (by Lee and Kocher, 2007; Lee et

al., 2005). Such flipping of the markers was also evident in the male-specific map produced by Lee et al. (2004) for *Oreochromis aureus*. Although the basic karyotype seems to be very similar and each linkage group can represent each chromosome by a series of DNA markers, the small scale difference of map orders could be due to a number of factors, for example number of individuals genotyped, genotyping accuracy/errors, different strains and species etc.

The distance of the QTL for SEX in the study of Lee and Kocher (2007) was 3.4 cM from UNH995. The flanking marker on the other side of the QTL in their study, Wt1b, was closer to SEX, only 2.5 cM away but excluded as candidate for sex determination because of the two recombinants identified in breakpoint analyses. In the present study, the Wt1b marker (modified as Wt1b_short) was found to be monomorphic and SEX was positioned in between UNH985 and UNH995, being 4.8 cM away from UNH995 (QTL lies at 9 cM, F_{stat} =355.1, Figure 4.2, results section).

Segregation of markers in three families of intraspecific *O. niloticus* was observed in order to map the sex determining region by Lee et al. (2003). Out of 105 markers, 80 markers successfully amplified and only two markers, UNH995 and UNH985, showed segregation differences between males and females in 2 of 3 families in BSA. Those two markers are also sex-linked in QTL analyses with the three families in the current study (Table 4.4, 4.5, Figure 4.2, results section) as UNH985(8.7)-SEX(9.0)-UNH995(13.8). From genotyping of individual fishes and subsequent scoring of the proportion of individuals whose phenotypic sex was consistent with the hypothesized Y chromosome by analysing the inheritance of multilocus haplotypes, Lee et al. (2003) showed that microsatellite markers GM201,

UNH995 and UNH104 were significantly associated with phenotypic sex. In their study, the markers had no association with the sex of fishes in the third family despite the fact that all three families came from the same stock (i.e., Stirling stock). Lee et al. (2003) suggested additional sex determining factors may be segregating in this stock and suggested analysing more families and strains from various sources for complete enumeration of the genes controlling sex in *O niloticus*. The current study was also designed to extend the analyses (using intraspecific Stirling Nile tilapia) of the segregation of markers in various families including type 'C' (as unknown type) and elaborated below for a precise understanding of the sex determining mechanism in this species.

Study on sex determining mechanism on another species of tilapia (blue tilapia, *Oreochromis aureus*) has identified markers linked with two sex-determining loci segregating in a single family of blue tilapia (Lee et al., 2004). Sex determination of blue tilapia is thought to be a WZ-ZZ (female heterogametic) system controlled by a major gene. Lee et al. (2004) searched for DNA markers linked to this major gene using the technique of BSA and identified 11 microsatellite markers on linkage group 3 which were linked to phenotypic sex. The putative W chromosome haplotype correctly predicted the sex of 97% of male and 85% of female individuals. They have suggested the W locus lies on LG3 (within a few cM of markers GM354, UNH168, GM271 and UNH131). Markers on LG1 also showed a strong association with sex in this species, and indicated the segregation of a male-determining allele in this region. Analysis of epistatic interactions among the loci suggested the action of a dominant male repressor (the W haplotype on LG 3) and a dominant male determiner (the Y haplotype on LG1). In the current study, SEX was positioned in LG1 with statistically highly significant F values (F_{stat} =355.1) and hence

LG3 markers were not used to observe the segregation difference in any of the three intraspecific same strain XYx XX families (type 'A').

The genetic map from the three families of cross type 'C' shows a map length of 38.5 cM considering four loci (Table 4.10, Figure 4.3a, results section). This falls in between the map by Lee et al. (2005), 33 cM and that by Lee and Kocher (2007), 42.2 cM, accounting the four markers, which are also built in same order with those two maps. SEX (28 cM, Table 4.11, Figure 4.3a, results section) was positioned only 0.5 cM away from UNH995 and UNH104 (both positioned at 27.5 cM). The male specific map of Lee et al. (2003) on *O. niloticus* also shows very similar positions of these two markers (Figure 4.4b, this section).

The genetic map from the six families considering type 'A' and type 'C' cross shows a map length of 35.2 cM considering four loci (Table 4.12, Figure 4.3b, results section) which can be compared to that produced by using genotypic data of three putative YY families of type C cross (38.5 cM). This map length is much closer to that of Lee et al. (2005), 33 cM. The marker orders are congruent with those of Lee et al. (2003, Figure 4.4b, this section), Lee et al. (2005, Figure 4.4c, this section) and Lee and Kocher (2007, Figure 4.4d, this section). SEX (17 cM) was positioned only 0.8 cM away (Figure 4.4a, this section) from UNH995 (16.2 cM) and 0.4 cM away from UNH104 (16.6 cM). This genetic map produced by the genotypic data of 6 families (consisting of family 'A' and 'C'; offspring N=276) supports the highest correspondence of phenotypic sex with two of three microsatellite markers as shown by Lee et al. (2003) and also an evidence for the two markers (UNH995 and UNH104) to be tightly linked (only 0.4 cM, comparable to the 0.7

cM by Lee and Kocher, 2007) and on the same side of the QTL for sex, which maps closer to these markers in the current study (0.8 cM from UNH995) compared to the distance of 3.4 cM in the study of Lee and Kocher (2007). The linkage maps from Lee et al. (2003), Lee et al. (2005) and Lee and Kocher (2007) are presented for a comparison of marker order and position of sex compared to that in the present study (Figure 4.4).

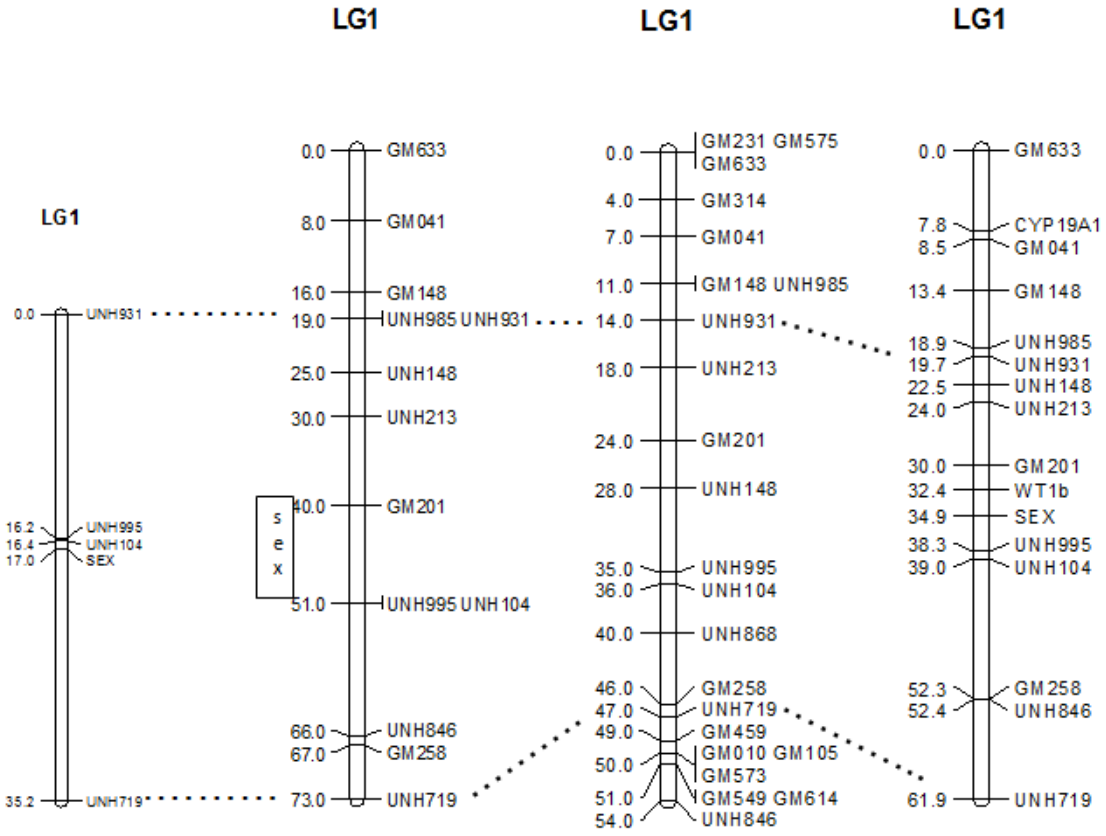


Fig 4.4a Fig 4.4b Fig 4.4c Fig 4.4d

Figure 4.4 Location of SEX in the present study (a) using four informative markers in six families (type 'A'+ type 'C') and comparing orders of map and/or location of sex with other works: b) Lee et al. (2003), c) Lee et al. (2005) and d) Lee and Kocher (2007)

The higher similarity of the map orders using different families (in combination) and mapping the QTL very close to UNH995 and UNH104 refines the location of the major sex determining region in LG1 in *Oreochromis niloticus*.

An observation of the allelic inheritance pattern of LG1 markers in families of cross type 'B' (Table 4.6) and 'C' (Table 4.7, 4.8 and 4.9) illustrated that microsatellite marker UNH995 was an informative (in terms of segregation of alleles) polymorphic marker in all families of cross type 'B' (family 4, 5 and 6) as well as two of the families of cross type 'C' (family 7 and 9) while another marker UNH104 was informative in all of the families in cross type 'C'. These two markers along with GM201 showed increasing association with phenotypic sex and predicted phenotypic sex with 95% accuracy in two out of three families in the study of Lee et al. (2003). Analysis of the inheritance of marker UNH995 and UNH104 in F7 (cross type 'C') in the current study showed that progeny that inherited one of the UNH995 alleles (i.e., 236) and UNH104 alleles (i.e., 190) from the male parent were all male, while inheritance of the other allele (allele 252 in case of UNH995 and allele 210 in case of UNH104) was associated with a mixture of male and female progeny. In the case of UNH931, allele 240 was predominant in male progeny of family 7. In family 8, only UNH104 showed such a pattern of allelic association with sex. In family 9, inheritance of marker UNH995 as well as UNH104 alleles followed the same pattern of allelic segregation with sex as that in family 7. This finding suggests that some alleles of the sex determining system in this species are "ambivalent" (symbolized as allele 'A')– thus the male parents of type 'C' families would be labeled as 'YA' rather than putative YY. The same is true to a lesser extent for families of type 'B', where one paternal LG1 haplotype was associated with only male progeny and the other with some female progeny.

Thus, the allele(s) giving close to 100% males are 'strong Y' (symbolised as "Y") and the allele(s) giving some female progeny are "weak Y" (tentatively symbolised as "Ɏ"). On the other hand, the allele(s) producing exclusively female individuals (as in clonal line females) can be considered as "strong X". The presence and actions of such multiple alleles (X, Y, Ɏ and A) at the same sex-determining locus of Nile tilapia are the unique findings in the present study.

A summary of LG1 haplotypes using UNH995 and UNH104 is given in Table 4.19 for type 'B' and 'C' families that show the 'ambivalent' and weak Y nature of some parental sex-determining alleles.

Table 4.19 Summary of segregation of UNH995 and UNH104 alleles in families of type 'B' and type 'C' with 'ambivalent' nature

Marker	Cross type	Sire genotype	Dam genotype	Male progeny		Female progeny	
				Genotype	No.	Genotype	No.
UNH995	Type B (all families)	184/236	184/184	184/184 184/236	35/68 33/68	184/184 184/236	22/22 0/22
	Type C (2 families)	236/252	184/184	184/236 184/252	22/41 19/41	184/236 184/252	0/43 43/43
UNH104	Type C (fam 7)	190/210	140/140	140/190 140/210	12/23 11/23	140/190 140/210	0/23 23/23
	Type C (fam 8)	140/190	140/140	140/140 140/190	8/16 8/16	140/140 140/190	23/23 0/23
	Type C (fam 9)	190/210	140/140	140/190 140/210	12/23 11/23	140/190 140/210	0/23 23/23

The discovery of "ambivalent" alleles at the main sex determining locus in this experiment could explain the failure to associate phenotypic sex and any region of the genome in one of the three families in the study of Lee et al. (2003). XA individuals seem to show a sex ratio of approximately 1:1 (see sex ratios in Table 4.19, for type 'C' families: with UNH995, progenies of genotype 184/236 are all male, whereas progenies of genotype 184/252 giving sex ratio approximately 1:1, giving 64-80% males overall), if the female parent was XX and the male was AA (homozygous for ambivalent 'A'). This hypothesis could also explain the single fully inbred gynogenetic clonal line with a sex ratio close to 1:1 (but expected to be all female) in the study of Sarder et al. (1999). If the founder mitotic gynogenetic female was AA, this ratio would be observed.

4.4.3 Association studies of markers from LG1 and genome wide scan

After an initial BSA investigation of association of markers from genome wide scan (GWS) with sex in three families (family 4, 5 and 6) of cross type 'B', an observation of the genotype data of the individual offspring was performed. The analysis showed that, in family 4, one marker from LG5 (UNH884) was informative in the sense that one allele (133) was less prominent in females. However, no significant departure of the two genotypes was observed against 1:1 (8:2, P=0.057). Females were predominantly 146/160 whereas males 133/160 and 146/160, two alleles (133, 146) being distributed approximately equally. Although the sample number of female individuals was low (type 'B' crosses had >90% ♂), an attempt was made to see if there was any interaction of this locus with one of the LG1 markers (UNH995) in association with sex (Table 4.17). It seemed allele 236 of locus UNH995 might interact with allele 133 of locus UNH884. On the other hand, an analysis of the genotype of 184/184 (UNH995) which showed a

segregation in female as well as in male could be made with the two types of genotypes predicted by UNH884. In the presence of allele 133 (UNH884), the fish of genotype 184/184 (UNH995) turns into male whereas with allele 146 (UNH884), individuals could be either male or female. Thus loci or allele/s from LGs other than LG1 that could show an interaction with sex determining alleles linked to 184/184 (UNH995) to establish female sex in those families (hypothesizing the male as YY) were not observed. An increased number of genotyped progeny with UNH884 for another family of type 'B' (family 5), was merely a further demonstration that there was no significant association between UNH884 and sex (Table 4.18).

4.4.4 Summary

A summary and comparison of the investigation and outcomes of the sex-linkage study in Nile tilapia (in the present work) can be done with those of others. Several studies have identified genetic markers linked to sex determination in tilapia, with either pure or hybrid strains. Lee et al. (2003) identified an XY system on LG1 in Nile tilapia, Lee et al. (2004) observed epistatic interactions between a WZ system on LG3 and the XY system on LG1 in a strain of blue tilapia. Two distinct QTL for sex determination in tilapias were reported on LG23 in a hybrid cross between blue tilapia and Mozambique tilapia (Cnaani et al., 2003, 2004). Eshel et al. (2009) provided evidence of association of a microsatellite marker on LG23 (UNH898) with sex in Nile tilapia, besides weak association of markers from LG1. A complex model augmenting the sex chromosome with an autosomal locus was suggested by Hammerman and Avtalion (1979) where simple genetic models did not explain all of the observed progeny sex ratios. New QTL for sex determination might emerge in hybrids due to the interactions of alleles from different species such as those

predicted by the autosomal theory (Hammerman and Avtalion, 1979). Cnaani et al. (2008) provided support for this model, in that both LG1 and LG3 contribute to sex determination in some families in *Oreochromis* spp. However, Mair et al. (1991b) and Shirak et al. (2006) reported significant complications in sex-linkage study in hybrid lines of tilapia because of the probability of interactions between multiple QTLs in the sex determination pathway. The sex-linkage analyses in the present study was based on pure strain Nile tilapia with a reference line of clonal females, and using three different types of crosses involving nine families (selected on the basis of sex ratios from varieties of families), so that a clear scenario of the sex-determination system in this species can be found. The genome wide scanning of markers and study on the inheritance of markers and sex in the present study did not support the theory of an autosomal modifier or interaction of loci in the families studied. Rather, another model, of segregation of multiple alleles at the same sex determining locus, similar to that proposed by Wohlfarth and Wedekind (1991), was more appropriate in the current study. The observation of polymorphism in a single sex determining locus (in XX/XY system) was the key finding in the sex determination studies in the Stirling strain of Nile tilapia.

Chapter 5
Marker-assisted selection in Nile tilapia (*O. niloticus*)

5.1 Introduction

The concept that it is possible to infer the presence of a gene from the presence of a marker tightly linked to the gene is the basis of marker-assisted selection (MAS). MAS refers to a selection process in which future broods are chosen based on genotypes using molecular markers. To implement MAS, researchers need to produce high-resolution linkage maps, understand the number of QTL affecting a given performance or production trait and their mode of inheritance and relative contribution, determine the linkage and potential interactions of different QTL for the trait and for other traits, and estimate the economic importance of each trait (Poompuang and Hallerman, 1997). Selection of one trait may be made at the expense of another, and a well-planned MAS program should take all economically important traits into consideration (Liu and Cordes, 2004).

In marker-assisted selection, breeders select animals or plants carrying beneficial genotypes and alleles of markers that are associated with or contribute to a trait of interest. Thus, as soon as knowledge about genes or QTL affecting essential traits becomes available and haplotypes carrying preferred alleles are flanked by molecular markers or causative mutations are found within the genes responsible for particular traits, MAS can be implemented (Rothschild and Ruvinsky, 2007). Successful implementation of MAS requires well-developed genomic tools, including information on genetic variations relevant to the QTL phenotype, mode of inheritance, interactions with other contributing QTLs and economical magnitude of the QTL studied (reviewed by Poompuang and Hallerman, 1997). MAS can be well applied to sort out potential broodstock by early selection before maturity and help reduce the time and effort in breeding programmes.

It is important to have high-density and high-resolution genetic maps, which are saturated by markers in the vicinity of a target locus (gene) that will be selected. It is generally expected that the maximum separation between markers and the QTL is no greater than a few centimorgans (generally 1-2 cM) (Chistiakov et al., 2005). Strategies to find markers tightly linked to the target gene are similar to those that are used for fine QTL mapping. When implementing flanking marker analysis, a large segregating population is screened with markers flanking the target interval (usually 5-10 cM) in order to identify individuals with a crossover within that interval (Dixon et al., 1995). In pooled sample mapping, DNA from individuals from a large segregating population that share a given phenotype is pooled (Churchill et al., 1993). DNA from each pool is analysed with markers flanking the target gene. Once a tight linkage is found between a molecular marker and a gene of interest, the inheritance of the gene can be traced in selective breeding programs.

Not all traits are equally suitable for MAS implementation. This is because the MAS technology should be economically justifiable in terms of the cost of development of technology and any potential gains. Assuming that this technology is based on relatively complex and expensive methods of sampling, DNA extraction, marker identification, and analysis on a mass scale, costs can initially be quite high until the marker is identified. Additional costs of running a MAS could be relatively small. There are several criteria affecting potential benefits of MAS that need to be considered. For examples, in a variety of situations, traits (e.g., meat quality) are not known or cannot be recorded prior to the required selection decisions. So, markers might be very valuable in improving the genotypes of hidden phenotypes of sires and dams in relation to the meat or flesh quality. Another example is resistance or susceptibility of animals to a certain disease or parasite,

which might occur only at the time of exposure. Here again MAS could be useful in promoting the most resistant genotypes. In some species (including numerous marine species) selection at the time of exposure could be an alternative option particularly when there is a major locus determining resistance (Notter and Cockett, 2005). MAS can be used in sex control in aquaculture species for desirable production performance. Young animals, which have yet to be involved in progeny-test schemes, present a better opportunity for MAS to be used than older individuals who might have a significant number of offspring with measured traits. As estimated breeding values (EBV: estimation of genetic worth of animals or animal's performance in a trait as compared to the base for that flock or breed. The value of an EBV in one individual is compared to all the animals in its linked gene pool that is within that flock, and across flocks, as well as across years, greatly increasing the accuracy of that information in predicting genetic superiority) of such young individuals can not be predicted, MAS using markers for the desired traits can be quite useful to preselect animals for further testing or to speed generation interval. In some instances, unfavourable haplotypes carrying alleles with the opposite effect on the breeding value might occur in a population. Such undesirable linkage disequilibrium can be better handled by MAS, which will facilitate identification of rare and desirable recombinant haplotypes. These recombinant haplotypes might present a new opportunity for selection and could be very beneficial. However, the initial investments in gene and QTL mapping, which are unavoidable, might be significant. For companies selling improved breeding stocks, active use of MAS can be viewed also as a marketing tool and may serve as evidence of the high genetic quality of their product to some customers. GeneSTAR® is a very suitable example of such situations to assess potential high meat quality in young bulls (Genetic Solutions, 2006). Buyers are willing to pay premium prices for animals with

presumed superior qualities. MAS has successfully been implemented in farmed animals, such as cattle (Maillard et al., 2003), pig (Rothschild, 2003), sheep (Notter and Cockett, 2005) and chicken (Malek and Lamont, 2003). In livestock, commercial implementation of MAS related to improvement of quantitative traits has been employed for removal of deleterious major genes, growth rate, meat quality, disease resistance, and reproductive traits in pigs and in other species such as cattle where markers are used routinely for improvement of protein percentage in milk and marbling and tenderness in beef cattle (Dekkers, 2004).

In aquaculture, most of the genetic improvements in quantitative traits to date have been performed following the use of traditional breeding approaches. The growing numbers of markers and the development of useful maps and candidate genes and markers in some species have allowed investigation of QTL(s) for a number of traits in some aquaculture species, for examples, in rainbow trout for thermal tolerance (Perry et al., 2001, 2005), spawning time (O'Malley et al., 2003), embryonic development (Robison et al., 2001), and disease resistance (Ozaki et al., 2001); in salmon, for body weight and condition factor (Reid et al., 2005); in arctic charr, for temperature tolerance (Somorjai et al., 2003); in catfish, for feed efficiency and performance traits (Karsi and Waldbeiser, 2005), and immune response (Karsi et al. 2005). However, in commercial aquaculture ventures, using markers in selection programmes is quite new. One good example of actual application of MAS is IPN (infectious pancreatic necrosis) disease resistance in salmonids (Houston et al., 2008)- which to the author's knowledge is the first commercial application of QTL in fish.

MAS can be applied in aquaculture species where sex control is important. One sex may grow faster than the other or has maturation features that are less desirable for production purposes. For example, in fish species such as Atlantic halibut, barfin flounder and half-smooth tongue sole, females grow faster and bigger than males while in tilapia species, the males grow faster, and therefore are of greater value. Monosex stocks have been developed in various fish species including rainbow trout, chinook salmon (Hunter et al., 1983), Nile tilapia (Mair et al., 1997) and halibut (Tvedt et al., 2006) typically combining sex reversal, progeny testing and selection depending on the sex determination system (XX/XY or WZ/ZZ) and the desired sex (all female or all male). Mixtures of genetic males and females are treated with androgens in order to masculinise the genetic females into functional males (except half smooth tongue sole, with WZ-ZZ system of sex determination). The identification of the two types of males (XY and XX) is commonly carried out by means of a test crossing of each individual with regular females (XX) and progeny testing of the sex of the offspring.

The generation and maintenance of monosex stocks require that genetic and phenotypic sexes are independently discernible. The inability to identify the genetic sex of neo-males could hamper the development of monosex stocks. Various molecular markers, including sex-specific and sex-linked genetic markers have been isolated and used for the assessment of the genetic sex of fish. For example, male (Y)-specific DNA markers were isolated and used for genetic sex identification in salmonids (Devlin and Nagahama, 2002; Felip et al., 2005). Also, male-specific RAPD markers were isolated from African catfish (*Clarias gariepinus*) (Kovacs et al. 2000). Two male-specific AFLP markers were also identified in the three-spined stickleback, *Gasterosteus aculeatus* (Griffiths et al., 2000), but were found

not to be applicable in the nine-spined *Pungitus pungitus* and 15-spined stickleback *Spinachia spinachia* (Griffiths et al., 2000). A sex-determining gene, *DMY*, was isolated from medaka (*Oryzias latipes*) (Matsuda et al., 2002); however, this gene, although sex-specific in medaka and *O. curvinotus*, is not the sex-determining gene in any other fish tested (Volff et al., 2003). Recently, sex-linked AFLP (Ezaz et al., 2004a) and microsatellite markers (Lee et al., 2003; Lee and Kocher, 2007) have been identified in Nile tilapia (*Oreochromis niloticus*). However, marker-assisted sex control has been reported only in salmonids (Devlin and Nagahama, 2002) and half-smooth tongue sole, *Cynoglossus semilaevis* (Chen et al., 2008).

Microsatellite markers are useful in early stages of MAS for the primary selection of parents for further crossing and subsequent genetic characterisation of progeny. For this, markers tightly linked to the target QTL are used. In the present study, one of the two tightly sex linked LG1 markers, UNH995, in Nile tilapia was utilised in MAS. Males were isolated on the basis of genotypes that were homozygous for the allele associated with the strongest male (sex) determination to designate them as "true" or "super (YY) males". These were stocked as 'true' YY red line male to add to the existing YY pedigree in Tropical Aquarium Facilities to advance YY line further or to assist production of genetically male Nile tilapia.

The objectives of the present study were:

i. To identify 'true' YY males on the basis of genotype data in sex linkage study

ii. To breed the 'true' YY males with clonal and outbred females for sex ratio study

5.2 Materials and methods

5.2.1 YY female broodstock

Two putative YY females were used in the study. They were selected based on the YY pedigree of the Tropical Aquarium Facilities at the Institute.

5.2.2 Validation of YY females with sex ratios

The selected YY females were crossed with different types of males to ensure their YY nature on the basis of progeny sex ratios and not on marker genotype at this stage. A total of nine males (3 putative YY males, 4 XY males and 2 XX neomales) were used to observe the progeny sex ratios in a total of 833 individuals (146 offspring with YY males, 562 with XY and 125 with XX neomales). The sexing of offspring started at 2.5 months old using the acetocarmine gonad squashing method described in Chapter 2.

5.2.3 Breeding of YY females to initiate MAS

The YY females, after a validation with sex ratios, were used to breed with two putative YY males and three XY males that were earlier confirmed using sex linkage studies (Chapter 4).

5.2.4 Sex reversal and Control group progeny

DES treatment (hormonal feminization) was carried out for half of the offspring from YY females x YY males and YY females x XY males. The details of the sex reversal technique were described in Chapter 2 section 2.4. Two replicates were set up (except two crosses) for each treatment and control group with 50 offspring/tank. Thus the intention was that half of the offspring from YY female x YY male crosses would be reversed to YY

neofemales and that treated fry from YY female x XY male crosses would result in a mixture of XY neofemales and YY neofemales. The YY neofemales would be separated (after genotyping) to stock in TAF YY pedigree as broodstock. The other half of the progeny groups were kept as controls, which should give putative YY male progenies from YY females x YY males, and a mixture of putative YY male and XY male progenies from YY females x XY males. The MAS would be used to separate 'true' YY males for increased production of all genetically male tilapia (GMT).

5.2.5 Rearing the offspring in recirculated tanks

The DES treated individuals were transferred from static plastic tanks to tanks in recirculating systems after three weeks of hormone treatment, where they were grown for six months. The control groups were also kept in the same kind of tanks. At the age of three months, one replicate from each of two types of crosses from DES treatment and control groups were sexed by gonad squash to observe the sex ratios.

5.2.6 PIT tagging of grow-out individuals (F_1)

A total of 47 fish from the control groups (including both types of crosses) were selected randomly, PIT tagged and fin biopsied. A total of 16 fish from the DES treated groups (including both types of crosses) were selected, PIT tagged and fin biopsied.

5.2.7 DNA extraction, PCR and genotyping

DNA was extracted from the fin clips, amplified and genotyped for only a single marker-UNH995 from LG1.

5.2.8 Separation of 'true' supermales (YY males)

On the basis of earlier studies on sex linkage and genotype profiles (Chapter 4), putative 'true' supermales were separated. Thus, the selected (and separated) YY males were homozygous for the strongest male-associated UNH995 allele (with genotype 236/236 at UNH995 locus).

5.2.9 Verification of 'true' YY males

Two first-generation selected YY 'true' supermales (selected with marker UNH995) were verified. One was crossed to a clonal line female and the other crossed to an outbred female to observe the sex ratio in progeny. The breeding plans for MAS are outlined in Figure 5.1.

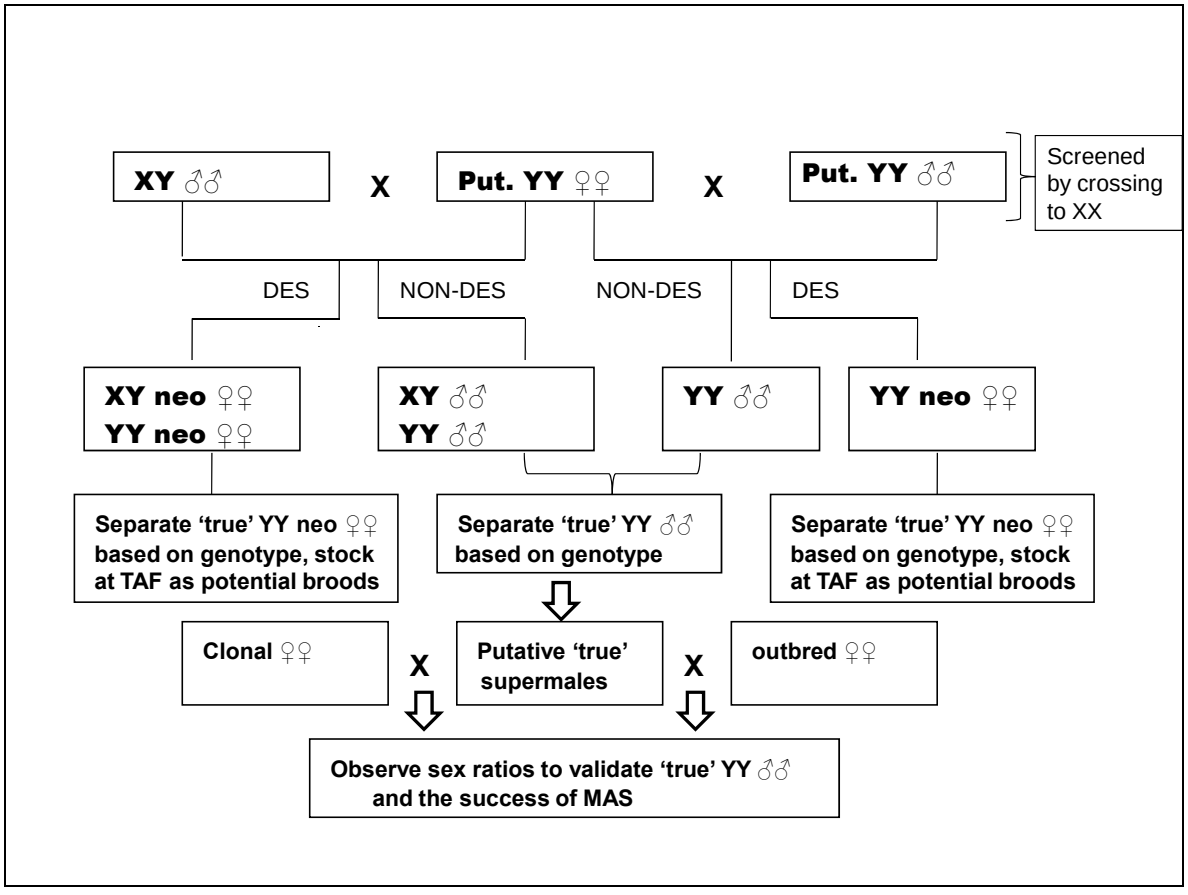


Figure 5.1 Flowchart of breeding strategies in marker-assisted selection of Nile tilapia

5.3 Results

5.3.1 Observation of sex ratios

Progeny sex ratios of putative YY females (selected from YY pedigree at TAF) crossed with different types of males are given in Table 5.1. In one cross between a putative YY female (00 068D 00AD) and a clonal neomale (00 064D 1843), a slight variation from expected progeny sex ratio was observed (94% male, N=117). Nevertheless, based on the observed progeny sex ratios (~100% male progeny) from crosses with two other types of males (putative YY and XY), the two putative YY females were used to initiate MAS.

Table 5.1 Sex ratios in putative YY females x different types of males

Males	**PIT tag no.**	**Sex ratios with putative YY females (% ♂ in parentheses)**	
		00 068D 00AD	00 068C F30D
Putative YY	00 013E 315C	M=25 F=0 (100)	M=54 F=0 (100)
	00 068C E167	M=37 F=0 (100)	-
	00 068D F8BB	M=30 F=0 (100)	-
XY	00 068C D9E3	M=179 F=1 (99)	-
	00 068C FBE2	M=34 F=0 (100)	-
	00 064E 46A8	M=66 F=0 (100)	M=121 F=1 (99)
	00 0638 D41C	M=160 F=0 (100)	-
XX neo	00 064D 1843	M=110 F=7 (94)	M=3 F=0 (100)
	00 064C EBEE	-	M=5 F=0 (100)

M=male progeny, F= female progeny

5.3.2 Observation of the rare females' genotype(s) with UNH995 in putative YY female x XX neomale

The genotypes of small number (N=7) of female offspring derived from a cross (Table 5.1) between a putative YY female (00 068D 00AD) and a clonal neomale (00 064D 1843 XX male). The other putative YY female crossed with two XX neomales produce very small number of viable progeny (n=8) and no females were present to be genotyped.

Table 5.2 Genotypes of the female progeny with UNH995 in cross between YY female and XX clonal neomale (Nine male progeny were also genotyped)

Cross type	Dam tag	Dam genotype	Sire tag	Sire genotype	Offspring genotype	
					Female progeny (n=7)	Male progeny (N=9)
(YY female x XX male)	00AD YY♀	236/252	1843 XX ♂	184/184	184/236 (N=0) 184/252 (N=7) *	184/236 (N=4) 184/252 (N=5) ns

* χ^2 (against expected ratio of 1:1 of each genotype): P<0.05; ns: Not significant: P>0.05

The genotype results can be compared to that obtained from cross type 'B' (putative YY males crossed with clonal XX females giving >90% male progeny) where all females had same paternal allele (allele 252). No female was found with allele '236' (Table 5.2). This result further justified the strategy of marker-assisted selection.

5.3.3 Survival and sex ratios in DES treated and control group progeny

The survival rates and sex reversal rates (in treatment groups) were variable in putative YY females x YY males and YY females x XY males and are shown in Table 5.3.

Table 5.3 Observation of progeny sex in sex-reversed group and control group

Cross type	Dam tag	Sire tag	R	Survival (no.) in DES-treated group (initial no.=50/R)		Survival (no.) in control group (initial no.=50/R)	
				3 mo	No. of ♂ and ♀ by HS in 6 mo	3 mo	No. of ♂ and ♀ by HS in 6 mo
Putative YY♀ x putative YY♂	F30D	315C	R1	20[a] ♂=12 ♀=8	-	38[b] ♂=38	-
			R2	18	♂=3 ♀=2	40	♂=26 ♀=0
	00AD	F8BB	R1	35	♂=26 ♀=2	35	♂=30 ♀=0
			R2	38	♂=29 ♀=3	35 *	-
Putative YY♀ x XY♂	F30D	D9E3	R1	12[a] ♂=7 ♀=5	-	33	♂=28 ♀=0
		46A8	R1	25*	-	36	♂=26 ♀=0
			R2	28	♂=12 ♀=6	34[b] ♂=34	-
	00AD	D9E3	R1	20	1 ♂=1	40	♂=35 ♀=0
		FBE2	R1	32*	-	38	♂=32 ♀=0
			R2	30	♂=12 ♀=10	35 *	-

[a] gonad-squashed batch from treatment group [b] gonad-squashed batch from control group HS=Hand sexing; R= Replication; * these batches were discarded at 4 months of age due to the shortage of tanks

The mean survival rates in DES treated and control groups in three months were 51.6% and 72.8% respectively. The low number of individuals obtained in six months (particularly in treatment groups) was due to the mortality by cannibalism and aggression. The mean sex reversal rates (male to female) in DES treated groups and controlled groups were 40.8% and 0% respectively in three months. Sufficient number of males (n=177) was obtained from control groups in six months to be tagged. The number of sex-reversed individuals from DES treated group was rather low (n=23).

5.3.4 Genotype of offspring with UNH995

The genotypes of 47 normal (control group) and 16 sex-reversed PIT-tagged F1 progeny (that were grown up to mature sexually) are summarized in Table 5.4 and 5.5.

Table 5.4 Genotypes of control group fish (F1) with UNH995

Cross type	Dam tag	Dam genotype	Sire tag	Sire genotype	No. of tagged progeny	Genotype
Control (YY female x YY male)	00AD YY♀	236/252	F8BB YY ♂	184/236	6	184/252 (N=2)
						236/252 (N=3)
						236/236 (N=1)
	F30D YY♀	184/252	315C YY♂	184/236	14	184/184 (N=1)
						184/236 (N=3)
						184/252 (N=7)
						236/252 (N=3)
Control (YY female x XY male)	00AD YY♀	236/252	FBE2 XY ♂	184/236	8	184/236 (N=2)
						184/252 (N=3)
						236/252 (N=2)
						236/236 (N=1)
	00AD YY♀	236/252	D9E3 XY ♂	184/236	5	184/236 (N=4)
						236/236 (N=1)
	F30D YY♀	184/252	Mixture of D9E3 XY ♂ and 46A8 XY ♂	184/236	14	184/184 (N=7)
						184/236 (N=3)
						184/252 (N=2)
						236/252 (N=2)

Table 5.5 Genotypes of treatment group fish (F1) with UNH995

Cross type	Dam tag	Dam genotype	Sire tag	Sire genotype	No. of tagged progeny	Genotype
DES treated (YY female x XY male)	00AD YY♀	236/252	FBE2 XY ♂	184/236	10	184/236 (N=2)
						184/252 (N=5)
						236/252 (N=3)
	F30D YY♀	184/252	46A8 XY ♂	184/236	6	184/184 (N=2)
						184/236 (N=2)
						184/252 (N=1)
						236/252 (N=1)

5.3.5 Separation of 'true' YY males

The three individuals having genotype 236/236 with UNH995 (anticipated as 'true' YY males, as described in section 5.2.8) were separated (from Table 5.4) and kept in individual tanks to breed further (Table 5.6). In sex-reversed group, putative 'true' YY neofemales with 236/236 genotype were not obtained (out of 16).

Table 5.6 Selection of 'true' YY males with UNH995

PIT tag	Genotype	Stocked in TAF as	Parents ID
00 038D 078C	236/236	Possible true breeder (YY supermale)	00AD YY♀ x D9E3 XY♂
00 068C F032	236/236	Possible true breeder (YY supermale)	00AD YY♀ x FBE2 XY♂
00 064E 2DD8	236/236	Possible true breeder (YY supermale)	00AD YY♀ x F8BB XY♂

5.3.6 Verification of 'true' YY supermales

The sex ratios obtained from crosses between selected 'true' YY males and clonal/outbred females are given in Table 5.7.

Table 5.7 Sex ratios in putative 'true' YY males x clonal and outbred females

Cross type	Sire tag	Sire genotype	Dam tag	Dam genotype	No. of male progeny	No. of female progeny	Sex ratio (M:F)
'True' YY male x clonal female	00 068C F032 YY♂	236/236	F2E0 clonal♀	184/184	99	2	98:2
'True' YY male x outbred female	00 038D 078C	236/236	2FB5 outbred ♀	184/184	75	0	100:0

5.4 Discussion

The study on sex linkage in Stirling Nile tilapia (Chapter 4) and observation of a 'strong' 'Y' allele linked to allele 236 of sex-linked marker UNH995 associated with mainly male progeny and the absence of that allele in rare females from crosses between YY females and XX neomales (Table 5.2) justify the utility of sex-linked markers (UNH995 and UNH104) in broodstock selection to improve GMT sex ratios. The selection of three F_1 progeny based on UNH995 genotype and breeding two of them with clonal and outbred females gave 98-100% male progeny. The presence of nearly all male progeny (in F_2) is clear evidence that a marker-assisted selection approach could be practical for monosex/genetically male tilapia production.

Genetic improvement in fish and other aquaculture species is a relatively new development. Most of the genetic improvements of aquaculture broodstock to date have been through the use of traditional selective breeding techniques such as selection, crossbreeding, and hybridization (reviewed by Hulata, 2001). To produce all-male or all-female progeny in aquaculture, two methods are commonly used. The direct method is inducing fry to differentiate toward males by androgen treatment or towards females by estrogen treatment. The indirect method includes the following steps: 1) Genotypic males are reversed to phenotypic females (neo-females) by estrogen treatment, or genotypic females are reversed to phenotypic males (neo-males) by androgen treatment, and cultured to adults; 2) All (genetically) male fry (in species with WZ/ZZ sex determination) and all (genetically) female fry (in species with XX/XY sex determination) are then obtained by crossing neo-females and normal males, and neo-males and normal females, respectively. Production of genetically all-male progeny in XX/XY sex determination requires production of 'YY' males which is conventionally achieved by the indirect method

(described in Chapter 1 section 1.3) but only confirmed after progeny sex ratio studies. Studies on inducing female fry to reverse to males with androgen treatment have been reported in several species, for example, rainbow trout (*Oncorhynchus mykiss*) (Johnstone et al., 1983), Japanese flounder (*Paralichthys olivaceus*) (Yamamoto, 1999), Atlantic halibut (*Hippoglossus hippoglossus*) (Hendry et al., 2003), blue tilapia, *O. aureus* (Desprez et al., 1995; Melard, 1995), Nile tilapia (Mair et al., 1997) and tongue sole *Cynoglossus semilaevis* (Zhou et al., 2005; Chen et al., 2007). The indirect method is considered to have greater value in terms of application than the first method because hormone treatment is not allowed in the culturing of commercial fish in many countries, the reason being the desire to have no residual pollution of hormones and no problem of food safety. Marker-assisted selection could be a very efficient technique to facilitate the development of the second method. For example, male-specific DNA markers have been isolated in some salmonids (Devlin and Nagahama, 2002) and are extensively used in sex control in these species. A female-specific AFLP marker (CseF305) has been isolated in half-smooth tongue sole *Cynoglossus semilaevis* (Chen et al., 2008) where females grow larger and faster than males. In this species, the female-specific CseF305 band of 160 bp was amplified in all 30 phenotypic females examined, but not in 30 phenotypic males. The availability of this female-specific marker in the sole lays a foundation for the development of a molecular marker-assisted sex control technique in the sole, and should prove to be very useful for controlling sex and producing monosex stock for half-smooth tongue sole. In addition, Chen et al. (2008) produced 130,000 fry from WZ neo♂ x WZ ♀ crosses. Genetic sex identification demonstrated that 73% of the neo-male progeny fry contained female-specific DNA markers. Three combinations of sex chromosomes (ZZ, ZW and

WW) were observed in the neo-male progeny. These super-females WW could be founders for all-female stock.

Apart from monosex production, one of the great utilities of genetic markers is to improve disease resistance in aquaculture species. Ozaki et al. (2001) identified QTL associated with resistance to infectious pancreatic necrosis (IPN), which is a well-known acute viral disease in rainbow trout. Two putative QTL affecting disease resistances were detected. These markers were of great potential for use in MAS for IPN resistance. MAS for IPN disease resistance was first applied in commercial salmon breeding programmes in Scotland and then in Norway (Houston et al., 2008). In tilapia, QTLs for a number of traits have been investigated, e.g., for body colour (Howe and Kocher, 2003), sex determination (Shirak et al., 2002), innate immunity, response to stress, biochemical blood parameters and body size (Cnaani et al., 2004), disease-related parameters (Shirak et al., 2006). Successful detections of QTL(s) could lead to the implementation of MAS. In shellfish species QTL and association studies are considerably more limited and have for the most part been confined to shrimp and prawns. Glenn et al. (2004) found SNPs in the cathepsin L gene and found suggestive associations with growth rate in both Pacific white shrimp and black tiger shrimp. Rocha et al. (2005) attempted to identify putative associations between DNA-markers and shrimp (*Litopenaeus vannamei*) production traits. Two SNP markers were found to be associated with statistically significant effects on an array of production traits, including harvest weight (HWT), test daily gain (TDG), biomass yield, grow-out survival, nursery, stocking and brood-stock weights, and several shrimp carcass and meat quality traits. Such QTL mapping in all these species can lead to MAS for efficient and precise selection, and significantly affect the aquaculture industry.

Some companies are using markers for other applications, such as parentage and selection decisions. One such example is Landcatch Natural Selection (http://www.swim-back.com/), which is a breeding company that is attempting to apply the latest methods in selective breeding technologies to its aquaculture operations worldwide. They use markers for traceability to parental stocks and some selection for trait improvement in salmon and advertise developments and expertise for markers in several other species. Another example is Aquagen AKVAFORSK Genetic Center (http://www.afgc.no/), which has been conducting genetic improvement with Atlantic salmon, rainbow trout, Atlantic cod, turbot, sea bass, and several other species. However, MAS implementation activities are in their infancy in aquaculture and fisheries industries and have limitations such as poorly developed genetic maps and knowledge of the relationships between the markers and traits of economic importance.

The present study has successfully shown how marker-assisted selection can enhance genetically male Nile tilapia production. On the basis of the association between departures from predicted sex ratios and alleles of markers in LG1, this study has also developed plans for upgrading the YY gene pool. The advancement of YY lines (along with a broadened gene pool) will help production of GMT on a sustainable basis. Figure 5.2 illustrates how GMT production by MAS and advancement of YY lines could be done.

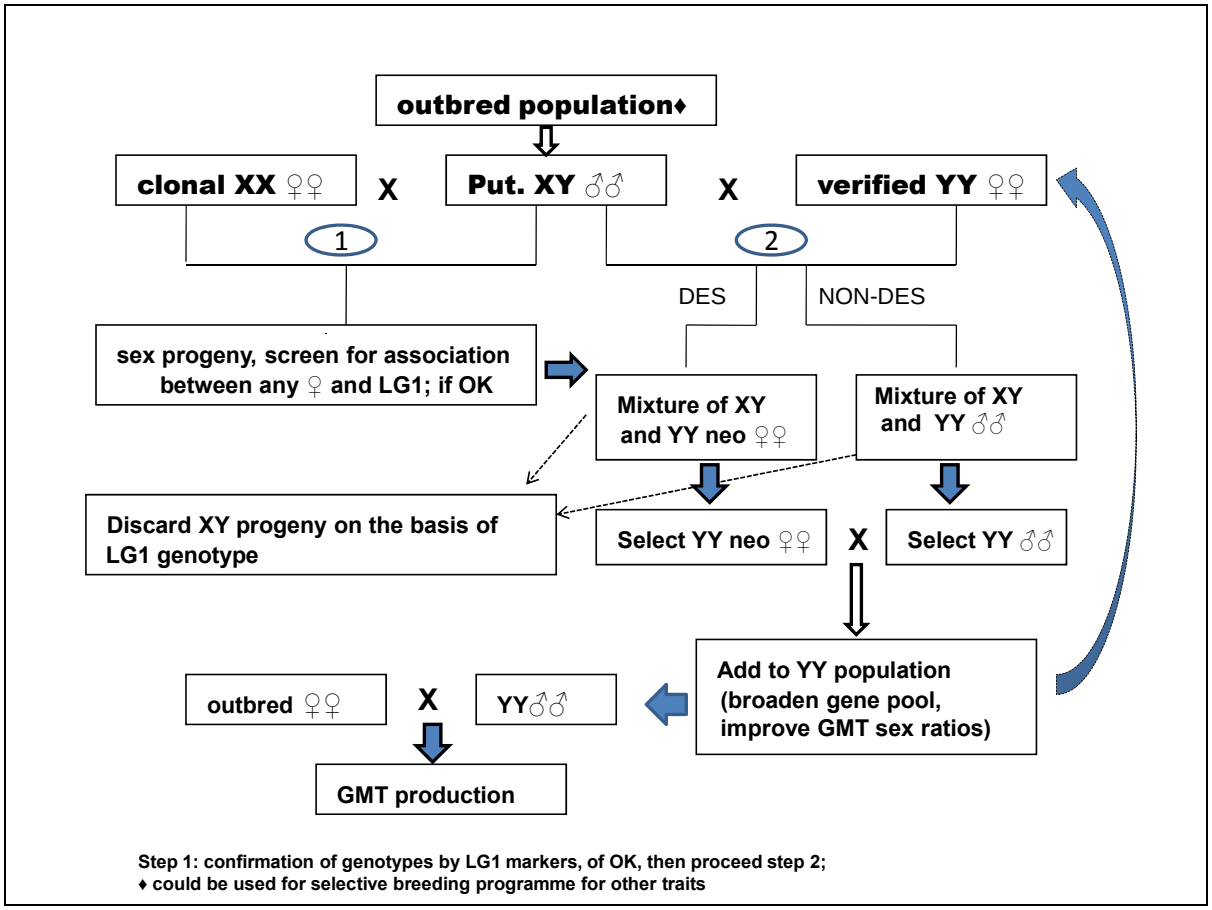


Figure 5.2 Advancing and broadening YY line through MAS, and increasing GMT production

Chapter 6
General Discussion

This PhD research has attempted to elaborate the mode of genetic sex determination in Nile tilapia (*Oreochromis niloticus*) by investigating inheritance of sex and sex-linked markers, and to facilitate marker-assisted selection in genetically monosex (male) production. An overall discussion on the experimental objectives and outcomes can briefly be given as follows:

6.1 Verification of clonal line females for sex determination studies

The clonal line of females was verified with microsatellite markers for sex determination studies in the Stirling Nile tilapia population. Such experiments are ideal to resolve the ambiguity if the female (parental) genetic components may also play a role in genetic variation affecting sex ratios in progeny. For example, Guan et al. (2000) suggested that female Nile tilapia may have a greater influence than males on variation of sex ratios because of the presence of two sex determining genes in tilapia that appeared to be different versions of the 'doublesex' gene first described in *Drosophila*. *Drosophila* has only one doublesex gene, and produces male and female products by RNA splicing after transcription. One of these genes is known to express in vertebrate testis (including Zebrafish). Tilapia has a variant of the same gene and is reported to express in ovaries (thereby known as female version of 'doublesex'). The expression of two doublesex genes is often mutually exclusive and therefore, it is anticipated additional genes or environmental factors may also control the expression of sex in this species. The use of a clonal line of females should reduce the female influence on sex ratios where interpretation on such sex determinants is complex, by acting as 'control' individuals in a predicted predominantly XX-XY sex determining mechanism. This makes male parents the

'hypothetical indicators' for any genetic variation inherited in progeny across a range of families in this study.

A number of studies have been performed to prove the gynogenetic and clonal line status in this particular species but with limited markers. Allozyme markers (ADA and EST for example), Multilocus DNA fingerprinting, RAPD, SSRa-PCR, AFLP and microsatellite markers have been used to investigate the clonal status (in terms of homozygosity) of either gynogenetic clonal founder and/or subsequent line of the progeny by a number of authors (Hussain et al., 1998; Jenneckens et al., 1999; Sarder et al., 1999; Ezaz et al., 2004a). Microsatellite markers were used for five fully inbred clonal lines by Ezaz et al. (2004b) and a single marker (UNH208) could differentiate each of the five lines of clonal females. They suggested, for other purposes (apart from identifying lines from each other), a multiplex of the five loci would serve as an identifying fingerprint for each clone. The clonal line of females used in the current PhD study is one of the several fully inbred clonal lines developed previously by gynogenesis (Sarder et al., 1999; Ezaz et al., 2004b) and showed good fertility compared to the others. The homozygous nature of this line with DNA markers (n=89) from across the whole genome provided very strong evidence of the fully inbred clonal nature of this line, that can be used as a reference line for different kinds of basic research including sex determination studies and quantitative genetics.

6.2 Sex linkage study in Stirling Nile tilapia population

Sex determination in tilapia species has been the subject of intensive research for approximately half a century. Approaches such as interspecific hybridization, sex reversal and progeny testing, chromosome set manipulations, morphological study of cell and

chromosome and molecular study have been performed for clarifying the basic sex determining system in tilapia. A low level of influence from environment or autosomes has been predicted in most of these approaches.

The present study investigated markers to screen a range of crosses to detect loci affecting sex ratio. The QTL for sex was positioned (in LG1) using genotype information from 6 different crosses and the pattern of allelic inheritance in association with sex in progeny was studied. Inheritance of LG3 and LG23 marker alleles was studied on the basis of some research works, where for example, LG3 markers were useful in association studies (between sex and markers) in *O. aureus* and *O. karongae* (Lee et al., 2004, 2005; Cnaani et al., 2008) and LG23 markers, in *O. aureus* x *O. mossambicus* (Shirak et al., 2006).

Three groups of animals selected in the present PhD study for sex linkage were screened based mainly on progeny sex ratios from a variety of crosses. The male parents of those groups were identified as normal XY (gave progeny sex ratios not significantly different from 1:1 in crosses to clonal line females), putative YY males (gave progeny sex ratios of >90% male in crosses to clonal line females) and males of unknown sexual genotype (gave intermediate progeny sex ratios, i.e., 60-80%). A monofactorial (chromosomal) sex determination system seemed to work in XY families (type A), but a small departure (<10%) in progeny sex ratios in YY families (type B) and a large departure (20-40%) in progeny sex ratios in putative YY families (type C) suggested that the sex determining system is not exclusively a monofactorial XX-XY. The effects of 'female components' and the influence of any environmental factors, particularly temperature were excluded in these groups because of using a standard clonal line as reference animals and because of running

the system in regulated temperature of 27.5±0.5 ^{o}C with all other water parameters in control. Therefore, the outcomes of the study were hypothesized to be genetic in any of the two possible modes: i) autosomal locus/loci interacts with major sex-determining locus (in LG1) to cause departure in the sex ratios, ii) variation at the major sex-determining locus with multiple alleles (allelic state at single locus, also known as complementary sex determination) to produce the departure from expected sex ratios.

The markers from LG1 were used in normal male group (type A, XY) followed by the other two groups (type 'B' and 'C') to observe any LG1-associated pattern of inheritance of phenotypic sex. The genotyping and association studies showed LG1 markers (UNH995 and UNH104) to be strongly associated with the QTL for sex in all three groups. The high association between sex and one of the markers in LG1 (UNH995) verifies earlier reports that the XX/XY locus is situated close to this microsatellite marker (Lee et al., 2003; Lee and Kocher, 2007; Cnaani et al., 2008). In the study of Lee et al. (2003) two such crosses from the Stirling population of Nile tilapia showed a very strong association between phenotypic sex and markers in LG1, while a third cross showed no association with screened markers from any of the 24 linkage groups, despite showing a sex ratio not significantly different from 1:1. All three crosses here showed a strong association between these LG markers and sex.

The genome wide scan with markers from other LGs to study the association with sex using bulked segregant analysis of female and male DNA pools and further analysis on DNA from individuals (where BSA initially showed an association, e.g. absence of one paternal allele, or strongly reduced allele peak height, in progeny DNA pool from one sex)

did not discover any sex-linked informative markers or any proper association that can support departed sex ratios in progeny sired by type B or type C male parents, rather specific LG1 markers (UNH995 and UNH104) were found to be informative to explain the genotypes in progeny sex in family type C. This demonstrated male progeny had approximately equal proportions ($P>0.05$ against H_0: 1:1) of each paternal UNH995 allele (in family 7 and 9), while female progeny had inherited one allele only. Considering the overall sex ratios of ~60-80% male in that family type ('C'), one allele (ambivalent 'A') was associated with 1:1 ♀♀: ♂♂, and the other (say 'P') with all male; thus males would be 2P: 1A (in an overall sex ratio of 75% males). However, testing such association was difficult in the type 'B' cross due to the lack of females. By repeating the crosses, some more female progeny were obtained for the analyses which also showed an association between LG1 (illustrated by UNH995) and sex where female offspring inherited only one of the paternal alleles.

The attempt to reveal any associations between markers from LG3 and LG23 with markers from LG1 in the experimental families of Nile population was based on the work of Lee et al. (2004) who found that sex in *O. aureus* appeared to be determined by interaction between the WZ/ZZ system in LG3 and a locus in LG1, and that loci in LG3 and LG23 appear to affect sex determination in some crosses in *O. niloticus* and in some other tilapias (Karayucel et al., 2004; Lee et al., 2005; Shirak et al., 2006). Limited heterozygosity expressed by the markers from these LGs in the males concerned (putative YYs) prevented a thorough investigation of such associations.

However, it is apparent from this work that the departures from the sex ratios predicted by using a "simple" XX/XY model (i.e. YY x XX should give all-male progeny) were

strongly associated with the XX/XY system itself, rather than being associated with loci in other LGs (e.g. LG3 or LG23). The allelic variation in this XX/XY model demonstrates some alleles could be stronger in effect (producing close to all male) while some others are weaker giving intermediate sex ratios in the progeny. The allele giving intermediate sex ratios- tentatively named as an 'ambivalent' is of scientific interest as it may explain, the failure of an association of phenotypic sex with any region of the genome in one out of three families in the study of Lee et al. (2003) (e.g. if the female parent was XX and the male AA, progeny would all be XA and expected to have a sex ratio of approximately 1:1). Likewise, this could explain the single fully inbred clonal line with a sex ratio close to 1:1 in the study of Sarder et al. (1999) (if the clonal line was AA). To verify these assumptions, some future works can be done. The males that produced male: female ratio approximately 70: 30 (denoted as YA) in sex linkage study can be crossed with clonal females (XX) to produce XY (male) and XA (supposed to be a mixture of males of females). The 'XA' female can be crossed with 'XA' male (these will be identified through genotyping on the basis of 'ambivalent' allele) to produce animals homozyogous for allele 'A'. The 'AA' line then can be advanced to cross with XX or putative YY to observe the progeny sex ratios. In addition, the interaction between these alleles and interactions with other QTL in the genome should be fine mapped with more markers to discover any other factors (other than multiple alleles) for sex determination in this species.

6.3 Marker assisted selection

The sex-linkage study in several families of Nile tilapia population was designed to find informative marker/s that can be used in a marker-assisted selection programme for producing increased male percentages in "genetically male" Nile tilapia. The knowledge

on the sex-linked informative marker/s along with the expression of multiple alleles and their mode of inheritance in progeny helped to identify 'true' 'Y' allele (strongest allele associated with only male progeny, a 'weak' Y allele and an 'ambivalent' allele (associated with equal numbers of male and female progeny) at a single locus. This knowledge was deliberately used to separate 'true' supermales (YY) in first generation offspring (segregation of 'strong' dominant homozygous alleles were hypothesized to form YY individuals from crosses between putative YY females and normal XY/putative YY males). The rearing of the putative 'pure supermales' (from the F_1) to maturity and subsequent breeding with clonal and outbred females produced enough progeny for sex ratio studies. The sex ratios in the F_2 generations sired by those selected YY males (avoiding alleles 184 and 252) produced very close to all-male progeny with both types of female parents. These findings were strong evidence of suitability of LG1 marker/s in separating putative 'pure' strains of supermales from normal males in Stirling Nile tilapia as well as producing all-genetically-male tilapia production.

6.4 Conclusions

The present PhD study was aimed to enhance male tilapia production without the direct use of hormones in direct food chain. It has demonstrated, by a thorough investigation of inheritance of sex (as QTL) and sex-linked as well as genome-wide markers using a (verified) fully inbred clonal line of females, novel allelic variation in the XX/XY system of the Nile tilapia, and showed that the marker assisted selection (MAS) in monosex production approach in this species could be practical. However, ideally such MAS would always be carried out using tightly linked informative markers on either side of the QTL in question. This seemed to be a limitation of the existing microsatellite markers in the tilapia linkage map – in some crosses, at least one marker was available to one side of the sex determining locus, but markers on the other side were either non-informative or a considerable distance from the QTL.

Although enough crosses were performed to obtain variable sex ratios for association studies (on QTL), there were some limitations of this work where future works are warranted. The clonal females used in this study were from a single line. More clonal lines of females could show genetic variation among them and should better reflect the variation in the XX/XY system. The number of individuals in the genome wide scan was rather low and prioritised BSA screening of sexed progeny can help understand more about any other QTL affecting establishment of sex in progeny. Furthermore, generation of additional polymorphic sex-linked markers and potentially sex-specific markers by RAD sequencing (Baird et al., 2008) would enhance MAS, both for mapped loci known to influence sex ratio and potentially to locate others in the genome.

Future work could be focused on further validation of MAS based on allelic variation in an XX/XY system to select for "strong Y" alleles giving 100% male progeny (or very close to this) and against "weak Y" or "ambivalent" alleles and to broaden the gene pool of YY males by making the process of selecting/adding new "strong Y" alleles from the general population of XY males more efficient (screening XY males and selecting YY males from XY x YY female crosses).

This research work has furthered our current knowledge on the sex determination system in Nile tilapia. The success of selecting and separating pure YY broodstock after sex linkage study and verifying them in pilot scale inspire undertaking MAS to improve monosex production approach. Further understanding of the sex determination system along with the current findings, using extended families and larger population of different strains will enhance genetically male tilapia production using a MAS approach, which should contribute to improving aquaculture practices worldwide, to help reducing hormone use in food fish and producing environment-friendly all male tilapia.

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Appendix I. Scientific and common names of the species used in the thesis

Anguilla rostrara: American eel

Apis mellifera: Honey bee

Apistogramma: Dwarf Cichlid

Aulopus japonicus: Japanese Thread-sail fish

Brachydanio rerio: Zebra fish

Caenorhabditis elegans: Round worm

Carassius auratus: Gold fish

Clarias gariepinus: African catfish

Ctenopharyngodon idella: Grass carp

Cynoglossus semilaevis: Tongue sole

Cyprinus carpio: Common carp

Dicentrarchus labrax: European Sea bass

Drosophila melanogaster: Fruit fly

Eigenmannia sp: Electric knife fish

Gasterosteus aculeatus: Three spine stickleback

Hoplias sp: Wolf fish

Hippoglossus hippoglossus: Atlantic halibut

Ictalurus punctatus: Channel catfish

Lates calcarifer: Asian Seabass

Leporinus sp: Black banded leporinus

Menidia menidia: Atlantic silverside

Nasonia vitripennis: Jewel wasp

Odontesthes bonariensis: Argentinian silverside

Appendix I (Cont'd). Scientific and common names of the species used in the thesis

Oncorhynchus mykiss: Rainbow trout

Oncorhynchus nerka: Sockeye salmon

Oreochromis andersonii: Three spotted tilapia

Oreochromis aureus: Blue tilapia

Oreochromis macrochir: Longfin tilapia

Oreochromis mossambicus: Mozambique tilapia

Oreochromis niloticus: Nile tilapia

Oreochromis spilurus spilurus: Sabaki tilapia

Oreochromis urolepis hornorum: Wami tilapia

Oreochromis variabilis: Victoria tilapia

Oryzias curvinotus: Hynann ricefish

Oryzias latipes: Medaka/Japanese killifish

Oryzias rhodorus: Amago salmon

Pagrus major: Red seabream

Paralichthys olivaceus: Hirame

Petromyzon marinus: Sea lamprey

Plecoglossus altivelis: Ayu

Poecilia formosa: Amazon molly

Poecilia latipinna: Sailfin molly

Pungitius pungitius: Nine spine stickleback

Appendix I (Cont'd). Scientific and common names of the species used in the thesis

Salvelinus sp: Charr

Sarotherodon melanotheron: Blackchin tilapia

Spinachia spinachia: 15 spine stickleback

Thalassoma bifasciatum: Bluehead wrasse

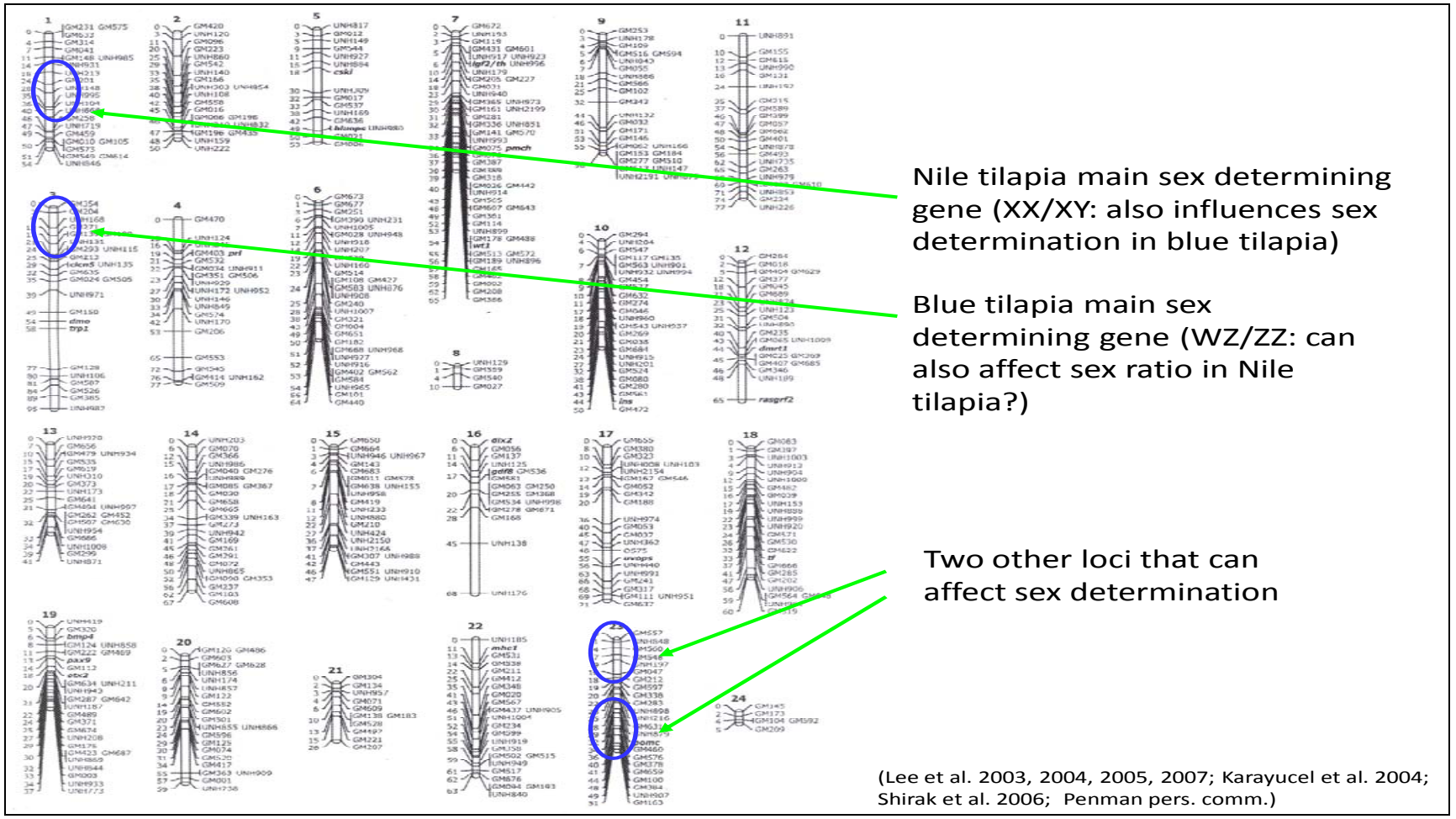
Tilapia rendalli: Redbreast tilapia

Tilapia zilli: Redbelly tilapia

Xiphophorus maculatus: Platy fish

Xiphophorus helleri: Sword tail

Appendix II. Putative sex determining gene(s) that can affect sex ratios in tilapia



Appendix III. Screened markers from Linkage group (LG)1 with oligo sequences

Number	Oligo name	Oligo sequence	Scale	Size	GC Content	Salt adjusted Tm	base stacking Tm*
1	GM633_L1_CAG_F	cagtcgggcgtcatcagtgtcccaagaaaaccagga	0.01	36	56%	57	76
2	GM633_L1_R	gacccaggactcatgtgctt	0.01	20	55%	42	65
3	UNH985_L1_F	gcgtcttgatgcaggataca	0.01	20	50%	40	63
4	UNH985_L1_God_R	catcgctgattcgcacattcccgacgagcaactgttat	0.01	38	50%	56	75
5	UNH931_L1_M13_F	ggataacaatttcacacaggacgttggtttgtcgggtaag	0.01	40	45%	55	73
6	UNH931_L1_R	taagtcagtgcgaccagacg	0.01	20	55%	42	65
7	UNH213_L1_M13_F	ggataacaatttcacacaggactgctcctcttgtttt	0.01	37	41%	52	70
8	UNH213_L1_R	tgtgataaggttaattaaagttagg	0.01	25	28%	38	58
9	GM201_L1_CAG_F	cagtcgggcgtcatcatattcaggctcttcttttgct	0.01	37	49%	55	74
10	GM201_L1_R	cagaatgaactccctccag	0.01	19	53%	39	60
11	UNH148_L1_M13_F	ggataacaatttcacacaggcttgaagttgcatttgc	0.01	37	41%	52	70
12	UNH148_L1_R	aaacactctcagctcaa	0.01	17	41%	30	57
13	UNH995_L1_God_F	catcgctgattcgcacatccagccctctgcataaagac	0.01	38	53%	57	75
14	UNH995_L1_R	gcagcacaaccacagtgcta	0.01	20	55%	42	66
15	UNH104_L1_CAG_F	cagtcgggcgtcatcagcagttatttgtggtcacta	0.01	36	50%	55	73
16	UNH104_L1_R	ggtatatgtctaactgaaatcc	0.01	22	36%	38	56
17	GM258_L1_M13_F	ggataacaatttcacacaggccttcacctccaccactttct	0.01	41	46%	56	74
18	GM258_L1_R	agatcgaacgtcgtcctctg	0.01	20	55%	42	64
19	UNH719_L1_God_F	catcgctgattcgcacataaaccattcatccttcactcg	0.01	39	46%	55	73
20	UNH719_L1_R	gaatgcttagtgcccatcaat	0.01	21	43%	39	62
21	UNH846_L1_CAG_F	cagtcgggcgtcatcatggagcagcttcttctacatca	0.01	38	53%	57	75
22	UNH846_L1_R	cacatgatggaagccgtgta	0.01	20	50%	40	63
* with adjusted Mg concentration at 1.5 mM as well as adjusted primer concentration (tail 20 nM, others 200-300 nM), in www.promega.com/biomath/calc11.htm							

Appendix IV. Screened markers from Linkage group (LG)3 with oligo sequences

Number	Oligo name	Oligo sequence	Scale	Size	GC Content	Salt adjusted Tm	base stacking Tm*
1	GM354_L3_M13_F	ggataacaatttcacacaggcgggagagcaggtcag	0.01	36	53%	56	74
2	GM354_L3_R	cacgttcagggttactgtgtt	0.01	21	48%	41	64
3	GM271_L3_F	gcagctggatcagtctctg	0.01	19	58%	42	63
4	GM271_L3_God_R	catcgctgattcgcacattgggaagtcgttcatacaaag	0.01	39	46%	55	73
5	UNH971_L3_F	ggtgggcagtgtgtgttttt	0.01	20	50%	40	65
6	UNH971_L3_CAG_R	cagtcgggcgtcatcattttcatccaggcctcagtt	0.01	36	53%	56	75
7	GM150_L3_F	gtctcagtttgtttggcttac	0.01	21	43%	39	60
8	GM150_L3_M13_R	ggataacaatttcacacaggaggtgattggcttagatgat	0.01	40	40%	53	71
9	GM128_L3_God_F	catcgctgattcgcacatatgatgagagaaagggaaaga	0.01	39	44%	54	72
10	GM128_L3_R	cattactgtgcctctgtgaag	0.01	21	48%	41	62
11	GM526_L3_M13_F	ggataacaatttcacacaggtcttcctcagcccatctgtt	0.01	40	45%	55	73
12	GM526_L3_R	caactgttggcagtgacagg	0.01	20	55%	42	65
13	UNH982_L3_CAG_F	cagtcgggcgtcatcatcaatactgtggtcccctcttt	0.01	38	53%	57	75
14	UNH982_L3_R	tctcagagcgctatcttcctg	0.01	21	52%	43	64
* with adjusted Mg concentration at 1.5 mM as well as adjusted primer concentration (tail 20 nM, others 200-300 nM), in www.promega.com/biomath/calc11.htm							

Appendix V. Screened markers from Linkage group (LG)23 with oligo sequences

Number	Oligo name	Oligo sequence	Scale	Size	GC Content	Salt adjusted Tm	base stacking Tm*
1	GM557_M13_F	ggataacaatttcacacaggcagctcgataaagggagacg	0.01	40	47%	56	73
2	GM557_R	gctgcattagcatcgtgtgt	0.01	20	50%	40	64
3	UNH848_CAG_F	cagtcgggcgtcatcatcccccgtaataaattaaacca	0.01	38	47%	55	73
4	UNH848_R	gcctgtgaataacaatgtatttcct	0.01	25	36%	41	63
5	UNH197_Godde_F	catcgctgattcgcacatcaggatggtgagatgttt	0.01	36	47%	54	73
6	UNH197_R	ttaagtggaagaagtcaatg	0.01	20	35%	34	56
7	GM597_F	acttgggtttgagcttggag	0.01	20	50%	40	63
8	GM597_M13_R	ggataacaatttcacacaggctctgtaatcccgcaccatt	0.01	40	45%	55	73
9	UNH898_F	gatgtccccacaaggtatgaa	0.01	21	48%	41	63
10	UNH898_CAG_R	cagtcgggcgtcatcataatccactcaccccgtttc	0.01	36	56%	57	75
11	UNH879_F	gcataaggtgactggctggt	0.01	20	55%	42	65
12	UNH879_Godde_R	catcgctgattcgcacatacaaaggggtcctgcaattt	0.01	38	47%	55	74
13	GM576_M13_F	ggataacaatttcacacaggccctggagaacagagtggtc	0.01	40	50%	57	74
14	GM576_R	cttggacttggctctgacct	0.01	20	55%	42	65
15	UNH907_CAG_F	cagtcgggcgtcatcacaggaccgactctgcaagat	0.01	36	58%	58	77
16	UNH907_R	gagctcttttgttgttcaaaatc	0.01	23	35%	38	60

* with adjusted Mg concentration at 1.5 mM as well as adjusted primer concentration (tail 20 nM, others 200-300 nM), in www.promega.com/biomath/calc11.htm

Appendix VI. Oligo sequences of modified SNP (Wt1b) primers

Modified SNPs	Oligo sequence	Scale	Size	GC Content
US001_F_M13 (Wt1b_1)	ggataacaatttcacacaggatgacagtgaccccatatg	0.01	39	44%
US001_R (Wt1b_R)	gcctctgaagtcttcgcaac	0.01	20	55%
US002_F_CAG (Wt1b_2)	cagtcgggcgtcatcaatgacagtgaccccatatc	0.01	35	54%
US001_R (Wt1b_R)	gcctctgaagtcttcgcaac	0.01	20	55%

Appendix VII. Oligo sequences of fluorescent labeled primers

Number	Oligo name	Oligo sequence	Size	GC Content	Salt adjusted Tm	base stacking Tm
1	M13R_Blue	ggataacaatttcacacagg	20	40%	36	57
2	CAGtag_Green	cagtcgggcgtcatca	16	63%	37	61
3	Godde_Black	catcgctgattcgcacat	18	50%	36	61

Appendix VIII. Oligo sequences of genome wide scanned markers (except LG1, 3 and 23)

No.	Oligo name	Oligo sequence	Size	GC Content
1	GM420M13F	ggataacaatttcacacaggTTAATTCTGGGTCTGGTGG	39	44%
2	GM420R	GGATAAGCGAATGGATGATAG	21	43%
3	UNH860GodF	catcgctgattcgcacatACTGTTTACCCACTGCGACA	38	50%
4	UNH860R	AGATGTGTCTGAGCCATCCA	20	50%
5	UNH159CAGF	cagtcgggcgtcatcaTTGTTTTAGGAGCTTCTTTTGTC	39	46%
6	UNH159R	ATATTCATCTGGATTTGGCTCTAA	24	33%
7	UNH170GodF	catcgctgattcgcacatTCCCAATTAGAGCTAGCAAAGTCC	42	48%
8	UNH170R	TATTGTAATTATGAAGAGATGTAG	24	25%
9	GM553CAGF	cagtcgggcgtcatcaGCTGGATTTGCATTGAGTGA	36	53%
10	GM553R	TAGACCGAGGCTGAAAGCTG	20	55%
11	UNH884CAGF	cagtcgggcgtcatcaGTAAATTGCTCGGGGCTCT	35	57%
12	UNH884R	ATCCTGCTCGGAGAGCTACA	20	55%
13	UNH309F	AGCGAGCGAGAGAGCTAGTG	20	60%
14	UNH309GodR	catcgctgattcgcacatGTGTCTTTCACGGACACCCT	38	53%
15	UNH980M13F	ggataacaatttcacacaggGAAGATATGCATGCGGACAC	40	45%
16	UNH980R	CACTCCCATTTCCTGTGTTG	20	50%
17	UNH948F	GCTCGCTCCAGAAAAATCAC	20	50%
18	UNH948GodR	catcgctgattcgcacatGTCAAAAAGGCATGGCAAAG	38	47%
19	UNH908F	CTTGCCATTCCTTTGTGCTT	20	45%
20	UNH908M13R	ggataacaatttcacacaggGTATGAACCTCCTGGCCTTC	40	48%
21	UNH968GodF	catcgctgattcgcacatACTGCTCCTCCTGTGTCTGG	38	55%
22	UNH968R	TCTTGCTGCTTCTCTCCACA	20	50%
23	GM440M13F	ggataacaatttcacacaggCTGCACTTTTACTGAGGG	38	45%
24	GM440R	TGGGAGATTAACAGAATAACA	21	33%
25	GM205CAGF	cagtcgggcgtcatcaAATGTAGCACTTTTAAGGAGA	37	46%
26	GM205R	AATGTAAGGAAATTTGTGTTT	21	24%
27	UNH899F	ACGTCACATGGAGGTGCTTA	20	50%
28	UNH899CAGR	cagtcgggcgtcatcaGCTAGACCTCTGTCCCCTGA	36	61%
29	GM027M13F	ggataacaatttcacacaggTGGCTCCAGTTAATCCTCT	39	44%
30	GM027R	TCTCATTCATTTACCCTGTTG	21	38%
31	UNH843CAGF	cagtcgggcgtcatcaCGTTCTACTCTGAAGAAAGACATGA	41	49%
32	UNH843R	CCACTCGACGGACGTTTTAG	20	55%
33	UNH886GodF	catcgctgattcgcacatACCCCTCCTAACTTGCTTCT	38	50%
34	UNH886R	TGCCTGCCACTAACTGTGAC	20	55%
35	GM343M13F	ggataacaatttcacacaggCCCTGCTGTTTCCTCCT	37	49%
36	GM343R	CCTTATCAGCTTTTCGTGTTC	21	43%
37	UNH132CAGF	cagtcgggcgtcatcaATATAAGAAACTGAGTCGGTGAG	39	49%
38	UNH132R	TGGAAATAGAGGGTGGGTGAG	21	52%
39	GM062GodF	catcgctgattcgcacatTTCAGTTTTTCAGCCAAATAC	39	41%
40	GM062R	CTGCAGCGTTAGAGTCCT	18	56%
41	UNH994M13F	ggataacaatttcacacaggCGCATGACCCTTACATACCC	40	48%
42	UNH994R	CAGCCAGCTTGGTTGTCATA	20	50%

Appendix VIII (Cont'd) Oligo sequences of genome wide scanned markers (except LG1, 3 and 23)

No.	Oligo name	Oligo sequence	Size	GC Content
43	UNH960CAGF	cagtcgggcgtcatcaCTGTCGATGTGTCCCTGTGT	36	58%
44	UNH960R	ACCCGGGACATACACTTGTC	20	55%
45	GM080GodF	catcgctgattcgcacatTGAATAATAAACCAGCGTGTA	39	41%
46	GM080R	TAGAAGCCCAGTGAGCA	17	53%
47	GM472M13F	ggataacaatttcacacaggCTAAATCTCCACGCAGTCC	39	46%
48	GM472R	TGTAATTGCTCCACAAATCTG	21	38%
49	UNH990F	GCCACAGGTGACCATGTTAG	20	55%
50	UNH990CAGR	cagtcgggcgtcatcaGGTGTCTGATTGCACTGACG	36	58%
51	UNH192GodF	catcgctgattcgcacatGGAAATCCATAAGATCAGTTA	39	41%
52	UNH192R	CTTTTTCAGGATTTACTGCTAAG	23	35%
53	GM215M13F	ggataacaatttcacacaggGGATAATGATGGCAGTGGT	39	44%
54	GM215R	TATTTTTCTTCCCAATGGTTC	21	33%
55	GM399CAGF	cagtcgggcgtcatcaCGCCCTGAGAGCAACA	32	63%
56	GM399R	AGTGTGCCGTTCCAAAAATAC	21	43%
57	UNH878GodF	catcgctgattcgcacatTTTCAGGAGGACGAGCAGTT	38	50%
58	UNH878R	CAGGCGGCAGATATTCATTT	20	55%
59	UNH979M13F	ggataacaatttcacacaggAGCTCACTGCCAACACACTG	40	48%
60	UNH979R	CATGTCTGGCAAAAGTGACG	20	50%
61	GM377CAGF	cagtcgggcgtcatcaACCAGCAGCAATACTCAAAC	36	53%
62	GM377R	ACAGGGACACAGATAGCAGAT	21	48%
63	UNH874GodF	catcgctgattcgcacatAGTAAAATGGGCGAACGTGT	38	47%
64	UNH874R	TGAAGCTGGGAGTTTCCTGT	20	50%
65	UNH1009M13F	ggataacaatttcacacaggCCATCTGCATGCTGTAAGACA	41	44%
66	UNH1009R	TCCCATTTGTCAGGTTCAGG	20	50%
67	*rasgrf2GodF*	catcgctgattcgcacatCTTGATCACCCCACCAAAAC	38	50%
68	*rasgrf2R*	TGGGTCTCCAAACATTCACA	20	45%
69	GM373M13F	ggataacaatttcacacaggGGCACCATCTCTAAGGAAA	39	44%
70	GM373R	TAAAGGGGACAAATGTGAAAT	21	33%
71	UNH954CAGF	cagtcgggcgtcatcaGGAAAACGTTTGGAGAGACG	36	56%
72	UNH954R	AAACGGAGCTCCTGTCTGAA	20	50%
73	GM070_L14_M13_F	ggataacaatttcacacaggccctgtgccagaatccat	38	47%
74	GM070_L14_R	ggcaaacagggtaaatgagag	21	48%
75	GM665_L14_CAG_F	cagtcgggcgtcatcatagttggtccctggttgctt	36	56%
76	GM665_L14_R	cagtgtttgttaggttctgcttg	23	43%
77	UNH865_L14_GodF	catcgctgattcgcacatacaaccccattcaccacact	38	50%
78	UNH865_L14_R	agcgttgcttgggaaaagta	20	45%
79	GM664_L15_F	gtgaactcagctcggactca	20	55%
80	GM664_L15_M13_R	ggataacaatttcacacaggacgcaatgggctgtaaaaat	40	40%
81	UNH880_L15_F	ggcagcagtataacaatcacca	22	45%
82	UNH880_L15_CAG_R	cagtcgggcgtcatcattctgacatccatccagcag	36	56%
83	GM129_L15_F	taataattgtgcgaggtgttt	21	33%
84	GM129_L15_God_R	catcgctgattcgcacataactagtgtgcaggtgcc	36	53%

Appendix VIII (Cont'd): Oligo sequences of genome wide scanned markers (except LG1, LG3 and LG23)

No.	Oligo name	Oligo sequence	Size	GC Content
85	GM056_L16_M13_F	ggataacaatttcacacagggacacaatgcctaaaaatctg	41	39%
86	GM056_L16_R	cctcaccgtccctctc	16	69%
87	GM168_L16_CAG_F	cagtcgggcgtcatcatcagaggggaaagtggaaaa	36	53%
88	GM168_L16_R	caacacgcgtagcagtaatga	21	48%
89	UNH176_L16_God_F	catcgctgattcgcacatgatcagctctcctctactta	38	47%
90	UNH176_L16_R	gatctgatttcttattactacaa	23	26%
91	UNH103_L17_F	caatgtccatccttcct	17	47%
92	UNH103_L17_M13_R	ggataacaatttcacacaggctgtctgactgcaaatgtaa	40	40%
93	UNH974_L17_F	gcacgtctgagagtgtggaa	20	55%
94	UNH974_L17_CAG_R	cagtcgggcgtcatcacagctttcacaccagcctaa	36	56%
95	UNH440_L17_F	acacatatggccaccagaca	20	50%
96	UNH440_L17_God_R	catcgctgattcgcacatgatctgctctccctgctgat	38	53%
97	UNH904_L18_M13_F	ggataacaatttcacacagggtcactgctgagccccttta	40	47%
98	UNH904_L18_R	gcattcagagtgccagagttc	21	52%
99	UNH888_L18_CAG_F	cagtcgggcgtcatcagatccgcccacctcaatta	35	57%
100	UNH888_L18_R	gcgccacctgggatataatac	21	52%
101	GM285_L18_God_F	catcgctgattcgcacattgcactttgggggatg	34	53%
102	GM285_L18_R	taatagctctgccgtttgttc	21	43%
103	UNH419_L19_F	tcccagcagccgtatagaat	20	50%
104	UNH419_L19_M13_R	ggataacaatttcacacaggggtgggatgttgctgaagtt	40	45%
105	UNH943_L19_F	ctgtccgccttaaagacctg	20	55%
106	UNH943_L19_CAG_R	cagtcgggcgtcatcagcgctcctgaggttactgtt	36	58%
107	UNH844_L19_F	gccacaatgtcaaggtttca	20	45%
108	UNH844_L19_God_R	catcgctgattcgcacatgcagctgctcacacactctt	38	53%
109	UNH174_L20_M13_F	ggataacaatttcacacaggtgaaaaatggaatttgg	37	35%
110	UNH174_L20_R	ttagatgagatatgaaactgc	21	33%
111	UNH866_L20_CAG_F	cagtcgggcgtcatcaactcccgctgttgctgttag	36	58%
112	UNH866_L20_R	gaggggagcctacaacgtaa	20	55%
113	GM363_L20_God_F	catcgctgattcgcacatccagtcccagtcatcct	35	54%
114	GM363_L20_R	agaaaacctgttgccattatc	21	38%
115	UNH957_L21_F	ctccgtgacaccaagctttc	20	55%
116	UNH957_L21_M13_R	ggataacaatttcacacaggatggcatccactacaagctg	40	45%
117	GM221_L21_F	tacagaagtcgaggcgagatg	21	52%
118	GM221_L21_CAG_R	cagtcgggcgtcatcagtggtggcgattgtgtcat	35	57%
119	GM531_L22_F	aaagccaacggtctgaattg	20	45%
120	GM531_L22_God_R	catcgctgattcgcacatagcagaggacacccctcat	37	54%
121	UNH905_L22_M13_F	ggataacaatttcacacaggtgatgacggtgaagtgaagc	40	45%
122	UNH905_L22_R	caagcagaaaatcctggagtg	21	48%
123	UNH840_L22_CAG_F	cagtcgggcgtcatcatttcctgttcacccagtttt	36	50%
124	UNH840_L22_R	gggctgagcagtctggtatt	20	55%
125	GM173_L24_God_F	catcgctgattcgcacattgaacttctagtctgcctctg	39	49%
126	GM173_L24_R	gtgttttgattcagggtatga	21	38%

Appendix IX. Parental and progeny DNA genotypes (in BSA) in clonal females x XY males (type 'A')

	F1♀parent (EA38 ClF)	F1♂parent D9E3 XY	F1♀pool	F1♂pool	F2♀parent (F2E0 ClF)	F2♂parent 46A8 XY	F2♀pool	F2♂pool	F3♀parent (EA38 ClF)	F3♂parent FBE2 XY	F3♀pool	F3♂pool	'P' in male parents
LG1													
GM633	206/206	214/214	206/214	206/214	206/206	214/214	206/214	206/214	206/206	214/214	206/214	206/214	***
UNH985	144/144	144/154	144/144	144/154	144/144	144/154	144/144	144/154	144/144	144/154	144/144	144/154	***
UNH931	227/227	227/261	227/227	227/261	227/227	227/261	227/227	227/261	227/227	227/261	227/227	227/261	***
UNH213	211/211	190/211	211/211	190/211	211/211	190/211	211/211	190/211	211/211	190/211	211/211	190/211	***
GM201	164/164	162/162	162/164	162/164	164/164	162/162	162/164	162/164	164/164	162/162	162/164	162/164	***
UNH148	172/172	182/182	172/182	172/182	172/172	182/182	172/182	172/182	172/172	172/172	172/172	172/172	**
UNH995	184/184	184/236	184/184	184/236	184/184	184/236	184/184	184/236	184/184	184/236	184/184	184/236	***
UNH104	147/147	147/197	147/147	147/197	147/147	147/197	147/147	147/197	147/147	147/197	147/147	147/197	***
GM258	142/142	148/148	142/148	142/148	142/142	148/148	142/148	142/148	142/142	148/148	142/148	142/148	***
UNH719	127/127	127/141	127/127	127/141	127/127	127/141	127/127	127/141	127/127	127/141	127/141	127/141	***
UNH846	187/187	187/199	187/199	187/199	187/187	187/199	187/199	187/199	187/187	187/199	187/199	187/199	***

Notes: P- Polymorphic locus, *polymorphism in one family, **polymorphism in two families, ***polymorphism in all three families of male

Appendix X. Parental and progeny DNA genotypes (in BSA) in clonal females x putative YY males (type 'B')

	F4♀parent 0073 clonal	F4♂parent (E167 YY)	F4♀pool	F4♂pool	F5♀parent 0B19 clonal	F5♂parent (315C YY)	F5♀pool	F5♂pool	F6♀parent DD59 clonal	F6♂parent (F8BB YY)	F6♀pool	F6♂pool	Polymorphism in male parents
LG1													
GM633	206/206	206/218	206/218	206/218	206/206	206/206	206/206	206/206	206/206	218/218	206/218	206/218	**
UNH985	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/157	144/157	144/157	M
UNH931	227/227	227/227	227/227	227/227	227/227	227/227	227/227	227/227	227/227	227/241	227/241	227/241	*
UNH213	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	M
GM201	164/164	164/164	164/164	164/164	164/164	164/164	164/164	164/164	164/164	164/164	164/164	164/164	M
UNH148	172/172	172/172	172/172	172/172	172/172	170/172	170/172	172/172	172/172	172/172	172/172	172/172	*
UNH995	184/184	184/236	184/184	184/236	184/184	184/236	184/184	184/236	184/184	184/236	184/184	184/236	***
UNH104	147/147	147/147	147/147	147/147	147/147	147/147	147/147	147/147	147/147	147/147	147/147	147/147	*
GM258	142/142	142/150	142/150	142/150	142/142	150/150	142/150	142/150	142/142	142/150	142/150	142/150	***
UNH719	127/127	141/141	127/141	127/141	127/127	141/141	127/141	127/141	127/127	141/141	127/141	127/141	***
UNH846	187/187	189/189	187/189	187/189	187/187	189/189	187/189	187/189	187/187	189/189	187/189	187/189	***
LG3													
GM354	142/142	142/142	142/142	142/142	142/142	142/142	142/142	142/142	142/142	142/142	142/142	142/142	M
GM271	134/134	134/134	134/134	134/134	134/134	134/134	134/134	134/134	134/134	134/134	134/134	134/134	M
UNH971	215/215	215/226	215/226	215/226	215/215	226/232	215/226/232	215/226/232	215/215	215/226	215/226	215/226	***
GM150	217/217	209/217	209/217	209/217	217/217	209/217	217/217	209/217	217/217	209/217	209/217	209/217	***
GM128	157/157	157/164	157/164	157/164	157/157	157/164	157/164	157/164	157/157	157/164	157/164	157/164	***
GM526	260/260	260/270	260/270	260/270	260/260	260/270	260/270	260/270	260/260	260/270	260/270	260/270	***
UNH982	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	M

Notes: M: monomorphic at locus, *polymorphism in one family, **polymorphism in two families, ***polymorphism in all three families of male

Appendix X. Parental and progeny DNA genotypes (in BSA) in clonal females x putative YY males (type 'B')-cont'd

	F4♀parent 0073 clonal	F4♂parent (E167 YY)	F4♀pool	F4♂pool	F5♀parent 0B19 clonal	F5♂parent (315C YY)	F5♀pool	F5♂pool	F6♀parent DD59 clonal	F6♂parent (F8BB YY)	F6♀pool	F6♂pool	Polymorphism in male parents
LG23													
GM557	268/268	227/227	227/268	227/268	268/268	227/227	227/268	227/268	268/268	227/227	227/268	227/268	***
UNH848	208/208	220/220	208/220	208/220	208/208	208/220	208/220	208/220	208/208	220/220	208/220	208/220	***
UNH197	205/205	198/198	198/205	198/205	205/205	196/205	196/205	196/205	205/205	196/196	196/205	196/205	***
GM597	151/151	155/155	151/155	151/155	151/151	151/155	151/155	151/155	151/151	155/155	151/155	151/155	***
UNH898	218/218	218/218	218/218	218/218	218/218	218/218	218/218	218/218	218/218	218/218	218/218	218/218	M
UNH879	238/238	213/213	213/238	213/238	238/238	213/238	213/238	213/238	242/242	213/213	213/242	213/242	***
GM576	242/242	218/218	218/242	218/242	242/242	218/242	218/242	218/242	242/242	218/218	218/242	218/242	***
UNH907	134/134	134/136	134/136	134/136	134/134	134/134	134/134	134/134	134/134	134/143	134/143	134/143	**

Notes: M: monomorphic at locus, *polymorphism in one family, **polymorphism in two families, ***polymorphism in all three families of male

Appendix XI. Parental and progeny DNA genotypes (in BSA) in clonal females x type 'C' males

	F7♀parent (0073 clonal)	F7♂parent 44F0	F7♀pool	F7♂pool	F8♀parent (0073 clonal)	F8♂parent F99B	F8♀pool	F8♂pool	F9♀parent (F2E0 clonal)	F9♂parent F5FC	F9♀pool	F9♂pool	'P' in male parents
LG1													
GM633	206/206	206/218	206/218	206/218	206/206	206/206	206/206	206/206	206/206	206/218	206/218	206/218	**
UNH985	144/144	144/144	144/144	144/144	144/144	132/144	132/144	132/144	144/144	144/144	144/144	144/144	*
UNH931	227/227	227/240	227/227	227/240	227/227	227/227	227/227	227/227	227/227	227/245	227/227	227/245	**
UNH213	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	211/211	M
GM201	164/164	160/160	160/164	160/164	164/164	164/164	164/164	164/164	164/164	160/160	160/164	160/164	**
UNH148	172/172	172/172	172/172	172/172	172/172	172/172	172/172	172/172	172/172	172/172	172/172	172/172	M
UNH995	184/184	236/252	184/252	184/236/252	184/184	188/234	184/188/234	184/188/234	184/184	236/252	184/252	184/236/252	***
UNH104	147/147	190/210	147/210	147/190/210	147/147	147/190	147/147	147/190	147/147	190/210	147/210	147/190/210	***
GM258	142/142	148/148	142/148	142/148	142/142	142/148	142/148	142/148	142/142	148/148	142/148	142/148	***
UNH719	127/127	121/141	121/127/141	121/127/141	127/127	127/141	127/127	127/141	127/127	121/141	121/127/141	121/127/141	***
UNH846	187/187	189/189	187/189	187/189	187/187	189/189	187/189	187/189	187/187	189/189	187/189	187/189	***
LG3													
GM354	142/142	142/142	142/142	142/142	142/142	132/142	132/142	132/142	142/142	142/142	142/142	142/142	*
GM271	134/134	134/134	134/134	134/134	134/134	145/145	134/145	134/145	134/134	134/134	134/134	134/134	*
UNH971	215/215	215/224	215/224	215/224	215/215	215/224	215/224	215/224	215/215	215/224	215/224	215/224	***
GM150	217/217	207/217	207/217	207/217	216/217	207/217	207/217	207/217	217/217	207/217	207/217	207/217	***
GM128	157/157	163/163	157/163	157/163	157/157	163/163	157/163	157/163	157/157	163/163	157/163	157/163	***
GM526	260/260	260/268	260/268	260/268	260/260	268/268	260/268	260/268	260/260	260/268	260/268	260/268	***
UNH982	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	M
LG23													
GM557	268/268	227/227	227/268	227/268	268/268	227/227	227/268	227/268	268/268	227/227	227/268	227/268	***
UNH848	208/208	220/220	208/220	208/220	208/208	220/220	208/220	208/220	208/208	220/220	208/220	208/220	***
UNH197	205/205	197/197	197/205	197/205	205/205	197/197	197/205	197/205	205/205	197/197	197/205	197/205	***
GM597	151/151	155/155	151/155	151/155	151/151	151/155	151/155	151/155	151/151	155/155	151/155	151/155	***
UNH898	218/218	218/222	218/222	218/222	218/218	218/226	218/226	218/226	218/218	218/222	218/222	218/222	***
UNH879	238/238	215/215	215/238	215/238	238/238	215/215	215/238	215/238	238/238	215/215	215/238	215/238	***
GM576	242/242	218/218	218/242	218/242	242/242	218/218	218/242	218/242	242/242	218/218	218/242	218/242	***
UNH907	134/134	134/143	134/143	134/143	134/134	134/134	134/134	134/134	134/134	134/143	134/143	134/143	**

Notes: P: Polymorphic, M: monomorphic at locus, *polymorphism in one family, **polymorphism in two families, ***polymorphism in all three families of male

Appendix XII. Parental and progeny DNA genotypes (in BSA) in clonal females x putative YY males (type 'B') with markers from genome wide selection (except LG1, 3 and 23 markers)

	F4♀parent 0073 clonal	F4♂parent (E167 YY)	F4♀pool	F4♂pool	F5♀parent 0B19 clonal	F5♂parent (315C YY)	F5♀pool	F5♂pool	F6♀parent DD59 clonal	F6♂parent (F8BB YY)	F6♀pool	F6♂pool	'P' in male parent
LG2													
GM420	137/137	137/137	137/137	137/137	137/137	137/137	137/137	137/137	137/137	137/137	137/137	137/137	M
UNH860	216/216	216/222	216/222	216/222	216/216	216/222	216/222	216/222	216/216	216/222	216/222	216/222	***
UNH159	251/251	251/257	251/257	251/257	251/251	251/257	251/257	251/257	251/251	247/257	251/257	247/251/257	***
LG4													
UNH170	162/162	162/162	162/162	162/162	162/162	162/162	162/162	162/162	162/162	162/162	162/162	162/162	M
GM553	257/257	294/294	257/294	257/294	257/257	294/294	257/294	257/294	257/257	257/294	257/294	257/294	***
LG5													
UNH884	160/160	133/146	133/146/160	133/146/160	160/160	133/146	133/146/160	133/146/160	160/160	133/146	133/146/160	133/146/160	***
UNH309	199/199	199/203	199/199	199/203	199/199	199/203	199/199	199/203	199/199	199/203	199/203	199/203	***
UNH980	233/233	220/220	220/233	220/233	233/233	220/220	220/233	220/233	233/233	220/220	220/233	220/233	***
LG6													
UNH948	197/197	197/199	197/199	197/199	197/197	197/197	197/197	197/197	197/197	197/197	197/197	197/197	*
UNH908	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	124/124	M
UNH968	226/226	226/244	226/244	226/244	226/226	244/244	226/244	226/244	226/226	244/244	226/244	226/244	***
GM440	275/275	275/275	275/275	275/275	275/275	275/275	275/275	275/275	275/275	275/275	275/275	275/275	M
LG7													
GM205	127/127	127/127	127/127	127/127	127/127	127/127	127/127	127/127	127/127	127/127	127/127	127/127	M
UNH899	158/158	158/158	158/158	158/158	158/158	158/158	158/158	158/158	158/158	158/158	158/158	158/158	M
LG8													
GM027	176/176	176/191	176/191	176/191	176/176	176/191	176/191	176/191	176/176	176/191	176/191	176/191	***
LG9													
UNH843	125/125	125/125	125/125	125/125	125/125	125/125	125/125	125/125	125/125	125/125	125/125	125/125	M
UNH886	185/185	185/185	185/185	185/185	185/185	185/185	185/185	185/185	185/185	185/185	185/185	185/185	M
GM343	191/191	191/193	191/193	191/193	191/191	191/193	191/193	191/193	191/191	191/193	191/193	191/193	***
UNH132	131/131	131/131	131/131	131/131	131/131	131/131	131/131	131/131	131/131	131/131	131/131	131/131	M
GM062	286/286	284/284	284/286	284/286	286/286	284/286	284/286	284/286	286/286	284/286	284/286	284/286	***

Notes: P: Polymorphic, M: monomorphic at locus, *polymorphism in one family, **polymorphism in two families, ***polymorphism in all three families of male

Appendix XII (Cont'd). Parental and progeny DNA genotypes (in BSA) in clonal females x putative YY males (type 'B') with markers from genome wide selection

	F4♀parent 0073 clonal	F4♂parent (E167 YY)	F4♀pool	F4♂pool	F5♀parent 0B19 clonal	F5♂parent (315C YY)	F5♀pool	F5♂pool	F6♀parent DD59 clonal	F6♂parent (F8BB YY)	F6♀pool	F6♂pool	'P' in male parent
LG10													
UNH994	235/235	235/239	235/239	235/239	235/235	235/235	235/235	235/235	235/235	235/239	235/239	235/239	**
UNH960	182/182	153/182	153/182	153/182	182/182	153/182	153/182	153/182	182/182	153/182	153/182	153/182	***
GM080	245/245	237/245	237/245	237/245	245/245	237/245	237/245	237/245	245/245	237/245	237/245	237/245	***
GM472	355/355	341/341	341/355	341/355	355/355	341/355	341/355	341/355	355/355	341/355	341/355	341/355	***
LG11													
UNH990	168/168	164/168	164/168	164/168	168/168	154/168	154/168	154/168	168/168	168/168	168/168	168/168	**
UNH192	156/156	156/156	156/156	156/156	156/156	156/156	156/156	156/156	156/156	156/156	156/156	156/156	M
GM215	223/223	217/217	217/223/227	217/223/227	223/223	223/227	223/223	223/227	223/223	227/227	223/227	223/227	***
GM399	273/273	263/263	263/273	263/273	273/273	263/273	263/273	263/273	273/273	263/273	263/273	263/273	***
UNH878	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	M
UNH979	271/271	271/271	271/271	271/271	271/271	271/271	271/271	271/271	271/271	271/271	271/271	271/271	M
LG12													
GM377	312/312	280/280	280/312	280/312	312/312	280/312	312/312	280/312	312/312	280/280	280/312	280/312	***
UNH874	214/214	210/210	210/214	210/214	214/214	210/210	210/214	210/214	214/214	210/210	210/214	210/214	***
UNH1009	173/173	192/192	173/192	173/192	173/173	208/208	173/208	173/208	173/173	208/208	173/208	173/208	***
Rasgrf	119/119	117/117	117/119	117/119	119/119	117/117	117/119	117/119	119/119	117/117	117/119	117/119	***
LG13													
GM373	318/318	312/318	312/318	312/318	318/318	318/318	318/318	318/318	318/318	312/318	312/318	312/318	***
UNH954	178/178	150/178	150/178	150/178	178/178	178/178	178/178	178/178	178/178	150/178	150/178	150/178	***
LG14													
GM070	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/144	144/144	M
GM665	238/238	238/238	238/238	238/238	238/238	238/238	238/238	238/238	238/238	238/238	238/238	238/238	M
UNH865	235/235	235/235	235/235	235/235	235/235	235/235	235/235	235/235	235/235	235/235	235/235	235/235	M
LG15													
GM664	245/245	245/245	245/245	245/245	245/245	245/245	245/245	245/245	245/245	245/245	245/245	245/245	M
UNH880	204/204	204/204	204/204	204/204	204/204	204/204	204/204	204/204	204/204	204/204	204/204	204/204	M
GM129	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	120/120	M

Notes: P: Polymorphic, M: monomorphic at locus *polymorphism in one family, **polymorphism in two families, ***polymorphism in all three families of male

Appendix XII (Cont'd). Parental and progeny DNA genotypes (in BSA) in clonal females x putative YY males (type 'B') with markers from genome wide selection

	F4♀parent 0073 clonal	F4♂parent (E167 YY)	F4♀pool	F4♂pool	F5♀parent 0B19 clonal	F5♂parent (315C YY)	F5♀pool	F5♂pool	F6♀parent DD59 clonal	F6♂parent (F8BB YY)	F6♀pool	F6♂pool	'P'in male parent
LG16													
GM056	243/243	243/243	243/243	243/243	243/243	243/243	243/243	243/243	243/243	238/243	238/243	238/243	*
GM168	336/336	336/352	336/352	336/352	336/336	336/352	336/352	336/352	336/336	336/338	336/338	336/338	***
LG17													
UNH103	232/232	225/225	225/232	225/232	232/232	225/232	225/232	225/232	232/232	225/225	225/232	225/232	***
UNH974	210/210	184/184	184/210	184/210	210/210	184/234	210/234	184/210/234	210/210	184/184	184/210	184/210	***
UNH440	198/198	176/198	176/198	176/198	198/198	198/198	198/198	198/198	198/198	176/198	198/198	176/198	**
LG18													
UNH904	184/184	174/184	174/184	174/184	184/184	174/184	174/184	174/184	184/184	174/174	184/184	174/184	***
UNH888	226/226	226/226	226/226	226/226	226/226	226/226	226/226	226/226	226/226	226/226	226/226	226/226	M
GM285	162/162	154/162	154/162	154/162	162/162	162/162	162/162	162/162	162/162	162/162	162/162	162/162	*
LG19													
UNH419	202/202	210/220	202/210/220	202/210/220	202/202	198/220	198/202/220	198/202/220	202/202	202/210	202/210	202/210	***
UNH943	155/155	134/155	134/155	134/155	155/155	134/134	134/155	134/155	155/155	134/155	Failed	Failed	***
UNH844	133/133	133/133	133/133	133/133	133/133	133/133	133/133	133/133	133/133	133/133	133/133	133/133	M
LG20													
UNH174	190/190	206/206	190/206	190/206	190/190	184/206	184/190/206	184/190/206	190/190	184/206	184/190/206	184/190/206	***
UNH866	167/167	167/167	167/167	167/167	167/167	167/167	167/167	167/167	167/167	167/176	167/176	167/176	*
GM363	210/210	210/210	210/210	210/210	210/210	210/220	210/220	210/220	210/210	210/210	210/210	210/210	*
LG21													
UNH957	192/192	192/192	192/192	192/192	192/192	182/182	182/192	182/192	192/192	172/178	172/178/192	172/178/192	**
GM221	197/197	197/197	197/197	197/197	245/245	238/245	238/245	238/245	197/197	197/197	197/197	197/197	*
LG22													
GM531	231/231	231/233	231/233	231/233	231/231	231/233	231/233	231/233	231/231	231/233	231/233	Failed	**
UNH905	168/168	168/171	168/171	168/171	168/168	158/168	158/168	158/168	168/168	158/168	158/168	158/168	***
UNH840	153/153	151/153	151/153	153/153	153/153	151/153	151/153	151/153	153/153	151/153	151/153	153/153	***
LG24													
GM173	285/285	242/242	242/285	242/285	285/285	242/285	285/285	242/285	285/285	242/242	242/285	242/285	***

Notes: P: Polymorphic, M: monomorphic at locus, *polymorphism in one family, **polymorphism in two families, ***polymorphism in all three families of male