Genetic and phenotypic characterisation of commercial dusky kob (*Argyrosomus japonicus*) cohorts

by

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Thesis abstract

Argyrosomus japonicus (dusky kob) is a large sciaenid finfish indigenous to South Africa, and as an emerging aquaculture species, current production practices rely on mass spawning of wild progenitors to produce first filial (F_1) - offspring for commercial trade. Economically, this approach can be quite lucrative, as the high fecundity of this species allow for the use of small breeding groups to generate thousands of offspring in a single spawn. In recent years, however, considerable efforts have been initiated to retain such animals with faster growth rate as breeders to the next generation, in order to improve growth related traits through selective breeding. Although mass spawning increases production outputs in the short term, under aquacultural conditions, however, mass spawning complicates the maintenance of pedigree records, thus hindering genetic improvement of the species due to the risk of inbreeding depression in subsequent generations. Therefore, the aim of this study was to genetically and phenotypically characterise commercial cohorts of dusky kob within the context of implementing a selective breeding programme. This aim was accomplished by achieving the following three objectives: to assess the genetic constitution and family structure within dusky kob broodstock and derived offspring cohorts; to estimate phenotypic and genetic parameters for growth traits in dusky kob; and to assess the frequency of genetic polymorphisms associated with growth in a large and small cohort of dusky kob using a candidate gene approach. This was done using 12 microsatellite markers, the genetic properties of a wild population (n = 34) and a commercially used broodstock cohort (n = 23) were compared to an offspring cohort comprised of individuals originating from two spawning events. Despite a heterozygous excess, likely due to the occurrence of a genetic bottleneck, the resulting offspring cohort displayed significantly reduced levels of allelic diversity with respect to both the wild samples and the commercially used broodstock ($P < 0.01$). This substantial reduction is primarily attributed to the relatively low numbers of breeders utilised in each spawning event (three and four respectively), but also the low participation among breeders, as parentage analysis indicated only two full-sibling families, one from each spawning event respectively. Therefore, the results of this study suggest that hormonal induction of adult breeders may not be sufficient to stimulate effective spawning in dusky kob.

The sample size of the study population was subsequently increased by combining pedigree and phenotypic data, for three growth related traits (weight, length and Fulton's conditioning index), from the current study with that obtained from a relevant study conducted on dusky kob in 2017. Consequently, estimates of narrow-sense heritability (h^2) were 0.40 ± 0.25 and 0.39 ± 0.25 for juvenile weight and length respectively, with a strong genetic correlation observed between these traits (1.00 ± 0.01). Although these estimates are likely biased due to detected environmental effects, small sample sizes (*i.e.* few families) and possible maternal effects, the observed result suggest a moderate to high heritable genetic component to phenotypic expression of growth related traits in dusky kob. Resultantly, a candidate gene approach was employed, with the aim of identifying molecular variants, single nucleotide polymorphisms (SNP's) in particular, that may be associated with increased growth rate. Sequencing of partial genes and subsequent genotyping *via* direct sequencing revealed two SNPs, one in the Ladybird homeobox 1 b gene (*lbx1b*_c.114) and one in the Tankyrase a gene (*tnksa*_c.69), that display statistical association with the trait of interest. Therefore, *lbx1*, which reportedly has important functions in neural and muscle development, and *tnksa*, which have key regulatory roles in glucose transport and insulin stimulated glucose uptake may be considered as putative candidate genes for investigating growth related traits in dusky kob, and may be useful candidates for marker-assisted selection.

Tesis opsomming

Argyrosomus japonicus (kabeljou) is ‘n groot Sciaenied vinvis, en wat as ’n opkomende akwakultuur spesie, huidiglik staatmaak op die massa-teling van wilde ouers om die eerste nageslag (F1) vir kommersiële handel te produseer. Ekonomies gesien, kan hierdie benadering winsgewend wees, aangesien die hoë vrugbaarheid van hierdie spesie toelaat dat duisende individue in ’n enkele kuit van ’n klein telingsgroep geproduseer kan word. Daar is onlangs ’n aansienlike poging aangewend om diere met ’n vinniger groeitempo as telers vir die volgende generasie te behou, om sodoende groeiverwante eienskappe deur selektiewe teling te verbeter. Alhoewel massa-teling produksie-uitsette op korttermyn verhoog, bemoeilik dit die instandhouding van stamboekrekords, en belemmer dit daardeur genetiese verbetering van die spesie, as gevolg van die verhoogte risiko vir intelingsdepressie in die daaropvolgende generasies. Daarom was die doel van hierdie studie om kommersiële kabeljou geneties en fenotipies te karakteriseer, binne die konteks vir die implementering van ’n genetiese verbeteringsprogram. Hierdie doelwit is bereik deur die volgende drie doelstellings: om die genetiese samestelling en familiestruktuur binne die teelstoet en afgeleide nageslagkohorte te evalueer; om fenotipiese en genetiese parameters vir groeienskappe in kabeljou te skat; en om die frekwensie van genetiese polimorfismes geassosieer met groei in 'n groot en klein kohort van kabeljou met behulp van 'n kandidaatgenbenadering te bepaal. Twaalf mikrosatelliet merkers was gebruik om die genetiese eienskappe van ’n wilde populasie (n = 34) en kommersiëel geproduseerde teeldiere (n = 23) te vergelyk met nageslag wat afkomstig is van twee broeigeleenthede (kuite). Ondanks ’n oormaat van heterosigositeit, wat heel waarskynlik veroorsaak is deur ’n genetiese bottelnek, het die nageslag betekenisvolle verlaagde vlakke van alleliese diversiteit getoon in vergelyking met beide die wilde populasie en die kommersiëel geproduseerde teeldiere ($P < 0.01$). Hierdie aansienlike afname word hoofsaaklik toegeskryf aan die relatief lae getal telers wat gebruik is in elke broeigeleentheid (drie en vier onderskeidelik), maar ook as gevolg van die lae deelname van hierdie telers, aangesien slegs twee volsib-families waargeneem is, een afkomstig van elke broeigeleentheid. Die resultate van hierdie studie dui dus daarop dat hormonale induksie van volwasse kabeljoutelers moontlik nie voldoende is om effektiewe deelname binne broeigeleenthede te stimuleer nie.

Die steekproefgrootte van hierdie studiepopulasie is vervolgens verhoog, deur die stamboek- en fenotipiese data vir drie groeiverwante eienskappe (gewig, lengte en Fulton se kondisioneringsindeks) van die huidige studie, sowel as ’n verwante studie op kabeljou, wat in 2017 uitgevoer is, te kombineer. Gevolglik, was die nou-sin oorerflikheid (h^2) bereken op 0.40 ±0.25 en 0.39 ±0.25 onderskeidelik vir gewig en lengte, met ‘n sterk genetiese korrelasie tussen hierdie eienskappe (1.00 ±0.01). Alhoewel die huidige oorerflikheidsberekeninge moontlik beïnvloed is deur waargenome omgewingseffekte, klein steekproefgroottes (min families) en moontlike moederlike effekte, dui die waargenome resultate op ’n gematigde tot hoë erflike genetiese komponent van die fenotipiese uitdrukking van groeiverwante eienskappe binne kabeljou. Gevolglik was ’n kandidaatgeenbenadering toegepas met die oog om molekulêre variante, meer spesifiek enkel nukleotied polifmorfismes (ENPs), te identifseer wat kan bydra tot verhoogde groeitempo. Die gedeeltelike volgordebepaling van kandidaatgene en gevolglike genotipering het twee ENPs, een binne die “Ladybird homeobox 1 b” geen ((*lbx1b*_c.114), en een binne die “tankyrase a” geen (*tnksa*_c.69), geïdentifseer wat statistiese assosiasie getoon het met groei. Daarom kan hierdie twee gene, *lbx1* wat volgens vorige studies ’n belangrike funksie in neurale en spierontwikkeling het, en *tnksa* met ’n regulatoriese rol in glukose-vervo en insulien gestimuleerde glukose opname, oorweeg word as moontlike kandidaatgene om groeiverwante eienskappe binne kabeljou verder te ondersoek, met toepassing vir merker bemiddelde seleksie.

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%	Percentage
>	Greater than
<	Less than
≥	Greater than or equal to
∞	Infinity
±	Plus-minus
5'	Five prime
3'	Three prime
A_e	Effective number of alleles
AI-REML	Average information restricted maximum likelihood
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
A_e	Effective number of alleles
A_n	Number of alleles
A_r	Allelic richness
BD	Body depth
BLUP	Best Linear Unbiased Prediction
bp	Base pair
°C	Degrees Celsius

CB	Commercial broodstock
CIs	Confidence interval
cm	Centimetre
CP	Cultured progeny
CTAB	Cetyl trimethylammonium bromide
CV	Coefficient of variance
DAFF	Department of Agriculture, Forestry and Fisheries
Df	Degrees of freedom
dph	Days post hatch
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
e.g.	*exempli gratia* (for example)
EST	Expressed sequence tag
EtBr	Ethidium bromide
et al.	*et alii* (and others)
F_1	First-generation
FAO	Food and Agriculture Organisation
F_{IS}	Wright's fixation index (individual relative to the sub-population, equal to inbreeding coefficient - *f*)
F_{IT}	Wright's fixation index (individual relative to the total population)
F_{ST}	Wright's fixation index (subpopulation relative to the total population)
g	Grams
H	Body shape index

h^2	Heritability (narrow-sense)
H_e	Expected heterozygosity
H_o	Observed heterozygosity
HW	Hardy-Weinberg
IAM	Infinite alleles model
i.e.	*id est* (that is to say)
kg	Kilograms
K	Fulton's conditioning factor
KW	Kruskal-Wallis
LD	Linkage disequilibrium
sL	Standard length
MAS	Marker-assisted selection
m	Meters
mm	Millimeters
mtDNA	Mitochondrial Deoxyribonucleic acid
ng	Nanograms
n	Sample size
N_e	Effective population size
N	Number of broodstock in tank
NGS	Next generation sequencing
PA_r	Private allelic richness
PCoA	Principal coordinate analysis

PCR	Polymerase chain reaction
PIC	Polymorphic information content
ppt	Parts per thousand
P-value	Probability value
QTL(‘s)s	Quantitative trait locus/loci
r	Relatedness
r	*Correlation coefficient*
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
r_g	Genetic correlation
r_p	Phenotypic correlation
RNA	Ribonucleic acid
s.d.	Standard deviation
s.e.	Standard error
SASSI	South African Sustainable Seafood Initiative
sL	Standard length
SNP(‘s)	Single nucleotide polymorphism
SPE	Spawning event
T_a	Annealing temperature
TL	Total length
uH_e	Unbiased expected heterozygosity
µl	Microliters

W	Bodyweight
WS	Wild samples

CHAPTER 1

Introduction: Literature Survey, Aims and Objectives

1.1) Species biology: An introduction to dusky kob (*Argyrosomus japonicus)*

1.1.1) *Argyrosomus japonicus*, a confusing taxonomy

The family Sciaenidae, one of the largest families belonging to the order Perciformes, contain approximately 70 genera and over 270 species globally (Chao, 1986). Commonly referred to as croakers and drums, in reference to the sounds produced by the males of most of these species (Griffiths & Heemstra, 1995; Trewavas , 2010). Members of the Sciaenidae family are morphologically diverse, which is further reflected in the different feeding modes, maximum size attained and life history patterns (Chao & Musick, 1977). *Argyrosomus*, one of the larger genera within this family (Farmer, 2008), is represented by at least nine recognised species (Griffiths & Heemstra, 1995) and display a high degree of interspecific morphology (such as otolith development, number of swim-bladder appendages and caudal/pectoral fin morphology). Consequently, sciaenids of the genus *Argyrosomus* have often been misidentified, likely due to their close phylogenetic relationship (Figure 1.1) and shared morphological characteristics, which in turn warranted the application, and extensive revision of the taxonomy for this genus (Griffiths & Heemstra, 1995). Based on the findings of an in-depth comparison of habitat distribution, morphometrics, otolith and anatomical structure by Griffiths and Heemstra (Griffiths & Heemstra, 1995), *Argyrosomus japonicus* which has been known by at least 13 different synonyms worldwide, had been inaccurately referred to as *Argyrosomus hololepidotus* (a species endemic to Madagascar) in both Australia and South Africa (Griffiths & Heemstra, 1995; Trewavas , 2010). Additionally, in South Africa, *A. japonicus* was also confused

for another member of this genus, *A. inodorus,* a species known to occasionally hybridise with *A. japonicus* (Mirimin *et al*., 2014).

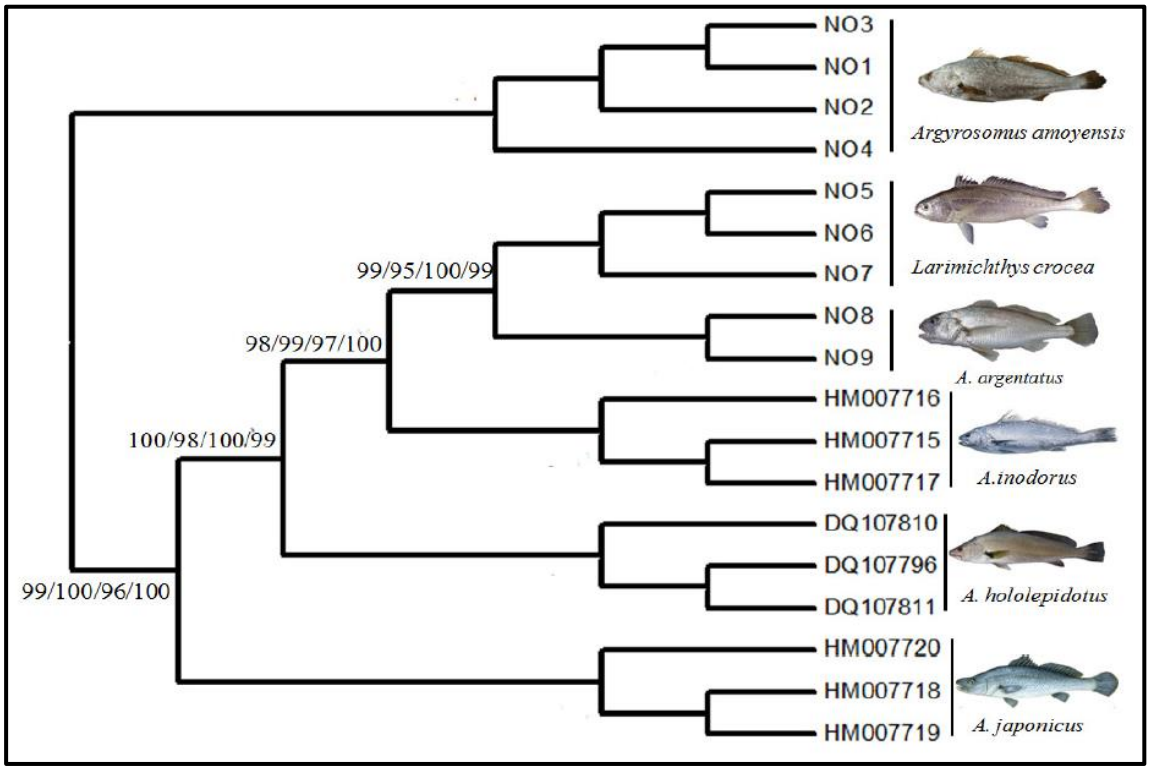

Figure 1.1: Phylogenetic tree of six sciaenid species based on DNA barcodes (obtained from Chao Chen, 2012).

Following the latest revision of this genus by Griffiths and Heemstra, wild populations of *A. japonicus* in South Africa, Australia and Japan could not be separated and were thus considered to be conspecific (Griffiths & Heemstra, 1995). Interestingly, the biology of this species has been extensively studied in both South Africa (Griffiths & Hecht, 1995; Griffiths & Heemstra, 1995; Griffiths, 1996;) and, more recently, in Australia (Silberschneider & Gray, 2008; Silberschneider *et al.*, 2009; Ferguson *et al.*, 2014) revealing significant differences in the life-history traits (*e.g*. growth, age at sexual maturity, time of spawning) amongst these geographical locations. Furthermore, mitochondrial DNA analysis revealed strong genetic differentiation between South African and Australian populations, suggesting that these populations have been isolated for quite some time and could potentially represent two different species (Klopper, 2005; Farmer, 2008). However, to date no studies have been reported supporting the distinctiveness of *A. japonicus* in Japan. A revision of the taxonomy *A. japonicus* is, therefore, justified. Regardless of this, the species of interest

in this thesis is the South African *A. japonicus*, commonly known as dusky kob. Aspects of the natural life-history and distribution of this species in South Africa are described below.

1.1.2) Ecology, life-history and distribution of *Argyrosomus japonicus*

The dusky kob, *Argyrosomus japonicus*¸ (Farmer, 2008) has been reported in four geographically-isolated regions of the Pacific and Indian Oceans, seemingly restricted to the latitudes of 20º and 40º, both north and south of the equator (Figure 1.2). In the northern hemisphere, dusky kob occurs along the coast of Pakistan continuing into north-west India as well as along the Chinese coast, from Hong Kong to southern Korea and Japan (Griffiths & Heemstra, 1995; Trewavas, 2010). Interestingly, these regions (*i.e.* Arabian and the East China Sea) are separated by a geographical barrier in the form of the Indian landmass, which extends into tropic equatorial waters and past the apparent physiological limit of the species distribution (*i.e.* 20º N). This bifurcated distribution may be maintained by the inability of eggs and larvae of *A. japonicus* to survive water temperatures that exceed 30°C (Battaglene & Talbot, 1994), a temperature commonly reached in equatorial waters.

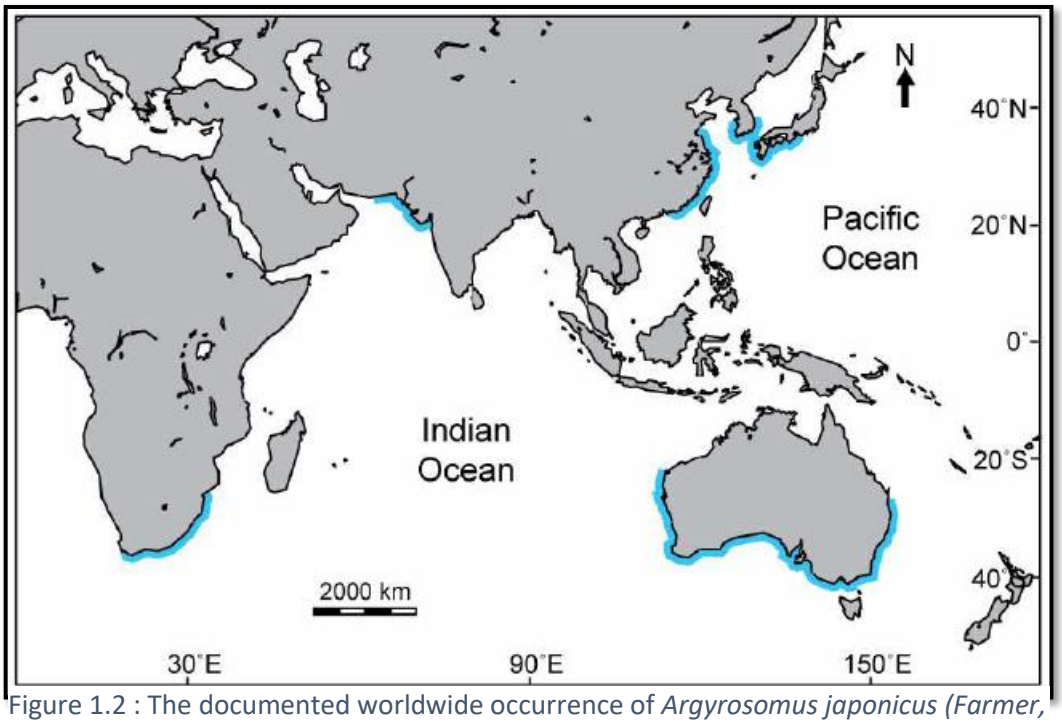

Figure 1.2 : The documented worldwide occurrence of *Argyrosomus japonicus (Farmer, 2008).*

In the southern hemisphere, this species is distributed along the entire southern seaboard of Australia, from Bundaberg in southern Queensland to North West Cape in Western Australia, and along the south-eastern coast of Africa, from the Cape of Good Hope to southern Mozambique (Marquet *et al.*, 1987; Griffiths & Heemstra, 1995; Trewavas, 2010). Due to the lack of *A. japonicus* encounters in waters deeper than 200m in either of these continents, it has been suggested that the vast expanse/depth of the Indian Ocean serves to isolate populations, acting as both a physical barrier to adult migration and the advection of eggs and larvae (Marquet *et al.*, 1987; Griffiths & Heemstra, 1995; Farmer, 2008).

In South Africa, *A. japonicus* is a continental coastal species, abundant from Cape Agulhas to northern KwaZulu-Natal where it reportedly represents a homogenous genetic stock (Mirimin *et al.*, 2016), and inhabit estuaries as well as the surf and nearshore zones of this region (Griffiths, 1996). Estuaries and their surrounding wetlands are bodies of water usually found where rivers meet the ocean, offering sheltered environments along the South African coastline, with varying turbidity, temperature and salinity conditions (Baird *et al.*, 1996; Kennish & Whitfield, 1999). Owing to the abundance of primary prey biomass such as zooplankton (*e.g.* calanoid copepods), crustacean (*e.g.* mysids), insects and teleost fish (Blaber, 1974; Griffiths, 1997a; Næsje *et al.*, 2007), estuaries are rich in biodiversity and are important nursing grounds for various larval and juvenile fish (Blaber, 1974) as well as feeding areas for many adult fish (Griffiths, 1997a; Griffiths & Griffiths, 2000). As a result, estuaries are essential in the life-history, productivity and diversity of estuary-dependent species, including dusky kob.

Juvenile dusky kob [<150 mm total length (TL)] appears exclusively in estuaries, with preference to the upper reaches of this environment due to favourable salinity [salinity 5 parts per thousand (ppt)], into which recruitment of post-flexion larvae and newly juveniles mostly occur at 20 mm – 30 mm TL (roughly one month of age) shortly after spawning (Griffiths, 1996; Ferguson *et al.*, 2008; Ferguson *et al.*, 2014). Early juveniles (<50 mm TL) predominantly prey on calanoid copepods whilst mysids are favoured by juveniles (<100 mm TL). Larger juveniles (100 mm – 149 mm TL) prey mostly on the late larvae and early juveniles of other species, whilst teleosts remain an essential prey taxon for dusky kob >100 mm TL (Griffiths, 1997a). Larger juveniles (approximately ≥150 mm TL) move from the upper reaches of the estuary to the middle/lower reaches (salinity 6 – 35 ppt), with some entering the adjacent marine surf zones (Griffiths,

1996). However, although more prevalent in the middle reaches, larger juveniles (150 mm - 400 mm TL) have been reported to make extensive use of the estuarine environment. These fish feed largely on early juvenile mugilids and on the small, pelagic, estuarine Round herring (*Gilchristella aestuaria*) (Griffiths, 1997a) along with several other teleost species and early juvenile con-specifics (*i.e.* cannibalism), which remain an important prey item for larger juvenile *A. japonicus* (>400 mm TL) (Griffiths, 1997a; Ferguson *et al.*, 2014). Juvenile *A. japonicus* grow rapidly and can attain roughly 220 mm - 350 mm TL within the first year (Griffiths, 1996; Ferguson *et al.*, 2014). Furthermore, tagging data indicated that dusky kob does not venture far from, and are likely to stay close to, their natural estuaries where they form localised populations until they reach sexual maturity (Griffiths, 1996).

Cannibalism of early juvenile is common in South African estuaries (Whitfield & Blaber, 1978; Marais, 1984; Griffiths, 1997a), and has been observed for many finfish species (Whitfield & Blaber, 1978; Marais, 1984; Smith & Reay, 1991; Pereira *et al.*, 2017), including dusky kob (Griffiths, 1997a,b). A study by Griffith, which relates the diet of *A. japonicus* to the distribution patterns of commonly consumed prey, suggested that conspecific predator avoidance may be a major contributing factor controlling the distribution of early juvenile dusky kob (Griffiths, 1997a). Furthermore, a study based on the feeding ecology of *A. japonicus*, with emphasis on the Eastern Cape surf zone (Griffiths, 1997b), further substantiated this claim as a lack of early juvenile dusky kob cannibalism was observed in the middle/lower reaches of the estuary. Therefore, it has been suggested that prey availability, as well as conspecific predator avoidance, are important factors that influence patterns of spatial distribution of juvenile dusky kob (Griffiths, 1997a; Næsje *et al.*, 2012). For these reasons, early juveniles (<150 mm TL) may be restricted to the upper reaches of the estuary, whereas larger juveniles have been reported to occur throughout this environment. This is not surprising considering that larger juvenile dusky kob (>400 mm TL) has few estuarine predators (Griffiths, 1996). On the contrary, *A. japonicus* are known euryhaline piscivores predators (Marais, 1984, 1988; Griffiths, 1997a; Ferguson *et al.*, 2008) and are regarded as opportunistic feeders (Griffiths, 1997a), as the diet of this species reportedly varies in accordance with prey species abundance (Whitfield & Blaber, 1978; Griffiths, 1997a,b).

As mentioned above, dusky kob are usually found in turbid water (Griffiths, 1996), where they retain several sensory advantages over their prey. In particular, the evolution of lateral line and auditory systems of *A. japonicus* (Griffiths & Heemstra,

1995) make this species adapt to turbid environments. Additionally, the capture of several blind dusky kob in Lake St Lucia by hook and line using cut bait (Griffiths, 1997b) suggest that *A. japonicus* possess enhanced mechano- and chemoreceptors respectively. Furthermore, the development of a larger wedge-shaped caudal fin and deep caudal peduncle (Griffiths & Heemstra, 1995), as well as a larger buccal cavity allow for rapid acceleration (Pauly, 1989; Lauder & Jayne, 1996; Flammang & Lauder, 2008) and effective suction feeding (Higham, 2006) respectively, and presents major advantages to the feeding ecology of this species. Therefore, the accumulation of these characteristics would suggest that dusky kob locate and stalk their prey using primarily non-visual cues, and that visual contact may only occur in the final stages of the attack. Therefore, turbid environments not only offer an advantage to the feeding ecology of this species (Van der Elst, 1998), they are also extensively used by various other larval and juvenile fish, as they provide adequate food supplies, and more importantly, increased protection from predators (Griffiths, 1996; Whitfield & Elliott, 2002; Gregory & Levings, 2004; Gregory, 2008).

Growth of *A. japonicus*, although reportedly faster in females than males (Griffiths & Hecht, 1995; Griffiths, 1996; Silberschneider *et al.*, 2009), declines significantly after reaching sexual maturity. Reportedly, in South Africa, fifty percent of females reach maturity at six years [≈1 000 mm standard length (SL)] of age and males at five years (≈950 mm SL). This, in turn, would suggest that a large proportion of adult energy is channelled into reproduction. As a result, all females at eight (>1 200 mm TL) and males at seven (>1 100 mm TL) years of age are sexually mature (Griffiths & Hecht, 1995). However, the length and age at which *A. japonicus* reach sexual maturity appear to differ regionally, where those in eastern Australia reportedly attain smaller size and age at sexual maturity (2 – 3 years of age) (Silberschneider & Gray, 2008; Silberschneider *et al.*, 2009; Ferguson *et al.*, 2014). Larger individuals (≥ 400 mm TL) generally remain in the inshore marine environment until they reach sexual maturity, and eventually venture offshore into deeper waters (Taylor *et al.*, 2006; Cowley *et al.*, 2008; Næsje *et al.*, 2012). As expected, the majority of fish caught inshore (estuaries and surf zones) are less than seven years of age, whereas those caught from offshore line-fishing are generally older than six years. Although individuals older than 27 years of age are rare, possibly due to exploitation, a 42-year-old (at 175 cm TL) *A. japonicus* has been recorded (Griffiths & Hecht, 1995; Griffiths & Heemstra, 1995).

Like many members of the family Sciaenidae, dusky kob produces drumming sounds by vibrating the swim bladder. This phenomenon has been associated with territorial display as well as spawning behaviour in sciaenids, and may reflect adaptation to spawning at night and in turbid habitats (Blaber, 2000). Furthermore, dusky kob are broadcast spawners, commonly forming spawning aggregations in the nearshore regions of estuaries (Chao & Musick, 1977; Griffiths, 1996; Silberschneider *et al.*, 2009; Ferguson *et al.*, 2014), where after spawning eggs and larvae are dispersed along the South African coastline, facilitated by the southward movement of the Agulhas Current (Beckley, 1995). Based on an extensive estuarine study of the South African east coast by Griffith (Griffiths, 1996), it was reported that adult *A. japonicus*, from the south-eastern and southern Cape, migrate to KwaZulu-Natal to partake in mass spawning events. The substantial increase of sexually mature *A. japonicus* with advanced gonadal conditions, caught in KwaZulu-Natal between August and November (late winter/spring), indicate that spawning of this species occurs within this time and coincides with the rainy season of this region, which is similar to observations in many sciaenid species (Griffiths, 1996; Ferguson *et al.*, 2008, 2014). This is to be expected, as freshwater inflow into estuarine environments has been reported to aid in early juvenile recruitment (Ferguson *et al.*, 2008, 2014). The early spawning of *A. japonicus*, which appears to happens at night, is presumably due to the favourable conditions associated with the warmer waters within the region (Griffiths, 1996), and a reduced risk of predation on eggs by zooplanktivores (Griffiths, 1996, 1997c). However, the presence of ripe and ripe/running gonads, in the south-eastern and southern Cape, indicates that spawning also occurs within this region from October to January (spring/summer). Therefore, it is likely that spawning is initiated in KwaZulu-Natal and continues as a portion of the spawning population returns to southern waters. The absence of *A. japonicus* with spent gonads in KwaZulu-Natal and the presence of such individuals in the south-eastern and southern Cape also suggests a return migration once spawning is completed (Griffiths, 1996).

The life history of *A. japonicus*, like many large sciaenids, is not only characterised by separate juvenile and adult phases in estuarine and marine habitats, but also by delayed reproduction in order to obtain large size/age at maturity. In essence, sexual maturity in such sciaenids, like *Argyrosomus*, are delayed in order to reach a larger size at first maturity (Griffiths, 1996; Ferguson *et al.*, 2008) in order to increase individual fecundity. Consequently, the increased pre-mature growth rate of these

species, in addition to attaining a larger size at maturity would allow increased longevity especially if natural mortality is low, as is the case for dusky kob (Griffiths, 1996). This life history strategy makes this species particularly vulnerable to exploitation in both estuarine and marine habitats. Overall, the life history of *A. japonicus* can be separated into four phases, where the last three are particularly vulnerable to exploitation:

(i) Egg and larval stages, which occur pelagically in nearshore marine environments;
(ii) Early juveniles, which are recruited into the upper reaches of estuaries;
(iii) Larger juveniles, which populate the upper to lower reaches of estuaries and surf zones;
(iv) Adults, which populate the nearshore marine environment, but frequent estuaries and surf zones.

Due to the habitable conditions, estuaries provide numerous fishing opportunities for both recreational and subsistence fisherman as well as a commercial fishery to a lesser extent (Lamberth & Turpie, 2003). As a matter of fact, due to the high abundance and variety of fish, estuaries are favoured fishing grounds for both recreational and subsistence fisherman (Baird *et al.*, 1996), where dusky kob is considered a major target. Additionally, adults forming spawning aggregations, particularly in KwaZulu-Natal, are heavily targeted by recreational and commercial line-boat fishermen, where dusky kob has sustained both commercial and recreational fisheries for decades (Brouwer *et al.*, 1997; Pradervand *et al.*, 2007). Consequently, intense exploitation of juvenile and adult populations of this species, especially considering having evolved a life-history strategy based on low natural mortality has intensified fishing pressure and ultimately led to recruitment overfishing of this species (Griffiths, 1996, 1997c; Ferguson *et al.*, 2014).

1.2 Dusky kob, an emerging aquaculture species

Due to the profitability and relatively high catch estimates of *A. japonicus* compared to other recreational angling species (Lamberth & Turpie, 2003), dusky kob is among the species most affected by fishing pressure in South Africa (Griffiths & Griffiths, 2000). Although the taxonomic confusion around the genus *Argyrosomus* has been resolved (Griffiths & Heemstra, 1995), *Argyrosomus spp* (like dusky- and silver kob) are still managed as *A. hololepidotus* along the south-eastern coast of Africa where minimum

legal catch size varies from 400 mm in KwaZulu-Natal, 500 mm west of Cape Agulhas and 600 mm east of Cape Agulhas (Sauer *et al.*, 2003). Notably, this minimal limit lies far below the size reached at sexual maturity (± 1 000 mm SL) and has subsequently resulted in gross overfishing of *Argyrosomus spp*, particularly dusky kob. Recruitment overfishing of dusky kob, resulting from combined fishing efforts in both estuaries and spawning aggregations, has depleted the spawner biomass per recruit to 1.0 – 4.5% of their pristine levels (Griffiths, 1997c), which is indicative of a severely overfished stock population, with levels below 20% considered as unsustainable. Currently, dusky kob is considered a threatened species and is listed as Red on the South African Sustainable Seafood Initiative's (SASSI) Consumer Seafood List (SASSI WWF, 2018) if caught from linefish or trawl.

Due to increased fishing pressure from a rapidly expanding seafood industry and the subsequent decline in wild stocks, farming of dusky kob has been initiated in South Africa (Bolton *et al.*, 2013), as this promotes a more sustainable alternative to the harvesting of wild stocks. Moreover, following the commencement of dusky kob aquaculture in South Africa, a great deal of research effort has been initiated to promote further understanding of the species biology, and gain insight into the culturing of this species (Daniel, 2004; Collett, 2007; Bernatzeder & Britz, 2007; Fitzgibbon *et al.*, 2007; Collett *et al.*, 2008a, 2008b; Bernatzeder *et al.*, 2010; Pirozzi *et al.*, 2010; Timmer & Magellan, 2011; Kaiser *et al.*, 2011; Musson & Kaiser, 2014). Culturing of dusky kob is reportedly in line with that of red drum (*Sciaenops ocellatus*), a well-established sciaenid species cultured in the United States (Lee & Ostrowski, 2001) and in China (Hong & Zhang, 2003), where species characteristics such as fast initial growth rate, tolerance to low salinity- and oxygen levels, good feed conversion ratio, disease resistance and high crowding densities all depicting dusky kob as a suitable candidate for aquaculture (Griffiths, 1996; Fitzgibbon *et al.*, 2007; Collett *et al.*, 2008; Collett *et al.*, 2011; Fielder *et al.*, 2011).

During a recent period (2001 - 2016), aquaculture production in South Africa experienced a steady growth of 5.8% per annum whilst marine catch is reportedly plateauing (DAFF, 2016; FAO, 2018). The South African marine finfish industry is currently in its infancy, with dusky kob being the only commercial species cultured (DAFF, 2011, 2016), whilst other marine finfish species (*e.g.* yellowtail, mangrove snapper, spotted grunter and yellow belly rock cod) are kept on farm facilities for research purposes (DAFF, 2016). Nevertheless, the finfish sub-sector in South Africa

is still an emerging industry with production reaching nearly 50 tons in 2012 and 160 tons in 2014 (DAFF, 2016) following a sizable capital investment in 2011 (42% of total aquaculture investment (DAFF, 2011)) into the farming of marine finfish. Therefore, not only is fish farming a viable method to produce more affordable and sustainable seafood to meet increasing demands, it also provides many employment opportunities on the training, research and management levels of aquacultural practices. Currently, there are three commercial hatcheries of dusky kob in operation in South Africa: these include two recirculation facilities situated in the Eastern Cape Province and a pond culture facility in KwaZulu-Natal, which receives its seed supply from one of the above-mentioned producers, and will be the focus of the present study.

Current production practices for dusky kob rely on mass spawning of captive broodstock (*i.e.* each male reproducing with many females and *vice versa*, in a single tank), held under temperature and photoperiod control, to allow for year-round progeny production (Battaglene & Talbot, 1994; Silberschneider & Gray, 2005). Prior to spawning, female broodstock are sedated and cannulated using a catheter, in order to collect viable oocytes. Interestingly, an individual female spawner can produce large amounts of eggs, ranging between 2 - 12 million in a single spawn, where oocytes of diameter ≥0.5 mm are considered suitable for successful spawning. Together with a rise in water temperature (>22 °C), male and female brooders are hormonally induced to commence spawning (Silberschneider & Gray, 2005), followed by the collection of viable (floating) fertilised eggs that are placed in incubation tanks to hatch. Ultimately, the production cycle is initiated with the collection of viable fertilised eggs, which hatch approximately 24-30 hours after spawning (Battaglene & Talbot, 1994).

Larvae are continually incubated, as they feed from their yolk sac for the first 48 hours, after which they are transferred to a larval rearing system (a partial recirculation system). Larval rearing continues with the introduction of live feeds (such as rotifers and brine shrimp) until they are fully weaned (at 20 days post hatch as per the current standard), and subsequently transferred to nursing tanks (juvenile stage). Juveniles are reared for several months (approximately 2 – 3 months), after which they are pooled and separated into independent size grades. Concurrently, slower growing juveniles are commonly culled, as an alternative to grading when tanks are limited, to minimize size disparities between progeny. Such practices do not only minimise detrimental social/behavioural effects (such as cannibalism), but also aid in maintaining standard growth rates throughout rearing onto harvest (which can range

from 400 g to 3 kg). Despite such practices, instances of aggressive behaviour and subsequent cannibalism is a common occurrence in dusky kob aquaculture and may occur as soon as 18 days post-hatch (dph) (Information, 2001; Musson & Kaiser, 2014). Similar findings have also been reported for other aquaculture species, including barramundi (Loughnan *et al.*, 2013), sharp-tooth catfish (Baras & Dalmeida, 2001), giant grouper (Hseu *et al.*, 2007), Japanese flounder (Dou *et al.*, 2004) and red drum (Chiu Liao & Chang, 2002). In addition, cannibalistic behaviour may be more conspicuous when progeny from multiple families, with differential growth rates, are reared in a communal environment (Baras & Jobling, 2002; Liu *et al.*, 2017). Nevertheless, environmental factors such as low feeding frequency, inadequate food source, stocking and crowding density and light intensity have been reported in altering aggressive behaviour with larger animals typically posing a greater potential threat (Qin & Fast, 1996; Kestemont *et al.*, 2003; Qin *et al.*, 2004; Fessehaye *et al.*, 2006; Collett *et al.*, 2008a; Timmer & Magellan, 2011).

Commercial production of dusky kob through aquaculture is undoubtable a more sustainable alternative to harvesting of wild stocks. Such breeding practices do not only relieve fishing pressure on wild populations, by significantly contributing to the commercial fish supply, but also preserve other marine species that are harmed during overfishing. Furthermore, unlike commercial fishing of wild populations, aquaculture is not limited by restrictive fishing practices nor seasonal availability of commercially fished species and can thus provide a larger and more consistent supply of fish. On the contrary, kob production still relies on genetically unimproved or wild-caught broodstock, where inconsistency in production performance and uncertainty concerning long-term spawner survival are commonly observed. Given such circumstance(s), dusky kob culture would immensely benefit from selective breeding programmes, which are being considered in South Africa.

1.3 Selective breeding in aquaculture

Genetic improvement programmes in livestock species have made a significant contribution to the productivity and sustainability of agricultural production worldwide. In fact, selective breeding and domestication in livestock species have led to the establishment of thousands of genetically distinct breeds/strains that are collectively termed ‘genetic resources’. Alternatively, despite numerous research efforts on optimising culturing conditions to increase production outputs in aquaculture, research

surrounding the use of selective breeding to improve the biological productivity of aquacultural species have been limited. Aquaculture reportedly lags far behind farmed livestock species with regards to the application of this technology, as many fish and shellfish producers use genetically unimproved stocks to produce marketable trade (Tave, 1993). On the contrary, only 10% of world aquaculture production is currently based on improved stocks (Gjedrem & Baranski, 2009). Additionally, selective breeding in such cases have resulted in significant genetic improvements and has subsequently facilitated the development of major aquaculture industries for a limited number of species, such as salmonids, tilapias, oysters and shrimp (Argue *et al.*, 2002; Eknath & Hulata, 2009; Gjedrem, 2012; Zak *et al.*, 2014; Chavanne *et al.*, 2016). Comparatively, the genetic gains achieved per generation, for traits such as growth rate and disease resistance, in aquaculture are reportedly four to five times higher (1 - 5% and 10 – 20%) than those obtained for livestock species (Gjedrem & Baranski, 2009). These substantial differences in response to selection and subsequent genetic improvements between farm animals and aquatic species are attributed to increased fecundity, and the existence of broad phenotypic- and genetic variation commonly observed in aquaculture. Both of which allow for high selection intensities resulting in elevated selection responses. Furthermore, aquatic animals have lower (more efficient) feed conversion ratios than larger livestock species (Fry *et al.*, 2018), likely due to metabolic requirements of ectothermic animals (Naylor *et al.*, 2009; Torrissen *et al.*, 2011). Moreover, a strong correlation between growth and feed conversion efficiency have been observed in aquaculture (Thodesen *et al.*, 1999; Ytrestøyl *et al.*, 2011), which indicates that individuals selected for fast growth rate would more effectively utilise feed resources than livestock species.

Mass selection, also termed phenotypic selection, refers to a method of artificial selection where a candidate is chosen solely based on their individual phenotypic performance. This approach has been widely applied in many aquatic species due to its simplicity and lower associated costs, as individual identification and/or the maintenance of pedigree records are unnecessary, and therefore requiring minimal infrastructural investments. Theoretically, mass selection can produce rapid improvement of economically important traits (such as growth rate), by exploiting the substantial genetic variation present for a number of traits in aquaculture, especially if the narrow-sense heritability (h^2) of the trait(s) under selection is high. Therefore, this method is particularly suited for traits that require records on individual performance

(such as weight and length) and is therefore not suitable for traits that require the slaughter of the animal (such as flesh quality or carcass yield) as selected individual are retained for breeding. In addition, mass selection has proven very effective in improving growth rate over relatively few generations of selection (Volckaert & Hellemans, 1999; Hussain *et al.*, 2002; Vandeputte *et al.*, 2009). However, the lack of pedigree records presents a threat to the sustainability of a selective breeding programme when employing such a method of selection. Considering the moderate to high narrow-sense heritability of body weight and length commonly observed for finfish species (Kause *et al.*, 2003; Vandeputte *et al.*, 2004; Saillant *et al.*, 2006, 2007; Wang *et al.*, 2008; Domingos *et al.*, 2013), individuals originating from the same family are likely to express similar phenotypic intensities for the trait of interest (*i.e.* increased growth). Under such circumstances, however, mass selection inadvertently increases the rate of inbreeding, which refers to the mating between individuals that are related to each other by ancestry. In the absence of pedigree information, related individuals are likely to be selected as breeders to contribute to the next generation (Huang & Liao, 1990; Blonk *et al.*, 2009; Gjedrem & Baranski, 2009; Knibb *et al.*, 2014), consequently accelerating the rate of inbreeding within a breeding population resulting in the ungoverned loss of genetic diversity. With this in mind, the selection of unrelated broodstock individuals is further constrained by the mass spawning behaviour of these species. Given that limiting- and variable contribution of breeders to progeny cohorts is a common observation in finfish aquaculture, and is particularly relevant when conducting a mass spawn for dusky kob (Mirimin *et al.*, 2016; Jenkins, 2018), the likelihood of selecting related individuals as breeding candidates increases. Furthermore, in fish- and other aquatic species with high fecundity, the risk of selecting related individuals as breeding candidates, and rapidly accumulating inbreeding, is exacerbated as few parental broodstock is needed to reproduce each generation (Bekkevold *et al.*, 2002; Campton, 2004; Fessehaye *et al.*, 2009). As a result, using a restricted number of breeding individuals to produce progeny cohorts can intensify the effects of random genetic drift, which leads to considerable fluctuations in allele frequencies between generations, and effectively contribute to a loss of rare alleles. Additionally, the rapid increase in homozygosity tends to ‘expose’ undesirable recessive genes, resulting in the depression of fitness-related traits (such as growth rate, survival and reproductive success) which diminishes the response to selection and decreased aquacultural productivity (Hulata *et al.*, 1986; Su *et al.*, 1996b; Pante *et al.*, 2001; Fessehaye *et al.*, 2009).

As an alternative, family-based breeding programmes (*i.e.* within- and between family selections) are often employed to minimise the occurrence of inbreeding. In such programmes, individual family relationships can be monitored through single pair- and/or strip spawning, followed by separate rearing and physical tagging once individuals reach a suitable size. Pedigree records can thus be obtained/maintained, allowing for the construction of more effective breeding schemes. As a result, phenotypically superior (*i.e.* faster growing) individuals across multiple families can be identified, and potentially selected as breeders to improve the trait of interest, whilst minimising inbreeding and associated deleterious effects. Regardless of this, the incorporation of both pedigree and phenotypic data from relatives allow for the estimation of parental breeding values for a variety of different traits, using best linear unbiased prediction (BLUP) procedures. Thus making it possible to estimate variance components for traits with low heritability as well as traits that require sacrifice of a particular animal, for which records can only be obtained from relatives (Meuwissen, 1997; Gjerde, 2005; Gjedrem, 2010). Therefore, not only do family-based breeding programmes provide the opportunity to preserve genetic gains through managing the homogenisation of the population through selective breeding, this method is also more flexible regarding the number and the type of traits that can be selected for.

To maximise genetic gains and limit inbreeding, the initial number of test families, and thus tanks required, must be large enough to ensure ample genetic variation, as this provides the basis for genetic improvement of traits through selective breeding. Moreover, previous studies have suggested that a base population comprising of 50 unrelated broodstock pairs (Gjerde *et al.*, 1996; Bentsen & Olesen, 2002; Fjalestad, 2005; Sonesson *et al.*, 2005) is necessary to attain sufficient genetic variation for selective breeding of commercial mass spawning species. However, establishing such base populations are not always possible for recently established hatcheries, especially for facilities that rely on species with late maturity (*i.e.* large size at maturity) such as dusky kob. On the contrary, housing a large base population as well as numerous test families is often a limiting factor in many finfish breeding programmes due to the significant infrastructural investments required to support separate rearing. As a result, separate rearing, as well as physical tagging once juveniles reach a suitable size, is often too labour intensive and financially strenuous to execute. In addition, separate rearing also introduces environmental (*i.e*. tank) effects common to full-sibling families, which can create bias in BLUP estimations, and slow genetic

improvements (Martínez *et al.*, 1999; Vandeputte *et al.*, 2011). Consequently, separate rearing has proven impractical not only in financial terms, but also due to biological constraints of the species involved, as is the case with natural mass spawning species, such as dusky kob.

Regardless of these obstacles, there is enormous potential to increase the aquacultural production efficiency through systematic genetic improvements. In fact, a key benefit of selective breeding is that genetic improvements obtained are permanent, as genetic gains achieved can be transferred from generation to generation, and when considering the high fecundity of aquatic species, can be expressed in thousands/millions of individuals per generation (Gjedrem & Baranski, 2009; Gjedrem, 2016). Therefore, the implementation of selective breeding programmes not only allow for the development of superior aquacultural product, but also has remarkable economic benefits as improved growth of cultured stock can reduce production- and maintenance costs due to a faster turnover rate (Gjedrem & Baranski, 2009). However, some basic conditions must be met for a selective breeding programme to function effectively. Firstly, it is pertinent for the trait in question to express some degree of phenotypic variation as this allows for an increased selection intensity, which provides the basis for improvement. Secondly, a portion of this variation must be attributed to additive genetic differences (sufficient h^2), since it is only the additive genetic variation that is transferred from one generation to the next. Essentially, this further highlights the importance of a large unrelated base population (maximizing N_e) with sufficient genetic variation, in order to maximise selection responses. Thirdly, the life cycle of the species in question must be known in order to manipulate the breeding behaviour and stimulate reproduction. Lastly, pedigree information must be available, as it is imperative to evaluate progeny for trait characters. Therefore, for late-maturing species such as the dusky kob, the establishment of selective breeding programmes lags behind those for species with shorter generational intervals, which alternatively allow for shorter production cycles, and especially, behind those where the loss of genetic diversity can be limited through single pair mating and/or strip spawning (Gjerde & Villanueva, 2003). Fortunately, with the advent of new molecular marker technologies, strategies now exist to evaluate the genetic variation, evaluate pedigree relationships and aid in conservational breeding strategies for commercially farmed aquaculture species with mass spawning reproductive modes.

1.4 Molecular markers in aquaculture

Following the establishment of the currently widely-accepted model of DNA structure (the double helix) in the early 1950's, various research endeavours have clarified uncertainties surrounding the structure and function of DNA and genes. Consequently, molecular research based on DNA cloning, sequencing and hybridisation in the 1970's, followed by DNA amplification and automated sequencing during the 1980's, have led to the development of various classes of DNA markers. Early molecular works for studying genetic variation at co-dominant Mendelian inherited loci were based on allozyme electrophoresis (Ferguson *et al.*, 1995), followed by the first mitochondrial DNA based population genetics studies in the early 1980's (Avise *et al.*, 1979). However, the development of DNA amplification using Polymerase Chain Reaction (PCR) has revolutionised molecular genetics research and given rise to a number of analytical techniques, ranging from isolation and sequencing of target DNA to methods analysing length polymorphisms, such as microsatellites (Hansen, 2003). Currently, numerous genetic marker types are available for studying aquatic species that are routinely used in phylogenetic and population genetic studies, where genetic markers are categorised under three general classes: (1) allozymes, (2) mitochondrial DNA, and (3) nuclear DNA (Okumu & Çiftci, 2003; Liu & Cordes, 2004; Chauhan & Rajiv, 2010; Maqsood & Ahmad, 2017).s

The term allozyme refers to a genetically distinct enzyme produced by an alternative allelic form at the same gene locus. Amino acid differences in the polypeptide chain produced by an alternative allele reflect changes in the underlying DNA sequence. Depending on the nature of the amino acid substitution, the resulting protein product could potentially manifest different properties (in size and charge) that allow for deferential segregation when performing electrophoresis. Despite the simplicity, speed and relative low costs associated with allozyme electrophoresis, the use of this technology in aquacultural genetics is relatively limited (Liu & Cordes, 2004). Because allozymes cannot be assumed to be selectively neutral (Karl & Avise, 1992), and considering their limited power in detecting genetic variability, they are not ideal for population genetic research nor parentage studies (Magoulas, 1998b). Additionally, the application of this method is further constrained by the invasive tissue sampling method (*i.e.* liver and heart tissue), often requiring the sacrifice of the fish, and the subsequent cryogenic storage to prevent enzymatic activity (Okumu & Çiftci, 2003).

A small portion (<1%) of the total DNA in eukaryotic cells are non-nuclear, and located within organelles in the cytoplasm called mitochondria. Mitochondrial DNA (mtDNA) is generally maternally inherited as a single haploid molecule, and unlike nuclear genomes, has a very high mutation rate resulting from a lack of repair mechanisms during cell replication (Wilson *et al.*, 1985). The entire mitochondrial genome is transcribed as a single unit (Okumu & Çiftci, 2003), except for the roughly 1-kb control region (D-loop) where replication and transcription is initiated (Chauhan & Rajiv, 2010). Additionally, non-coding regions like the D-loop generally exhibit elevated levels of variation compared to the coding regions, which make up the majority of the mitogenome (Brown *et al.*, 1993). One consequence of its non-Mendelian transmission is that the effective population size of mtDNA is smaller than that of nuclear DNA, therefore, making it a more sensitive indicator of population phenomena such as bottlenecks (Okumu & Çiftci, 2003), and detecting hybridisation between species or their subspecies when used in combination with nuclear markers (Nijman *et al.*, 2003). Therefore, mtDNA is more effective in evaluating genetic variability at the species or intra-specific level, and is, therefore, not as effective when assessing genetic variability within commercial stocks. Nevertheless, mitochondrial DNA has predominantly been used in phylogenetic projects and has been indispensably utilised in bar-coding studies (Krishnamurthy & Francis, 2012; Taylor & Harris, 2012).

The advancement of DNA magnification through PCR has resulted in the development of many PCR-based techniques for evaluating genetic variation, ranging from multiple arbitrary primer markers like Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphisms (AFLP), to sequence targeted PCR-based techniques like Single Nucleotide Polymorphisms (SNP) and microsatellite markers (Okumu & Çiftci, 2003; Maqsood & Ahmad, 2017). RAPD and AFLP markers are based on the random amplification of anonymous regions of nuclear DNA. These methods have been widely used in fisheries research, historically, as they are considered to be simple, relatively affordable and have high levels of polymorphism (Magoulas *et al.*, 1998a, 1998b; Okumu & Çiftci, 2003; Liu & Cordes, 2004; Maqsood & Ahmad, 2017). One of the major advantages of RAPD and AFLP markers is that primers are commercially available and do not require prior knowledge of the target DNA sequence or genome organisation (Okumu & Çiftci, 2003; Chauhan & Rajiv, 2010). However, a major disadvantage of RAPD's and AFLP's is that they are dominant markers, therefore, heterozygotes and homozygotes for the null alleles cannot be differentiated.

Furthermore, results from RAPD's and AFLP's are often difficult to interpret or replicate (Liu & Cordes, 2004). Currently, SNP and microsatellite markers are the most widely used molecular markers in aquaculture genetics. Both markers are inherited as co-dominant markers and are ubiquitous throughout the genome. Single Nucleotide Polymorphisms refer to incidences of point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus. SNP markers have gained substantial popularity in molecular genetics as they are the most abundant polymorphism in any organism's genome, in both coding and non-coding regions, and tend to reveal hidden polymorphism not detected with other markers and methods (Liu & Cordes, 2004; Maqsood & Ahmad, 2017). Theoretically, a single point mutation can produce as many as four alleles, each containing one of the four bases at a given SNP site. However, most SNPs are usually restricted to one of two possible base pairs and are therefore, regarded as bi-allelic. Alternatively, due to their hyper-variability, microsatellites regularly show tens of alleles at a locus that differ from each other in the number of the repeats (Hoshino *et al.*, 2012). Additionally, microsatellite markers are considered to be selectively neutral, occurring in both coding regions (*e.g*. Expressed Sequence Tags, EST's) and non-coding regions (Li *et al.*, 2002). Therefore, the versatility of microsatellite markers has made them applicable to many facets of aquacultural genetics, where they are particularly suited for studying genetic multiplicity as well as parentage scrutiny.

In contrast to microsatellites, SNP's on the basis of traditional DNA sequencing, although considerably popular, have been rarely used in population genetic studies, mainly due to difficulties associated with their characterisation and genotyping in non-model organisms (Helyar *et al.*, 2011). In recent years, however, the use of SNP's have exponentially increased (Guichoux *et al.*, 2011) as a result of newly developed high throughput sequencing techniques (*e.g.* exome sequencing, RNA-sequencing, whole genome sequencing, RAD-sequencing, *etc*.) that can be applied to a wide range of organisms. These techniques allow for the identification of thousands to millions of SNP's, as well as the simultaneous estimation of SNP frequencies across the genomes of individuals, populations and species (Metzker, 2010; Davey *et al.*, 2011; Schlötterer *et al.*, 2014). However, although such technologies exist, the availability of sufficient funds, and thus DNA markers for specific aquatic species, is commonly related to the economic value or scientific importance of the target species (Hayes & Andersen,

2005). Therefore, the availability of SNPs for non-model species such as dusky kob is lacking and merits further research.

1.5 Application of molecular markers in aquaculture

The use of molecular markers has gained considerable momentum and has shown significant promise in various aquacultural applications .In addition, with the help of genetic markers, allelic variation in genetic elements can be linked to variation in traits of economic interest. Thus, providing DNA level information on the inheritance of such traits. Furthermore, microsatellite markers, in particular, have proven quite useful in aquaculture, and can provide valuable information on various population genetic parameters, by:

(i) Genetic identification and discrimination of hatchery stocks;
(ii) Monitoring inbreeding or other changes in the genetic variation;
(iii) Assignment of progeny to parents (pedigree inference);
(iv) Identifying quantitative trait loci (QTL), and the use of such markers in selection programmes (Magoulas *et al.*, 1998a, 1998b; Davis & Hetzel, 2000; Fjalestad *et al.*, 2003; Subasinghe *et al.*, 2003).

1.5.1 Assessing genetic diversity

Due to the highly ubiquitous and hyper-variable nature of microsatellites, microsatellite loci frequently display a high level of allelic variation, making them excellent markers for evaluating within-species genetic heterogeneity (Okumu & Çiftci, 2003). In general, microsatellite markers are justifiably considered as the ideal marker for a wide range of applications in fisheries and aquaculture, as they provide valuable information on population genetic structure, population bottlenecks, and effective population size (N_e) in a number of aquaculture species such as Atlantic Salmon (Houston *et al.*, 2012), catfish (Tan *et al.*, 1999; Yue *et al.*, 2003), tilapias (Palti *et al.*, 2001; Carleton *et al.*, 2002; Streelman & Kocher, 2002) and common carp (Tanck *et al.*, 2001). Furthermore, microsatellites have also demonstrated applicability in evaluating several other population genetic parameters such as gene flow, population differentiation and inbreeding (Archangi, 2008; André *et al.*, 2011; Senanan *et al.*, 2015; Li *et al.*, 2017).

In addition to being an effective tool in defining the geographical and spatial scales for population differentiation, microsatellites are also suitable for identifying the origins of individuals in mixed stocks of migratory fish (Okumu & Çiftci, 2003). Thus, making it possible to assess population demographic history, and to identify geographical centres of genetic diversity from which broodstock can be sourced. As previously mentioned, microsatellites are predominantly selectively neutral markers, thus making it possible to assess levels of neutral genetic diversity amongst potential broodstock candidates. This offers a major advantage in the establishment of a genetically diverse base population, as genetic relatedness can be significantly lowered (Sekino *et al.*, 2004; Sriphairoj *et al.*, 2007; Loughnan *et al.*, 2015). On the contrary, increased genetic variation during the initial phases of selective breeding presents an opportunity to preserve genetic variants that could allow for adaption to changing environments (*e.g*. rearing conditions, diseases), and could provide unique variants for traits of current and future interest (Elliott, 2000; Hayes *et al.*, 2006).

Microsatellite markers can also be used to evaluate differences in estimates of general genetic diversity (*via* changes in the relative number and frequency of alleles) between broodstock and succeeding cultured generations, and potentially reveal processes leading to such observed differences. In fact, commonly employed breeding practices such as mass spawning, size grading, culling and differential breeding schemes, in addition to commercial rearing of different families, have negatively impacted levels of genetic variability as well as reduced the effective population size (N_e) in many aquaculture species (Sekino *et al.*, 2003; Brown *et al.*, 2005; Lind *et al.*, 2009). High fecundity, differential broodstock contributions, low survival rates and cannibalism are typical factors that can potentially result in the reduction in the genetic variance of F1-generation cultured populations, as have been reported for a number of fish species including brown trout (Hansen, 2002; Was & Wenne, 2002; Aho *et al.*, 2006), Japanese flounder (Sekino *et al.*, 2003, 2004; Dou *et al.*, 2004), barramundi (Qin *et al.*, 2004; Loughnan *et al.*, 2013; Domingos *et al.*, 2014) and also dusky kob (Mirimin *et al.*, 2016). Recent studies have shown that cultured F1 populations suffered significant losses in all aspects of genetic diversity when using captive broodstock that only represents a snippet of the genetic variability found in the wild (Mirimin *et al.*, 2016; Jenkins, 2018). Subsequently, broodstock chosen from these cohorts are more likely to be related, where mating within such a population would increase incidences of inbreeding and random genetic drift, and potentially lead to inbreeding depression. Nevertheless,

incidences of inbreeding and loss of genetic variation over successive generations are almost unavoidable when establishing a uniform breeding population. Therefore, whether the production goal is for commercial or restocking purposes, it is imperative to not only capture, but also to maintain, as much of the naturally occurring variation as possible within cultured populations.

Although microsatellite markers have proven immensely useful in population genetic studies, and are often assumed to reflect the genome-wide diversity of taxons (Selkoe & Toonen, 2006), challenges regarding the accurate interpretation of microsatellite data are often misjudged (Putman & Carbone, 2014). In fact, the efficiency of microsatellite markers can be substantially reduced if high number of null alleles (Yu *et al.*, 2008), and low heterozygosity (Tokarska *et al.*, 2009) which may arise from inbreeding, persist throughout the population. Elevated levels of inbreeding is not uncommon in aquacultural breeding programmes (Blonk *et al.*, 2009; Doyle, 2016; de los Ríos-Pérez *et al.*, 2017), and in general, is expected to increase over generations in captive aquaculture stock. Furthermore, whether or not a limited number of microsatellites markers accurately reflect the extent of genome-wide diversity and/or inbreeding in a population remains an ongoing issue. Furthermore, genotype accuracy may vary between laboratories, and especially between microsatellite markers (Ellis *et al.*, 2011) as scoring of such variable markers are not very reproducible, especially for some loci with multiple peaks and complex patterns.

Compared to the hypervariability of microsatellites, the bi-allelic state of SNP markers severely limit their information content per locus. However, as previously mentioned, SNP's are more prevalent compared to other marker types (Liu & Cordes, 2004; Maqsood & Ahmad, 2017) and therefore, the high number of SNP's generated through Next generation sequencing (NGS) techniques may offset the aforementioned problems. Additionally, the mutation rate of microsatellite markers, although influenced by numerous factors such as repeat type, repeat copy number and marker location (Ellegren, 2000), are several orders of magnitude higher and much more variable than for SNP's (Fischer *et al.*, 2017). Therefore, fewer microsatellites than SNP's are often required in population genetic studies (Väli *et al.*, 2008; Glover *et al.*, 2010; Premachandra *et al.*, 2019). As a result, microsatellite-based studies typically sample a constricted portion of the genome with unusually high mutation rates (Ellegren, 2004), which is further aggravated when only the most informative markers are retained for further study after the initial screening of a few individuals from a

population. Under such circumstances, estimates of genetic diversity and differentiation may suffer from ascertainment bias (Haasl & Payseur, 2011; Queirós *et al.*, 2015), and may not accurately reflect genome wide patterns or variation. Positive correlations between expected microsatellite heterozygosity and SNP diversity have been reported at the population level in salmon (Ryynänen *et al.*, 2007; Glover *et al.*, 2010; Ozerov *et al.*, 2013) and several farmed species (Väli *et al.*, 2008; Ciani *et al.*, 2013), however, most of these studies utilised only a limited number of SNP's (ranging from tens to a few thousand). The outcome of these comparative studies was strongly affected by the number of SNP's utilised. Studies in which a relatively low number of SNP's (< 300) were compared to microsatellites revealed that the latter had more resolving power to infer differences in genetic summary statistics (Väli *et al.*, 2008; Coates *et al.*, 2009; Ciani *et al.*, 2013; Defaveri *et al.*, 2013; Granevitze *et al.*, 2014), or found similar results when the number of SNPs were slightly increased (± 400 SNPs) (Miller *et al.*, 2014). On the contrary, studies that utilised larger numbers of SNPs (± 3 000), generated *via* genotyping arrays for SNP detection, found that SNP's performed better than microsatellites (Glover *et al.*, 2010; Gärke *et al.*, 2012; Ozerov *et al.*, 2013). However, the use of pre-selected marker screening methods such as arrays and SNP chips may cause ascertainment bias as a consequence of the over representation of common alleles, whilst rare alleles are undetected. Nevertheless, for non-model species such as dusky kob, SNP markers, and the use of such high throughput genotypic techniques are largely unavailable.

1.5.2 Inferring pedigree

Due to their multi-allelic nature, microsatellites have been proclaimed to be the most powerful type of markers to assess genetic parentage (O'Connell & Wright, 1997), which have already been isolated and characterised in several aquacultural species including salmon (O'Reilly *et al.*, 1996; Gilbey *et al.*, 2004;), Japanese flounder (Sekino *et al.*, 2003, 2004; Dou *et al.*, 2004), trout (Liljedahl & Gall, 1996; Su *et al.,* 1996a, 1996b, Hansen, 2002; Was & Wenne, 2002), tilapia (Bentzen *et al.*, 1991; Kocher *et al.*, 1998) and dusky kob (Archangi *et al.*, 2009; Mirimin *et al.*, 2013; Mirimin *et al.*, 2016; Jenkins, 2018). Determining pedigree relationships using microsatellite markers are tremendously useful in aquacultural operations, particularly under communally reared populations (Liu *et al.*, 2012; Vandeputte & Haffray, 2014; Jenkins, 2018). By evaluating progeny produced during a mass spawn, for the presence/absence of similar alleles, it is possible to identify successful breeding candidates and determine

their relative contributions to the next generation. Additionally, with the help of Maximum Likelihood (ML) methods, unknown parental genotypes can even be reconstructed. Furthermore, parentage assignment could reveal major differences in gender reproductive performance, which in turn would aid in the development of more effective breeding schemes, potentially enhancing the genetic variability of selective breeding programmes (Liu *et al.*, 2012; Vandeputte & Haffray, 2014; Bright *et al.*, 2016).

Differential juvenile rearing, until progeny reach suitable physical-tagging size, presented a major obstruction to the application of effective selective breeding programmes. Not only is differential family rearing costly and labour intensive, but it also restricts the number of families available for selection and can introduce environmental effects common to members of the same family (Doyle & Herbinger, 1994). Marker-assisted parentage assignment has subsequently solved this problem, as multiple families can be reared communally soon after hatching, and pedigree relation determined at any time through molecular means. Therefore, microsatellite markers can also aid in the selection of unrelated broodstock candidates in a two-step selection process called walk-back selection (Doyle & Herbinger, 1994). Typically, selection for replacement commercial broodstock occurs prior to harvest, where candidates are identified based on superior phenotypic performance. Although similar to mass selection, walk-back strategies only retained the best performing candidates for continued breeding if they bear no or little relation to an individual already selected. Therefore, it addition to improving both space and labour constraints, this strategy also minimises potential environmental effects on the expression of commercially valuable traits (Robinson *et al.*, 2010; Sonesson, 2007), as resulting progeny cohorts can be reared communally.

Additionally, progeny testing presents a feasible approach to assessing parental breeding values in aquaculture. Progeny testing is considered to be a more accurate method, compared to family or combined selection, for estimating parental breeding values given that a number of half-sib families can be tested under communal rearing settings (Gjedrem, 2000, 2005). Therefore, such a method of selection may be particularly suited for late maturing species such as dusky kob, where genetically unimproved broodstock with unknown phenotypic performance are repeatedly spawned, and where selective breeding programmes are being considered and/or implemented. Importantly, the numbers and sizes of the families produced from mass

spawning should also be evaluated throughout the production cycle, as progeny cohorts often represent potential future breeding candidates. Therefore, by evaluating communally reared progeny cohorts originating for captive stock, superior breeders can be identified and utilised to improve the trait of interest.

Utilising pedigree information inferred from marker-assisted parentage assignment, together with phenotypic measurements of production traits allow for the estimation of narrow-sense heritability (h^2, the phenotypic variation attributed to additive genetic factors). Estimation of heritability components, as well as genetic correlations between traits, allow for the prediction and subsequent assessment of future genetic gains and is, therefore, a prerequisite for the establishment of efficient selective breeding programmes. Moderate to high heritability estimates have already been reported for aquacultural growth traits, body weight and length, as well as strong genetic correlations between these traits in common carp (Vandeputte *et al.*, 2004), rainbow trout (Kause *et al.*, 2003), sea bass (Saillant *et al.*, 2006; Wang *et al.*, 2008), red drum (Saillant *et al.*, 2007) and barramundi (Domingos *et al.*, 2013). Furthermore, heritability estimates have also been estimated for other body traits, such as Fulton's conditioning factor (K), a trait derived from both body weight and -length and an indicator of the "well-being" of a fish (Kause *et al.*, 2003, 2007; Saillant *et al.*, 2007; Domingos *et al.*, 2013;), in addition to disease resistance (Domingos *et al.*, 2013), flesh colour (Norris & Cunningham, 2004) and body deformity related traits (Bardon *et al.*, 2009). Due to the communal rearing of multiple families, and the resulting absence of between-family environmental variance, heritability estimates obtained are often elevated and more accurate than observations under separate rearing conditions (Ninh *et al.*, 2011, 2013). Regardless of this, competition effects (such as cannibalism) on the expression of growth traits should not be disregarded (Vøllestad & Quinn, 2003; Muir, 2005;). Therefore, in order to avoid bias in estimates, large sample sizes are thus required, especially if the number of families is low and sizes are highly skewed.

Whilst the high polymorphic information content (PIC) and fast mutation rate of microsatellites make them ideal for resolving fine-scale population structure (Putman & Carbone, 2014), these markers may not be as effective for inferring genome-wide or individual-level patterns in genetic diversity (Väli *et al.*, 2008). Furthermore, if incidences of inbreeding persist, microsatellite markers are likely to become less efficient in identifying relatives, particularly in populations with prior population bottlenecks and lack of gene flow, which limits allelic diversity. Resultantly, given that

SNP markers are ubiquitous throughout the genome, employing SNP's to develop pedigrees have become more popular (Clarke *et al.*, 2014; Harney *et al.*, 2018). Although SNP's are also subject to the same concerns as microsatellites, specifically difficulties with null alleles, low heterozygosity and ability to form pedigrees from inbred stock, the utilisation of SNP's appear to offer several advantages. Firstly, as previously mentioned, employing NGS techniques such as restricted-site associated DNA sequencing (RAD-seq) and partial genome sequencing, thousands of loci can be screened for variants at moderate cost (assuming the technological infrastructure is already in place) (Nguyen *et al.*, 2018). Secondly, SNP's can be used to form standard SNP chips with thousands of SNP's, allowing for fully automated high throughput and standardised genotyping platforms (Ha *et al.*, 2014). Consequently, SNP-based pedigrees have been reported for numerous farmed species, specifically cattle (Gorbach *et al.*, 2010; Fernández *et al.*, 2013; Panetto *et al.*, 2017; Strucken *et al.*, 2017) and sheep (Clarke *et al.*, 2014; Heaton *et al.*, 2014), whilst in aquaculture, SNP-based pedigrees are rare and have only been reported for the major cultured fish species of salmon (Hauser *et al.*, 2011; Holman *et al.*, 2017), rainbow trout (Abadía-Cardoso *et al.*, 2013; Liu *et al.*, 2016) and yellowtail (Premachandra *et al.*, 2019).

1.5.3 Quantitative trait loci mapping, marker associations and marker-assisted selection

A major application of molecular markers technologies lies in the construction of genomic linkage maps by mapping polymorphic DNA markers (*e.g*. SNP's, microsatellites and mtDNA markers *etc*.) to chromosomal regions based on their segregation relationship. More importantly, because complex traits of economic importance are often attributed to small additive effects of many genes, linkage maps can be used in combination with breeding programmes to identify such genetic elements, known as Quantitative Trait Loci (QTL). Construction of linkage maps generally requires large numbers of variable markers across multiple families, thus ubiquitous hypervariable makers (such as SNP's and microsatellites) are ideal (Gilbey *et al.*, 2004; Liu & Cordes, 2004; McCouch *et al.*, 2010; Yue, 2014). To date, linkage maps have been developed for many aquaculture species such as salmonids (McClelland & Naish, 2008; Moen *et al.*, 2008; Rexroad *et al.*, 2008), tilapia (McConnell *et al.*, 2000; Lee *et al.*, 2005) and common carp (Sun & Liang, 2004) and have facilitated the improvement of many commercially important traits. In fact, the construction of linkage maps and subsequent QTL mapping in model species have

significantly accelerated genetic improvements *via* marker-assisted selection (MAS) (Davis & DeNise, 1998), and continues to demonstrate elevated selection responses of complex traits that are difficult to select for through classical methods of selection (Davis & Hetzel, 2000). However, the construction of high-density linkage maps and subsequent QTL analysis is a laborious time-consuming process and frequently a long-term objective. Moreover, the large number of molecular markers required presents a limitation to the application of such technologies in non-model species. Therefore, the assembly of linkage maps, QTL analysis and subsequent implementation of selective breeding programmes are delayed for many emerging aquacultural species, as such funds and prerequisites to utilise such technologies are largely unavailable.

Alternatively, employing a candidate gene approach presents a promising alternative to the discovery of genetic elements (*e.g.* SNP's) that may have significant influences on economically important traits. As such, SNP's have been extensively used to screen candidate genes for growth related traits in several aquatic species (Tao & Boulding, 2003; Glenn *et al.*, 2005; Xu *et al.*, 2006). Candidate genes generally refer to genes with known biological function that are either directly or indirectly involved in the developmental processes of the traits of interest. Therefore, candidate gene linked markers, unlike the discovery of QTL's, are more precisely targeted and can detect variation in genes that relate directly or indirectly to the trait of interest (Lynch & Walsh, 1998; Zhu & Zhao, 2007). Furthermore, a statistical association between specific molecular haplotypes (or alleles) of a candidate gene and the trait of interest is taken as evidence that the gene is either directly involved in the genetic control of the trait, or that the functional polymorphism is sufficiently close to the marker so that the two loci are in linkage disequilibrium (Lynch & Walsh, 1998). Therefore, the identification of such molecular variants, and subsequent exploitation in breeding programmes *via* MAS, could prove very useful for achieving genetic improvement of commercially values traits. Furthermore, MAS also presents a major advantage to the selection of potential broodstock candidates in established breeding programmes, as high performing candidates can be identified, and selected during early stages of rearing, with little to no harm to the animal itself (Yue, 2014). As a result, rates of genetic gain are expected to be much higher for traits for which breeding value predictions rely solely on the measurement of relatives (as is the case with family-selection). In consequence, MAS repeatedly demonstrate accelerated genetic improvements, as productivity gains have often increased in earlier generations compared to classical

selection alone (Davis & DeNise, 1998). Additionally, MAS provide a unique opportunity to select for traits that are often difficult or expensive to measure (such as feed conversion), can only be measured in a particular sex (such as fecundity), measurable after sexual maturity (such as reproductive traits) and traits where the sacrifice of the fish is required (such as flesh quality) (Davis & Hetzel, 2000). In addition, MAS could prove particularly useful for improving growth rate in fish with extremely long generation intervals, as measurable selection responses may be delayed, and those that are currently produced from unimproved (wild-caught) broodstock, as superior breeders can be readily identified.

1.6 Study rationale, aims and objectives

1.6.1 Problem statement and rationale

Dusky kob is an estuarine dependent sciaenid finfish indigenous to South Africa, naturally occurring in estuaries and surrounding wetlands along the eastern and southeaster coastline. Estuaries and adjacent natural habitats of dusky kob are, however, subjected to increased fishing pressures due to recreational and subsistence fisherman where dusky kob is considered a major target. Furthermore, due to the profitability and relatively high catch estimates of dusky kob compared to other recreational angling species, catch harvests of this species has supported a lucrative fisheries sector for decades. This, in combination with unsustainable harvesting and poor management legislations, has subsequently resulted in the collapse of natural populations. Therefore, the overfished status of dusky kob, together with intensifying pressure to meet the growing market demand for the species has promoted a shift from harvesting wild stock, toward aquaculture production. Consequently, the farming of commercial dusky kob has since been established, where focus is slowly shifting toward the implementation of selective breeding programmes to aid in the potential improvement of commercially valued traits such as growth rate. However, current production practices for dusky kob rely on mass spawning of captive wild-caught broodstock, which impedes on the acquisition of pedigree information needed to record individual family relations and phenotypic performance for the subsequent calculation of genetic parameters.

Microsatellite markers have proven to be an effective tool for evaluating differences in performance between hatchery- and wild stock, assigning parentage to cultured progeny cohorts, and monitoring fluctuations in genetic variation between generations under aquacultural conditions. As a result, several authors have subsequently developed microsatellite loci for dusky kob to aid population genetic data and parentage studies (Archangi *et al.*, 2009; Mirimin *et al.*, 2013; Barnes *et al.*, 2014). Mirimin *et al.* only successfully assessed parental contributions to first-generation (F_1) cultured individuals of dusky kob produced via mass spawning and also compared estimates of genetic diversity of the cultured cohort to that of the wild progenitors (Mirimin *et al.*, 2016). However, this study was limited in terms of the F1 cohort investigated (n = 50) that was produced from a breeding population with variable, albeit unknown sizes and sex ratios. Similarly, in addition to assessing genetic diversity, Jenkins (2018) effectively determined parental contributions in multiple progeny cohorts (half- and full-siblings) as well as estimate broodstock participation under mass spawning (Jenkins, 2018). Consequently, it was observed that although eight females and four males were present, not all broodstock participated in the spawn (seven out of twelve) and that the relative contributions of the broodstock were severely skewed, with one full-sib family dominating the progeny cohort. For this reason, heritability estimates for growth traits (weight and length) were observed to be imprecise. Therefore, some aspects relating to broodstock sex ratio in breeding schemes, and the effectiveness of standard hormonal induction procedures on dusky kob still remain unclear, and warrants further investigation across multiple spawning events. Generally, moderately high numbers of breeders are used to conduct mass spawns in aquacultural environments, therefore, it is unclear how the relative contributions of breeders to progeny would be affected when employing alternative mating designs (*e.g.* smaller breeding groups). Furthermore, the extent to which such breeding schemes would affect levels of genetic diversity and family compositions in the resulting progeny merits investigation, as this would undoubtedly influence the initial compositions and phenotypic performance of families that will be available for the estimation of heritability components, which is a prerequisite for the establishment subsequent selective breeding. Additionally, for dusky kob, traditional phenotypic-based selection has been utilised to achieve genetic improvement of commercially values traits such as increased growth, whilst the molecular mechanisms underlying the expression of such complex traits remain unaddressed. Investigation into such molecular elements by using a candidate gene approach may prove vastly informative.

Therefore, not only can this approach provide new insight into the genetic basis of growth related traits, but the identification of molecular variants associated with such traits could also prove immensely useful in achieving accelerated genetic improvements *via* marker-assisted selection (MAS).

1.6.2 Aims and objectives

The aim of this study is thus to genetically and phenotypically characterise commercial populations of dusky kob within the context of implementing a selective breeding programme. A population genetic analysis, coupled with DNA based parentage analysis, was performed (in *Chapter 2*) to assess the genetic diversity and family structure of two mass spawned cohorts of dusky kob. Furthermore, the phenotypic (growth) performance of an additional four mass spawned cohorts was evaluated to exploit the potential for selection for growth rate and body shape (*i.e*. Fulton's K-index). Modern quantitative genetic theory (*via* linear mixed modes and Restricted Maximum Likelihood) was employed to estimate phenotypic and genetic parameters (*i.e*. heritability and genetic correlations) of dusky kob growth. Additionally, through a candidate gene approach, the population prevalence and frequency of genetic polymorphisms (SNP's) in genes associated with growth in aquacultural species will be assessed (in *Chapter 3*). The obtained results will subsequently be interpreted and discussed in terms of broad managerial recommendations related to the development of genetic improvement strategies for the South African dusky kob (in *Chapter 4*).

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CHAPTER 2

Assessing broodstock contributions, genetic diversity and phenotypic performance in a commercial population of mass spawning dusky kob, *Argyrosomus japonicus*

Abstract

Argyrosomus japonicus (dusky kob) is a large sciaenid finfish indigenous to South Africa, and as an emerging aquaculture species, current production practices rely on mass spawning of wild progenitors to produce F_1 - offspring for commercial trade. Although this technique allows for increased production outputs in the short term, mass spawning under aquacultural conditions complicates the maintenance of pedigree records, thus hindering the genetic improvement of the species due to the risk of inbreeding depression in subsequent generations. Considering recent efforts to retain F_1 - offspring, with desirable growth traits, as potential future broodstock, the absence of pedigree records in mass spawned cohorts threatens the sustainability of possible breeding programmes. Therefore, the present study aimed to evaluate the effect of such breeding practices on the genetic constitution of commercial dusky kob and also assess phenotypic performance (growth) between families, in an attempt to estimate genetic parameters for dusky kob growth traits [weight (g), length (mm) and Fulton's conditioning factor]. Using twelve microsatellite markers, the genetic diversity estimates of a wild population (n = 34) were compared to that of commercially used broodstock (n = 23) and derived offspring cohorts (n = 411). Parentage analysis of the produced offspring presented with only two full-sibling families, with significant differences in phenotypic performance. Overall, the commercial cohorts (broodstock and offspring cohorts) displayed reduced estimates of genetic diversity [allelic richness (A_r) and private allelic richness (PA_r)], effective population size (N_e) and presented significant differentiation from the wild population with a global F_{st} of 0.05 ($P < 0.001$). Furthermore, estimates of heritability (h^2) were 0.40 ± 0.25 and 0.39 ± 0.25 for juvenile weight and length, respectively, with a genetic correlation of 1.00 ± 0.01 (and phenotypic correlation of 0.997) between the traits, while estimates for the

morphometric ratio K (a phenotypic index combining traits weight and length) were not significantly larger than zero (0.001 ± 0.11) and displayed negative genetic correlations with weight (-0.73 ± 0.42) and length (-0.80 ± 0.31) respectively. In conclusion, although estimates of h^2 are likely inflated due to maternal and/or environmental effects and lack of a robust experimental design, results of the current study illustrate that selective breeding for increased growth rate has the potential to be successful in dusky kob. Findings also indicate that the expression of K may differ to a great extent temporally compared to the expression of juvenile weight and lengths, and may, therefore, not be a reliable predictor of fish body weight in juvenile dusky kob.

2.1) Introduction

Mass spawning is a common aquacultural practice generally employed for species that naturally spawn in groups (Domingos *et al.*, 2014; Chavanne *et al.*, 2016). *Argyrosomus japonicus* (dusky kob), is an emerging aquaculture species in South Africa, where current production practices rely on mass spawning of wild broodstock to produce F_1 - offspring for commercial trade. Economically, this practice is advantageous and potentially lucrative, as dusky kob is a highly fecund broadcast spawning species, which would allow for increased production outputs in the short term. However, whilst this mass spawning is seemingly an advantage for production purposes, it can introduce potential problems for the implementation of selective breeding programmes.

Broodstock diversity tends to represent only a comparatively small proportion of the total genetic variability when compared to their wild progenitor populations. Spawning within such broodstock populations creates a genetic bottleneck, which significantly contributes to the loss of unique or rare alleles (Loughnan *et al.*, 2013). Although higher numbers of unrelated broodstock may reduce such genetic losses (Bentsen & Olesen, 2002), the founder effect, which limits genetic variability in the breeding pool, increases the probability of genetic deterioration in later generations through inbreeding and random genetic drift (Aho *et al.*, 2006; Lind *et al.*, 2009; Lind *et al.*, 2012). Alternatively, maintaining detailed pedigree records is key to circumventing this problem. For many aquaculture species (Nagler *et al.*, 2000; Fessehaye *et al.*, 2006; Kan *et al.*, 2011), this can be achieved through individual family spawning in separate tanks, subsequently allowing for controlled mating schemes, broodstock contribution assessment, and differential family rearing. However, for commercial mass spawning species, such as the dusky kob, single-pair mating designs and separate rearing are challenging, due to mating behaviour of the species and infrastructural requirements. Thus, there is limited control over the relative contributions of the broodstock and the practice of communal rearing of offspring complicates pedigree record keeping. Consequently, in a typical mass spawn, it is quite common for broodstock to display skewed contributions to the offspring cohort (Gold *et al.*, 2010; Loughnan *et al.*, 2013; Domingos *et al.*, 2014; Rhody *et al.*, 2014; Jenkins, 2018). For this reason, disparities in family size and phenotypic performance amongst families are highly variable and frequently observed in aquacultural environments (Sekino *et al.*, 2003; Aho *et al.*, 2006;

Mirimin *et al.*, 2016; Jenkins, 2018). This threatens the sustainability of selective breeding programmes by hindering the selection for next generation broodstock; candidates may yield from a limited number of families, subsequently increasing the probability of excessive inbreeding. This is concerning considering its counterproductive consequences to the productivity of captive stocks and further emphasising the need for maintaining pedigree records to manage these effects.

In addition to this, due to the increased variation in juvenile size, differential family growth has been associated with the promotion of aggressive behaviour and cannibalism (Smith & Reay, 1991; Timmer & Magellan, 2011), which is frequently observed in communally reared offspring cohorts. Smaller, less developed offspring are commonly preyed upon by larger, more aggressively dominant individuals, further promoting juvenile size disparities, as such individuals receive an increased feed ration (Qin & Fast, 1996; Al-Hafedh & Ali, 2004; Pereira *et al.*, 2017;). Therefore, such individuals are frequently culled to promote more uniform variance in size between cohorts (Macbeth *et al.,* 2002). Furthermore, culling individuals with unfavourable phenotypes (smaller individuals) may unintentionally exclude unique (family specific) genetic variants associated with other traits of interest (*e.g.* disease resistance and/or fertility), before selection for new breeders is possible, and could inadvertently alter family representation even further. Thus, when selecting phenotypically superior offspring for broodstock replacement, selection is again constrained by a limited number of families and subsequently increasing the risk of inbreeding. Another juvenile grow-out practice, namely size grading, is commonly employed to minimise size disparities, reduce cannibalism and produce a more uniform cohort for stocking purposes (Gunnes, 1976; Barki *et al.*, 2000; Ahvenharju & Ruohonen, 2007). During grading, juveniles from different families are pooled and divided into independent size grades depending on body size. This promotes a more uniform growth rate within cohorts as well as significantly reducing the non-genetic (*i.e.* environmental) component of phenotypic variance among families (Sekino *et al.*, 2003; Fu, Shen *et al.*, 2013; Luo *et al.*, 2014), as they are reared in a common environment.

Selecting fish with economically desirable traits, such as increased growth rate, is of major interest to dusky kob aquaculture (De-Santis & Jerry, 2007; Datta *et al.*, 2013; Difford *et al.*, 2017). In addition to producing larger offspring, improving growth rate reduces operational costs associated with production efficiency, resulting in an increased turnover rate. In this regard, research efforts have previously been focussed

on the effect of water temperature (Bernatzeder & Britz, 2007; Collett *et al.*, 2008), salinity (Bernatzeder *et al.*, 2010), light intensity (Collett *et al.*, 2008) as well as dietary requirements on optimal juvenile rearing (Daniel, 2004; Woolley, 2009; Kaiser *et al.*, 2011; Adesola *et al.*, 2018;). However, research into the genetic improvement of commercial traits in this species is limited (Jenkins, 2018) compared to other finfish (Vandeputte *et al.*, 2004; Charo-Karisa *et al.*, 2006; Dupont-Nivet *et al.*, 2008; Gheyas *et al.*, 2009;), but remains an important and reasonable strategy as genetic improvement is both cumulative and permanent. Therefore, it is fitting to assess the genetic variances within and between populations, as these parameters enable the prediction of response to selection in progeny cohorts as well as prediction of relative breeding values of commercially used broodstock (Gjerde, 2005). Provided phenotypic measurements on individual growth performance and pedigree information for such individuals will also enable the estimation of the additive genetic (*i.e.* heritability) component underpinning the phenotypic variance for the desired trait(s). Another important trait to consider is Fulton's conditioning factor (K), a trait derived from both body weight and length. Fulton's conditioning factor reflects the body composition and shape of fish and could be of major interest in dusky kob aquaculture if body conformation has an impact on market acceptance. Furthermore, moderate to high estimates of heritability for K are frequently observed in several other aquacultural species (Nilsson, 1994; Kause *et al.*, 2003), not only illustrating that selection for increased weight would inadvertently lead to selection for K, but also suggests that similar or closely linked genes may be responsible for the expression of both traits. The application of molecular markers, such as microsatellites, are not only ideal for assessing the genetic diversity in captive stocks to maximise variation, they have also proven particularly useful in monitoring inbreeding levels (Loughnan *et al.*, 2013; Domingos *et al.*, 2014) where pedigree information was lacking. Furthermore, given sufficient data, microsatellite markers can also be used to reconstruct family pedigrees, evaluate parental contributions to fast- and slow-growing progenies and identify genetically superior breeders (Luo *et al.*, 2017), as well as allow for the estimation of genetic parameters (*e.g*. heritability and genetic correlations) of commercial traits.

Therefore, the aim of the present study was to evaluate the genetic properties of commercially produced dusky kob and compare such estimates to the progenitor population as well as wild individuals to evaluate the effect of breeding practices on the genetic constitution of this species in an aquacultural environment. Furthermore,

this study aimes to assess variance components as well as phenotypic performance between families, in an attempt to estimate genetic parameters for dusky kob growth.

2.2) Materials and methods

2.2.1) Study population and genotyping

A total of 23 broodstock (CB) was sampled at a pond culture facility, coded as Mtz, located in KwaZulu-Natal, South Africa. All broodstock were housed in smaller breeding groups of three to six individuals per tank rather than a single communal broodstock tank. Prior to sampling, which took place over a four day period, two spawning events (SPE's) were induced, each from a distinct breeding group. The first SPE (SPE1) included two wild males and one wild female, potentially spawning one half-sibling- and two full-sibling families. Similarly, the second SPE (SPE2), which took place two months after SPE1, also included two wild males, but also included two F_1 females, potentially spawning four full- and half-sibling families respectively. Even though only two SPE's were recorded at the time of sampling, all 23 broodstock individuals were included for analysis as these individuals represent the extent of genetic diversity at this facility. Furthermore, all progeny cohorts (CP, from SPE1 and SPE2) were combined and subsequently, graded into three size grades (small, medium and large), each consisting of two rearing pools each (as per standard farm practice), prior to sampling. Therefore, family relations were not taken into account during sampling. A set of 75 individuals were sampled from each of the small and medium grade rearing ponds, however, only 61 and 50 individuals were sampled from the large grade rearing ponds. Therefore, a total of 411 offspring of approximately four and six months of age were collected. Ethical approval for samples collection was obtained from the Research Ethics Committee: Animal Care and Use (Protocol #:6569) at Stellenbosch university. Additionally, as the farm produce dusky kob for commercial trade and many ponds were available allowing for relaxed stocking densities, culling of juveniles was not performed. A wild-caught broodstock database (WS, n=34) from a previous study (Mirimin *et al.*, 2013), that is representative of the wild progenitor population was also included for analysis.

Fin clip tissue from all broodstock and cultured individuals were preserved in 70% ethanol and stored at room temperature. Genomic DNA extractions was performed on each sample as a single extraction using a standard CTAB DNA extraction protocol

(Saghai-Maroof *et al.*, 1984). Following extraction, DNA quantity and quality was evaluated using a NanoDropTM ND 1 000 spectrophotometer (ThermoFisher Scientific) and normalised to a final working concentration of 20 – 30ng/µl. Multi-locus genotypic data was obtained by amplifying 17 microsatellite markers, previously developed and optimised for dusky kob (Archangi *et al.*, 2009; Mirimin *et al.*, 2013), in four multiplex reactions (Table 2.1). Genotyping accuracy was evaluated by amplifying markers *AJAP34* and *AJAP37* used in multiplex 3 and 2 respectively, which amplifies the same locus but differed in size by 23bp and thus providing an internal control.

Table 2.1: Seventeen microsatellite loci, grouped into four multiplex reactions, used to genotype the wild, commercial and cultured specimens. Each marker is reported along with the respective repeat type, size range, and fluorescent tag as well as the source citation of each marker.

Multiplex	Marker name	Repeat type	Dye	Size range (bp)	Source citation
I	*AJAP06*	$(GGAT)_n$	FAM	154 – 206	(Mirimin *et al.*, 2013)
	UBA05	$(CT)_n$	PET	117 – 170	(Archangi *et al.*, 2009)
	UBA06	$(CA)_n$	VIC	131 – 183	(Archangi *et al.*, 2009)
	UBA42	$(TGC)_n$	NED	112 – 200	(Archangi *et al.*, 2009)
II	*AJAP12*	$(ATCT)_n$	PET	103 – 188	(Mirimin *et al.*, 2013)
	AJAP14	$(ATCT)_n$	FAM	80 – 230	(Mirimin *et al.*, 2013)
	AJAP37	$(AGC)_n$	NED	130 – 248	(Mirimin *et al.*, 2013)
	UBA40	$(CA)_n$	VIC	133 – 199	(Archangi *et al.*, 2009)
	UBA851	$(AG)_n$	FAM	203 – 253	(Archangi *et al.*, 2009)
III	*AJAP05b*	$(AGAT)_n$	PET	110 – 189	(Mirimin *et al.*, 2013)
	AJAP34	$(CAG)_n$	VIC	167 – 245	(Mirimin *et al.*, 2013)
	UBA50	$(GT)_n$	FAM	120 – 260	(Archangi *et al.*, 2009)
	UBA854	$(TG)_n$	NED	120 – 260	(Archangi *et al.*, 2009)
IV	*AJAP24*	$(AGAT)_n$	PET	245 – 306	(Mirimin *et al.*, 2013)
	UBA44	$(GT)_n$	NED	158 – 208	(Archangi *et al.*, 2009)
	UBA853	$(GA)_n$	FAM	162 – 200	(Archangi *et al.*, 2009)
	UBA53	$(CA)_n$	VIC	187 - 227	(Archangi *et al.*, 2009)

Microsatellite amplification was performed with the KAPA2G™ Fast Multiplex PCR Kit in total reaction volumes of 10µl. Each reaction mixture contained 2X KAPA2G Fast Multiplex Mix, 0.2µM stock solution of each primer and 20 - 30ng of DNA. All multiplex

reactions were carried out using similar PCR cycling parameters with an initial denaturing step at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 20 seconds and a final extension step at 72°C for 7 minutes. Alternatively, amplification of broodstock DNA was performed at an annealing temperate of 58°C for all multiplexes. PCR amplification was verified using gel electrophoresis on a 1.5% agarose gel and fragment lengths estimated by capillary electrophoresis. Allele scoring was performed using GeneMapper v4.0 (Applied Biosystems).

2.2.2) Population genetic analysis pedigree reconstruction:

The software program Microsatellite Toolkit v3.1 (Minch *et al.*, 1996) was used to identify the presence of possible redundant samples (as an internal quality control check), calculate allele frequencies, polymorphic information content (PIC) and to convert the molecular data to appropriate input file formats for downstream analyses. Micro-checker v2.2.3 was used to evaluate the presence of potential genotypic errors, allelic dropout, allele stuttering and null alleles in the microsatellite data set (1 000 randomisations, Bonferroni-adjusted at 5% nominal level) (Van Oosterhout *et al.*, 2004). The presence of null alleles was assessed with the Brookfield 1 estimator (Brookfield, 1996). Marker neutrality was evaluated by using an F_{ST}-outlier test in Arlequin v3.5 (20 000 simulations, 100 dames simulated per group, min and max heterozygosity as 0 and 1 respectively; $P < 0.05$) (Excoffier *et al.*, 2005). Exact probability tests (under the Markov Chain method, 10 000 dememorisations, 500 batches and 5 000 iterations per batch) was performed in GenePop v3.4 (Rousset, 2008) to determine whether genotypic frequencies across loci were consistent with those expected under Hardy-Weinberg (HW) equilibrium expectations. General indices of genetic diversity including: number of alleles (A_n), effective number of alleles (A_e), observed and expected heterozygosities (H_o and H_e, respectively) and per locus F_{IS} were estimated for each cohort using GenAlEx v6.501 (Peakall & Smouse, 2012). Allelic richness (A_r) and private allelic richness (PA_r) were also calculated for each group using HP-Rare v1.1 (Kalinowski, 2005), implementing the rarefaction technique for a minimum of 40 alleles (20 diploid individuals) to standardise groups to a uniform sample size. Statistical differences in all the above-mentioned measures of genetic diversity were tested between cohorts by means of a Kruskal-Wallis test (KW; $P < 0.05$).

To evaluate population differentiation between the cultured progeny (CP), commercial broodstock (CB) and wild sample (WS) cohorts, pairwise F_{ST} values (1 000 permutations, significance: $P < 0.05$) as well as a locus-by-locus Analysis of Molecular Variance (AMOVA; 1 000 permutations, significance: $P < 0.05$) was performed in Arlequin v3.5.2.2 (Excoffier *et al.*, 2005; Excoffier & Lischer, 2010). Similarly, a principal coordinate analysis (PCoA; standardised variance mode) was performed to generate a visual representation of population distinctiveness. GenAlEx was also used to estimate mean relatedness (r) among the three cohorts, using the Queller and Goodnight estimator (999 permutations at a 95% confidence interval) (Queller & Goodnight, 1989). Effective population size (N_e) was calculated for all three cohorts respectively using the Linkage disequilibrium (LD) method (random mating, lowest allowed allele frequency: 0.05), in NeEstimator v2.01 (Do *et al*., 2014). To test for signatures of recently reduced N_e within each group, the Wilcoxon signed rank test (Cornuet & Luikart, 1996) for heterozygote excesses was used, assuming the infinite alleles model (IAM, applying 10 000 iterations at 5% nominal level) in Bottleneck v1.2.02 (Piry *et al.*, 1999).

Broodstock contribution to each cohort was evaluated through microsatellite-based parentage analysis: firstly, by genotypic exclusion using all available broodstock genotypes, in Vitassign v8-2.1 (genotyping error rate: 0 - 10%) (Vandeputte *et al.*, 2006) and, secondly, using a full-pedigree likelihood method in COLONY™ v2.0.5.0 (Jones & Wang, 2010). For the latter, allele frequencies and marker error rates were calculated for all the cultured individuals and their respective parents, implementing a genotypic error rate of 0.01 for each marker, and assuming polygamous mating systems in both sexes.

2.2.3) Phenotypic performance analysis

Estimation of genetic parameters for growth related traits

During sample collection, each individual was phenotyped for (wet) weight (W), standard body length (sL) and Fulton's conditioning factor (K), calculated as $K = 10^5 * (W/sL^3)$ where W is weight in grams and sL is length in millimetres. Additionally, due to differences in age between the spawning events, the mean growth rate for trait W, sL and K was also calculated by dividing each growth parameter (g, mm, or K) by the exact age in days post hatch (dph) for each individual sampled. Phenotypic and pedigree data from the present study was combined with that of Jenkins (2018), to

improve the accuracy of the analysis, where a similar methodology was used to evaluate growth performance. The additional dataset includes phenotypic and molecular marker data from four offspring cohorts collected from each of two additional aquaculture facilities, coded as Oc and PO, were included in downstream analysis. The first offspring cohort of the Oc facility (Oc_1) was sampled at one month (32 dph), five months (152 dph), and 24 months (796 dph) of age (from weaning to marketable size) and represents five families in total, whilst the second offspring cohort (Oc_2) was sampled before harvest at 393 dph and represents only one full-sibling family. Similarly, the first offspring cohort of PO (PO_1) was also collected pre-harvest (at 484 dph) whilst the second (PO_2) was collected at four months of age (121 dph), and represents one family- and three families respectively (Jenkins, 2018).

Phenotypic data was then assessed for normality and homogeneity of variances using a Shapiro-Wilk test and a Leven's test, respectively in XLStat v12.11.22 (threshold significance of 5%) (Carr, 2002). Additionally, where phenotypic data did not meet a normal distribution, log-transformation was required prior to the estimation of (co)variance components. Variance components (for additive genetic and residual effects) and standard errors for growth traits W and sL and the morphometric ratio K was estimated using Average Information Restricted Maximum Likelihood (AI-REML) (Jensen *et al.*, 1997), univariate as well as bivariate animal linear mixed models in DMU AI software v6.5.2 (Maia *et al.*, 2014) as:

$$y = Xb + Za + e \qquad (1)$$

where y is the vector of the observed phenotypes of each trait, X is the incidence matrix relating observations to the fixed effects (hatchery of origin, sampled tank and age of each sampled individual), *b* is the vector of fixed effects, Z is the incidence matrix relating observations to the additive genetic effect, *a* is the vector of the random additive genetic effects ~ $(0,A\sigma^2_a)$ where σ^2_a is the additive genetic variance and A is the pedigree derived numerator relationship matrix among the animals and e is the vector of residual effects ~ $(0, \sigma^2_e)$ where σ^2_e is the error variance. The narrow-sense heritability (h^2) for traits W, sL and K was, therefore, estimated as the ratio of the additive genetic variance to the total phenotypic variance (Falconer & Mackay, 1996; Milner*et al*., 2000): $\sigma^2_A / (\sigma^2_A + \sigma^2_e)$, where σ^2_A and σ^2_e are the variances attributed to additive and residual error effects respectively. Phenotypic- (r_p) and genetic correlations (r_g) between traits was also calculated. Bivariate models similar to

univariate models (1) were used to obtain covariance components for the traits: the genetic correlation between the traits were calculated as $r_g = \sigma_{A1A2} / (\sqrt{(\sigma^2_{A1})} \sqrt{(\sigma^2_{A2})})$, where σ_{A1A2} is the estimated additive genetic covariance component between the traits.

Additionally, the mean phenotypic performance of full-sibling families in cohorts Oc_1, Oc_2, PO_1 and Mtz, sampled across multiple tanks at the Oc, PO and Mtz hatcheries respectively, was evaluated as a measure of environmental (tank) effects by means of a Kruskal-Wallis test. Similarly, a Bayesian two-factor analysis of variance (ANOVA) performed in JASP (JASP & JASP Team, 2019) was used to evaluate mean growth rate of families simultaneously occurring across multiple tanks as a measure of gene (family) by tank (environment) interaction, where a Tukey's test was used for post hoc analysis when required.

2.3) Results

2.3.1) Marker genotyping

From a total of seventeen microsatellite markers, fifteen were successfully amplified and genotyped for 90% of all the individuals in the present study. Two of the markers (*UBA44* and *UBA53*) in multiplex IV were excluded from subsequent analyses as these markers had an amplification success rate of <85%. No redundant samples within the dataset was found and none of the markers displayed non-neutral behavior. However, genotypic errors due to null alleles were detected at markers: *UBA851* and *AJAP24*; which were subsequently excluded from downstream analyses. Genotypic consistency between *AJAP37* and *AJAP34* had an error rate of 0.12. Therefore, following the exclusion of the internal control (marker *AJAP34*), results based on 12 markers were reported. Additionally, out of the 411 offspring sampled, 43 individuals (10.5%) were excluded from further analyses for missing more than 15% (>2 out of 12 loci) of the data at the remaining markers. Thus, 368 individuals (89.5%) were retained in the dataset for further analysis.

2.3.2) Genetic diversity

All twelve microsatellite markers across all three cohorts were polymorphic, with high polymorphic information content (PIC>0.5) ranging from 0.52 (*AJAP37*) to 0.79 (*UBA853*) (Table A2.1). Global exact probability tests revealed that genotypic frequencies of the WS conformed to HW equilibrium, whereas those of the CP and the

CB presented significant deviation from HW expectations ($P < 0.01$). The mean number of alleles (A_n) across all loci were lowest in the cultured offspring (CP, 4.83 ±1.27), moderate in the commercial broodstock (CB, 6.17 ±0.94) and highest in the wild samples (WS, 9.50 ±2.50, Figure 2.1). This trend was consistently observed as the CP and CB cohorts displayed significantly reduced estimates in all measures of genetic diversity, with the exception of H_o (KW: $P < 0.05$), when compared to the WS. Allelic richness (A_r) ranged from 5.54 – 12.10 and 4.00 – 7.00 in the WS and CB respectively (Table A2.1), whilst A_r in the CP ranged from 3.00 - 6.95 and was observed to be significantly different between all cohort comparisons (KW: $P < 0.05$). Similar results were observed for measures of private allelic richness (PA_r) between all cohorts, with estimates ranging from 0.00 – 7.12, 0.01 – 1.88 and 0.00 – 0.11 in the WS, CB and CP cohorts respectively (Table A2.1, KW: $P < 0.05$).

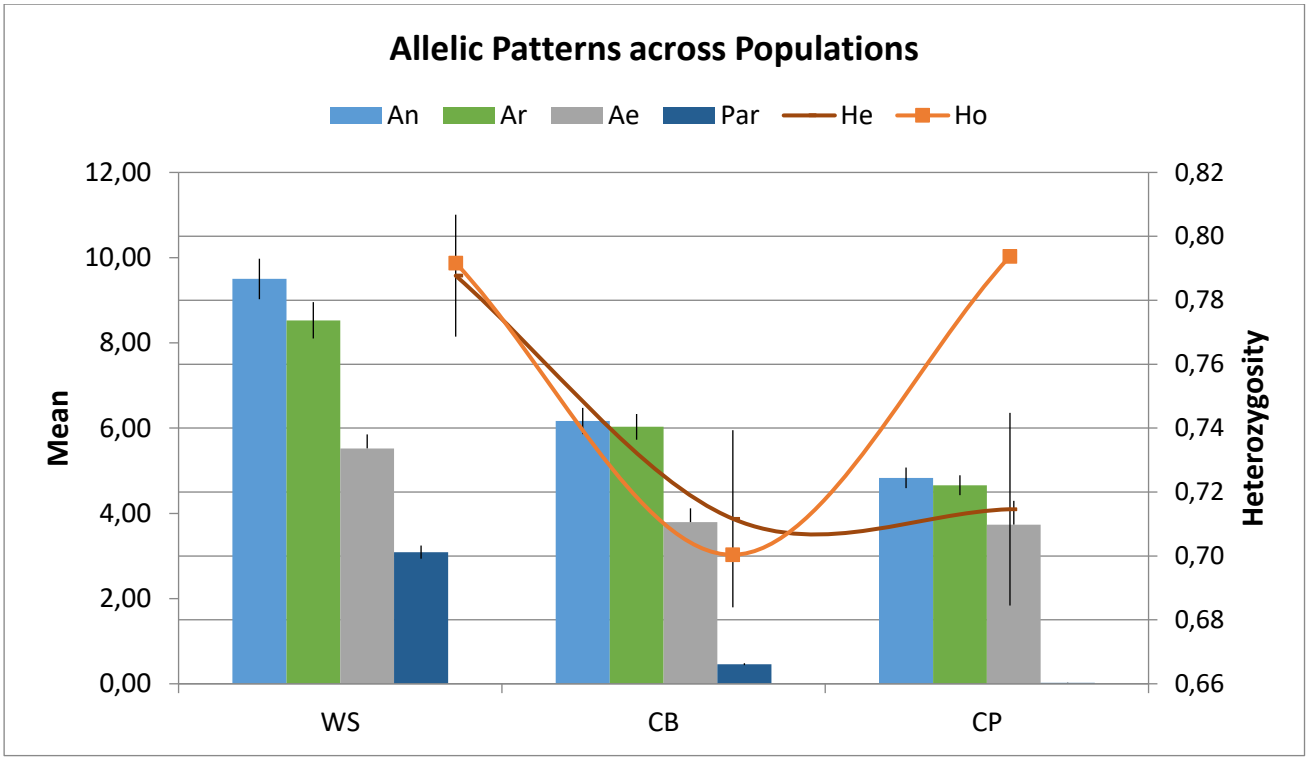

Figure 2.1: Summary of genetic diversity indices, for the wild samples (WS), commercial broodstock (CB) and cultured progeny (CP) cohorts, expressed as mean number of alleles (A_n), allelic richness (A_r), effective number of alleles (A_e), private allelic richness (PA_r), expected heterozygosity (H_e) and observed heterozygosity (H_o).

Pairwise F_{ST} estimates were significant ($P < 0.01$) among all cohort comparisons with the least amount of differentiation observed between the CB and CP ($F_{ST} = 0.031$), moderate differentiation between the CB and WS ($F_{ST} = 0.037$) and the most

differentiation being observed between the WS and CP (F_{ST} = 0.052, Table 2.2). This minimum differentiation between the CP and CB is also evident from the PCoA results (Figure 2.2), illustrating substantial overlap between these cohorts. Similarly, the PCoA also supports the significant deviation of the CP, which forms two clusters at opposite quadrants (blue clusters), from the WS (grey cluster), which mostly cluster in the first and second quadrants. Additionally, the CP cohort clusters (PCoA, Figure 2.2) are relatively distinct and can be ascribed to the low within population and high within individual genetic diversity that accounts for 0% and 96% respectively of the observed variation (AMOVA, Table 2.3). Furthermore, the AMOVA also supported significant genetic differentiation with a global F_{ST} of 0.05 and amongst population variation at 4%.

Table 2.2: Pairwise F_{ST} estimates (shaded) and corresponding Bonferroni corrected P-values (unshaded) for 12 microsatellite loci. Significant differentiation between all cohort comparisons are observed with the highest genetic distance observed between the wild and cultured cohorts.

Groups	**WS**	**CB**	**CP**
WS	-	*<0.001*	*<0.001*
CB	0.037	-	*<0.001*
CP	0.052	0.031	-

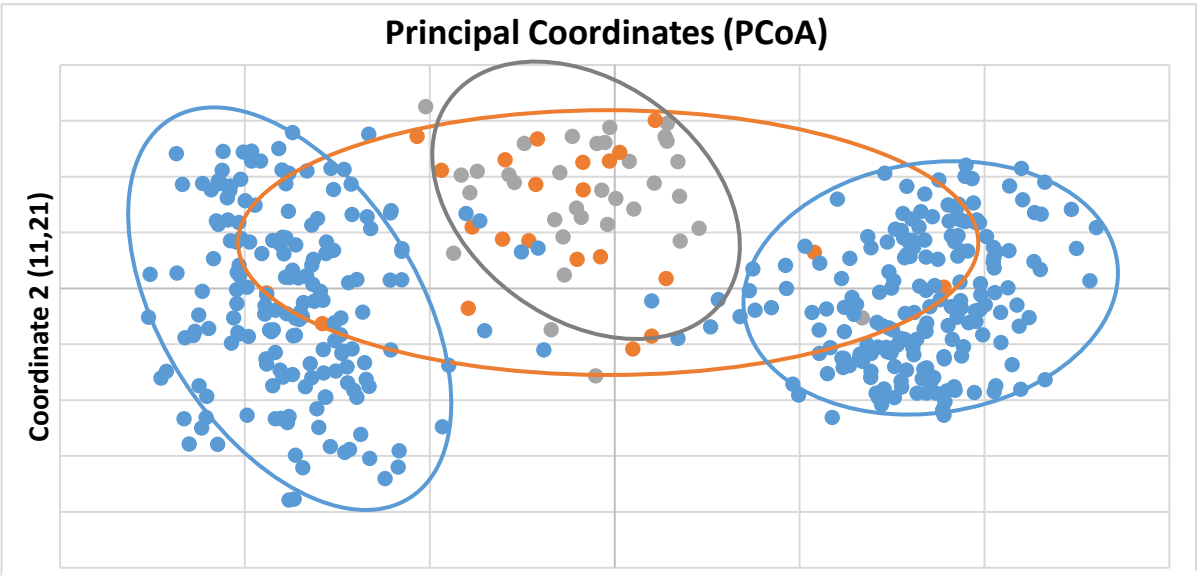

Figure 2.2: Principle coordinate analysis (PCoA) illustrating differentiation between sample groups. The cultured cohort forms two distinct clusters (blue) on opposite sides of the plot with the commercial (orange) and wild (grey) cohort groups mostly clustering in the first and second quadrants.

Table 2.3: Locus-by-locus AMOVA results depicting significant global differentiation between all cohorts. Within population variation is low with the majority of variation attributed to differences between individuals.

Source of variation	Sum of squares	Variance components	% of variation
Among populations	51.870	0.214	4%
Among individuals/ Within populations	1690.867	0.000	0%
Within individuals	1999.000	4.704	96%
Total	3741.738	4.918	-
F_{ST}:	0.047	0.001	
F_{IS}:	-0.080	1.000	
F_{IT}:	-0.029	0.989	

Mean relatedness (r) for the WS cohort was observed to be non-significant. However, for both the CB and CP cohorts the relatedness coefficient was significantly larger than zero, with the CB displaying the highest value (Figure 2.3). Additionally, mean relatedness for the CP cohort was observed to be significantly different from the other cohorts, as the observed value falls outside confidence intervals. Effective population size (N_e) estimates revealed a low N_e for the CP cohort (LD = 1.90), followed by CB (Table 2.4). Correspondingly, the Wilcoxon signed-rank test under the infinite allele model (IAM) patterns of mutation for recent reduced effective population size also indicated significant deviation from mutation-drift equilibrium within the CP, suggesting the occurrence of a genetic bottleneck (Table 2.4; $P < 0.05$).

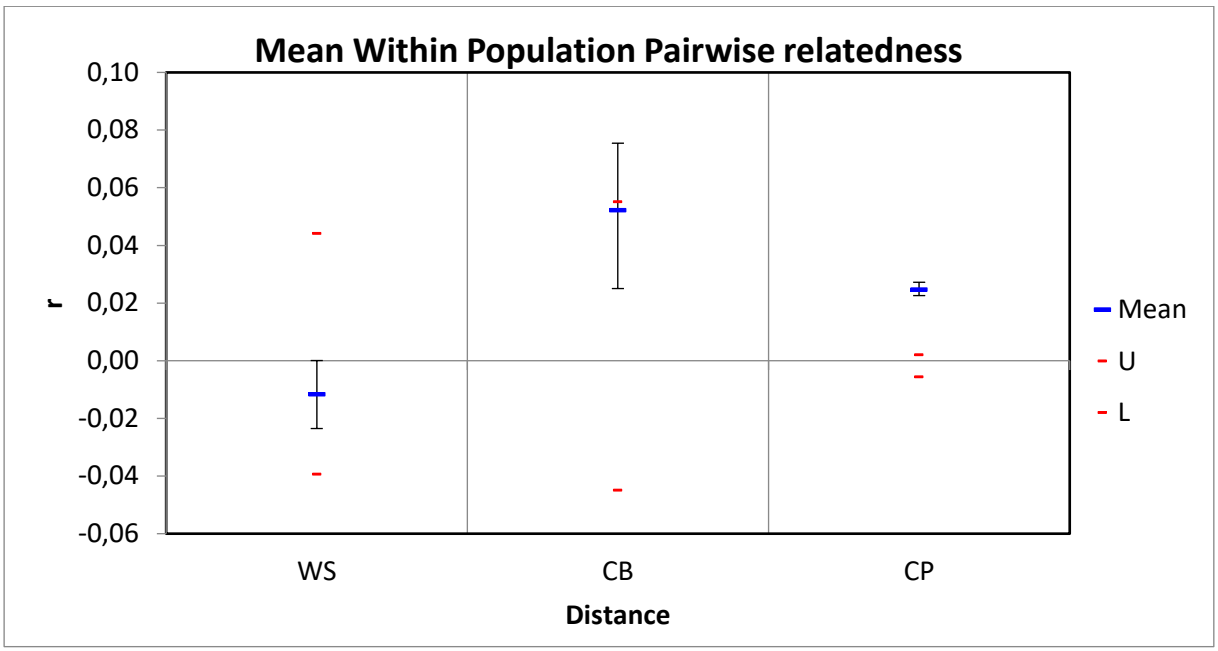

Figure 2.3: Mean within population pairwise relatedness (r) values among wild and commercial (progenitors and cultured offspring) based on the Queller and Goodnight estimator. Error bars indicating 95% confidence intervals about the respective means. Upper (U) and (L) bounds in red indicating 95% confidence levels for the null hypothesis of no difference between the cohorts.

Table 2.4: Bottleneck (Wilcoxon) test results under the infinite allele model (IAM) as well as estimates of effective population size (N_e) caluclated using the linkage disequilibrium (LD) method, where 95% confidence intervals (CIs) are included.

Parameters	**WS**	**CB**	**CP**
Sample size (n)	34	23	368
Wilcoxon test			
IAM	ns	ns	<0.01
N_e			
LD method	85.90	28.50	1.90
95% CIs	(61.00 – 137.80)	(19.40 – 47.50)	(1.70 – 2.10)

2.3.3) Parental contributions

Genotypic exclusion-based parentage assignment in Vitassign revealed that four parents successfully contributed to the CP cohort. These breeders were subsequently identified as a single mating pair in both SPE1 and SPE2 respectively. Therefore, only a single mating pair in each SPE successfully contributed to the progeny cohort, which consists of two full-sibling families spread across three size grades from six sampling pools (Figure 2.4). These results were subsequently confirmed in COLONY™ using a maximum likelihood approach, revealing only two full-sibling families of approximately equal size. From a total of 368 offspring successfully genotyped, 171 originated from SPE1 and 178 originated from SPE2. However, contrary to progeny assigned to SPE1, six individuals from SPE2 failed to assign to a candidate father, but accurately assigned to and clustered alongside the relevant female and other kin in the first and fourth quadrants (PCoA; Figure 2.2). Additionally, 19 individuals failed to assign to any SPE, although five and 14 individuals clustered with the SPE1 breeding pair, in quadrants two and three, and SPE2 family respectively (PCoA; Figure 2.2). However, these 25 individuals were subsequently excluded from further analyses, as they were lacking full pedigree information.

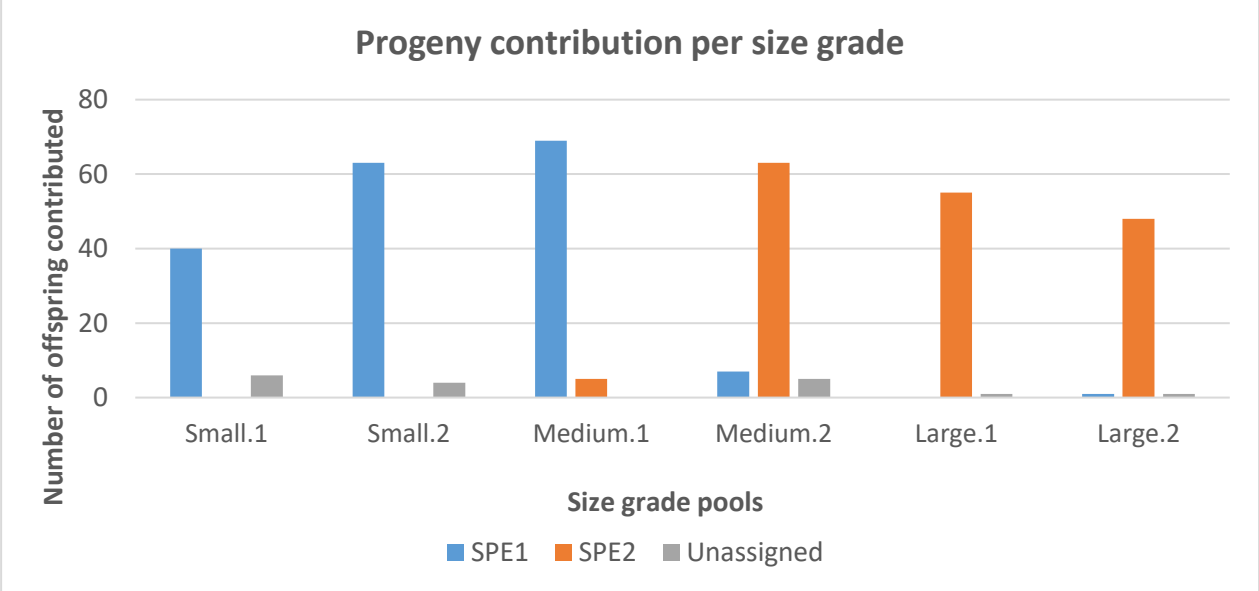

Figure 2.4: Number of offspring contributed to each size grade. The SPE2 full-sibling family (blue) occurring predominantly in the smaller grades and the SPE1 full-sibling family occurring in the larger grades.

2.3.4) Phenotypic data and genetic parameters of growth traits

Statistical descriptions, in term of growth rate, for each growth related trait [weight (W/dph), standard length (sL/dph) and conditioning factor (K/dph)] recorded at eight different ages from 32 dph (one month) to 796 dph (approximately two years) originating from three hatchery facilities is shown in Table 2.5. Additionally, all statistical descriptions are also displayed as phenotypic performance (Table A2.2) as well as growth rate (Table A2.3) per tank sampled at each respective hatchery. Phenotypic data obtained from Jenkins (2018) is represented across 14 tanks (tank 1 - 14) where tank one to six represents a single SPE from weaning to marketable size (Oc_1), whilst individuals from tanks seven to 10 represent an additional SPE at this particular hatchery samples at approximately one year of age. Contrary to the Oc hatchery, samples originating from the first SPE at the PO hatchery (PO_1) were collected from a non-graded, small grade and large tanks representing tanks 11, 12 and 13 respectively, whilst sampled originating from the second SPE (PO_2) were collected from a single tank (tank 14). Additionally, phenotypic data obtained in the present study is represented across six tanks (tank 15 – 20) representing the small (tank 15 and 16), medium (tank 17 and 18) and large (tank 19 and 20) grades respectively.

Mean growth rate for traits W, sL and K were not comparable between cohorts of similar ages across hatcheries (Table 2.5). Individuals sampled at 152 dph from Oc_1 experienced a 1.5X higher growth rate compared to individuals sampled from Mtz at 175 dph (SPE1; Table 2.5) whilst the PO_2 cohort, sampled at 121 dph, experienced a two-fold increase in growth rate compared to those sampled at Mtz at 112 dph (SPE2). Overall, individuals obtained from the PO hatchery displayed the highest growth rate followed by the Oc hatchery (Table 2.5). Furthermore, when comparing the two SPE's sampled in the present study, the SPE1 family experienced a 1.6X higher growth rate when compared to the SPE2 family. Nevertheless, individuals sampled from PO_1 specifically experienced the highest growth rate whilst, overall, cohorts originating from this hatchery had lower estimates of K (Table 2.5). Similarly, within each respective hatchery, an inverse relationship between age (and thus increased size) and K is observed.

Table 2.5: Mean and median growth rate, associated standard deviation (s.d.) and variances for dusky kob growth traits weight (W/dph), standard length (sL/dph) and Fulton's conditioning factor (K/dph) as well as the covariation and correlation between each respective trait. Each sampling cohort is indicated along with the hatchery of origin, the number of tanks sampled for each cohort, the age of the cohort (in days post hatch) as well as the number of individuals sampled.

				Traits											
				Weight (g/dph)				**sLength (mm/dph)**				**K/dph**			
Hatchery	***Cohorts***	***AGE***	***N***	***Median***	***Mean***	***s.d.***	***Variance***	***Median***	***Mean***	***s.d.***	***Variance***	***Median***	***Mean***	***s.d.***	***Variance***
Oc	Oc_1	32	103	0.011	0.016	0.013	0.000	0.794	0.866	0.222	0.049	0.062	0.0637	0.013	0.000
		152	100	0.273	0.277	0.084	0.007	0.901	0.916	0.104	0.011	0.010	0.0100	0.001	0.000
		796	100	0.616	0.633	0.212	0.045	0.408	0.410	0.052	0.003	0.002	0.0018	0.000	0.000
	Oc_2	393	98	0.719	0.768	0.333	0.111	0.700	0.684	0.099	0.010	0.004	0.0038	0.000	0.000
PO	PO_1	484	100	0.818	0.860	0.297	0.088	0.682	0.683	0.074	0.005	0.002	0.0023	0.000	0.000
	PO_2	121	102	0.165	0.192	0.085	0.007	0.983	0.986	0.142	0.020	0.011	0.0106	0.001	0.000
Mtz	Mtz	112	172	0.085	0.095	0.061	0.004	0.723	0.718	0.148	0.022	0.017	0.0167	0.002	0.000
		175	171	0.123	0.157	0.098	0.010	0.629	0.641	0.143	0.021	0.010	0.0101	0.001	0.000

Hatchery	***Cohorts***	***AGE***	***N***	***Weight / Length***		***Weight / K***		***sLength / K***	
				Covariance	***Correlation***	**Covariance**	***Correlation***	**Covariance**	***Correlation***
Oc	Oc_1	32	103	0.003	0.961	0.000	0.036	0.000	-0.143
		152	100	0.008	0.926	0.000	0.029	0.000	-0.308
		796	100	0.011	0.971	0.000	-0.310	0.000	-0.499
	Oc_2	393	98	0.032	0.964	0.000	0.038	0.000	-0.154
PO	PO_1	484	100	0.020	0.909	0.000	0.242	0.000	-0.149
	PO_2	121	102	0.012	0.964	0.000	0.224	0.000	0.020
Mtz	Mtz	112	172	0.008	0.942	0.000	-0.381	0.000	-0.536
		175	171	0.013	0.956	0.000	-0.359	0.000	-0.563

Mean phenotypic performance of a single full-sibling family in cohorts Oc_1 and Oc_2, each sampled from four tanks, revealed significant differences in growth between tanks (Table A2.2; KW: $P < 0.05$). Additionally, similar results were observed when comparing the phenotypic performance of both full-sibling families obtained from the Mtz hatchery, whilst individuals obtained from the PO hatchery, sampled from tanks 11, 12 and 13 respectively, revealed no significant differences on growth rate (KW: $P > 0.05$; Table A2.2). Furthermore, comparison of the mean growth rate of families co-occurring in only 3 tanks (tank 17, 18 and 20; Figure 2.4) sampled from Mtz revealed significant family (genetic) by tank (environment) interaction, where the model of best fit (Table 2.6; Tank + Family + Tank ⁕ Family) displays a model average (R^2) of 0.63 [95% CI (0.44 – 0.74)] in comparison to the hypothesis of no difference. Furthermore, subsequent post hoc test confirms significant tank and family effects (Table 2.7; $Log(BF_{10,U}) < 1$ indicates strong evidence) as the average growth rate of SPE1 is observably higher that SPE2 across all three tanks (Figure 2.5).

Table 2.6: Bayesian two-factor ANOVA results indicating the model of best fit, Tank + Family + Tank x Family, in comparison to the other models relating tank and family effects.

Model Comparison					
Models	**P(M)**	**P(M|data)**	**Log(BF $_M$)**	**Log(BF $_{10}$)**	**error %**
Tank + Family + Tank ⁕ Family	0.200	0.999	8.206	0.000	
Tank + Family	0.200	0.001	-5.433	-6.820	9.962
Tank	0.200	9.682e -14	-28.580	-29.965	9.889
Family	0.200	2.908e -54	-121.886	-123.271	9.889
Null model	0.200	8.421e -55	-123.125	-124.510	9.889

Table 2.7: Post hoc comparisons table depicting significant differences in phenotype performance of across tanks 17, 18 and 20 for both the SPE1 and SPE2 family.

Post Hoc Comparisons - Tank					
		Prior Odds	**Posterior Odds**	**Log(BF $_{10, U}$)**	**error %**
17	18	0.587	0.175	-1.210	1.050e -6
	20	0.587	9.903e +24	58.087	6.096e -28
18	20	0.587	1.823e +33	77.118	7.557e -38
Post Hoc Comparisons - Family					
		Prior Odds	**Posterior Odds**	**Log(BF $_{10, U}$)**	**error %**
SPE2	SPE1	1.000	3.454	1.239	1.216e -6

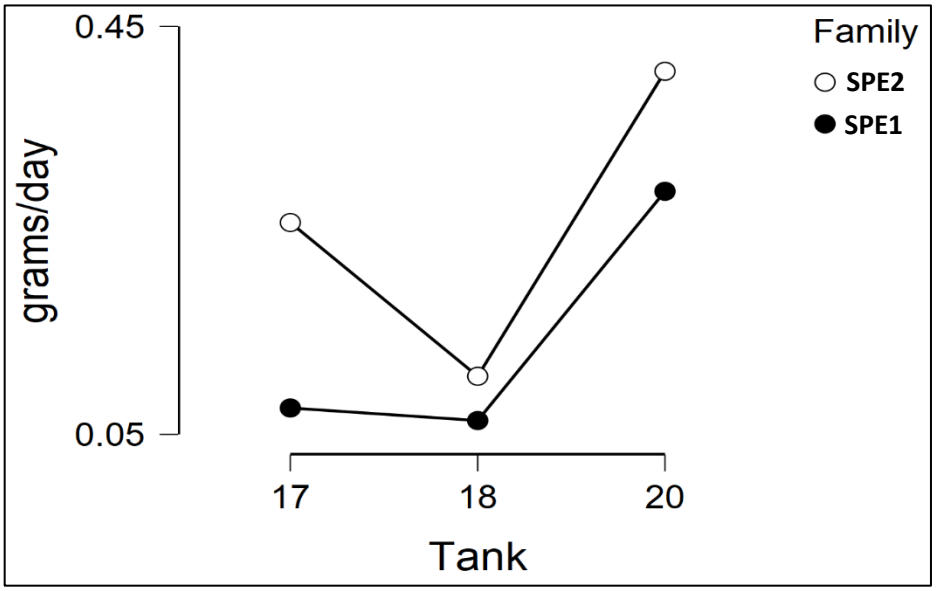

Figure 2.5: Mean growth rate of family SPE1 and Family SPE2 across three ponds.

Heritability estimates were calculated using both phenotypic performance (Table A2.2) and growth rate (Table A2.3), however, similar results were displayed. As expected, utilising data obtained in the present study alone yielded heritability estimates for dusky kob growth traits W, sL and K were 0.61 ±0.49, 0.62 ±0.40 and 0.05 ±2.62 respectively, under the bivariate model. However, under the univariate model estimates were notably larger for W (0.66 ±0.42) and smaller for sL (0.60 ±0.40) and K (0.03 ±0.16). Consequently, estimates for heritability for W, sL and K, following the incorporation of phenotypic and pedigree data obtained from Jenkins (Jenkins, 2018), were 0.40 ± 0.25, 0.39 ±0.25 and 0.001 ±0.11 utilising a bivariate approach (Table 2.8). Similar results were obtained using a univariate approach where, overall, estimates for W and sL were higher and more precise (standard error < h^2) than estimates obtained for the morphometric ratio K. Furthermore, heritability observed for W were, in general, higher than that observed for sL under both approaches with a genetic correlation of 1.00 ±0.01 between these traits. Removal of families with five and fewer assigned offspring resulted in higher estimate of heritability for each trait (Table 2.8, as indicated in brackets). Similarly, heritability estimates observed for W and sL following the removal of small families were similar under both approaches, whereas in contrast to W and sL, heritability for K were 0.24 ±0.28 and 0.10 ±0.12 as estimated using the univariate and bivariate models respectively. Contrary to the similar positive genetic correlation observed between W and sL before, and after, small families were removed (1.00 ±0.01 *vs* 0.99 ±0.01), genetic correlation between W and K, and sL and K were -0.73 ±0.42 and -0.80 ±0.31 respectively, after the removal of small families.

Table 2.8: Genetic parameters (heritability; h^2 and genetic correlation; r_g) ± standard errors and phenotypic correlations (r_p) for dusky kob growth traits, weight (W) and length (sL). Estimates in brackets represent heritability calculated after families with five or fewer offspring were removed from the dataset.

Analysis	Univariate	Bivariate
h^2 W	0.40 ± 0.25 (0.46 ±0.29)	0.40 ± 0.25 (0.46 ±0.29)
h^2 sL	0.39 ± 0.24 (0.41 ±0.27)	0.39 ±0.25 (0.41 ±0.27)
h^2 K	0.001 ± 0.10 (0.24 ±0.28)	0.001 ±0.11 (0.10 ±0.12)
Weight vs. Length		
r_g	1.00 ±0.01 (0.99 ±0.01)	
r_p	0.997	
Weight vs. Conditioning factor		
r_g	-0.94 ±80.93 (-0.73 ±0.42)	
r_p	0.636	
Length vs. Conditioning factor		
r_g	-0.97 ±87.48 (-0.80 ±0.31)	
r_p	0.691	

2.4) Discussion

The improvement of stock performance through cumulative genetic gains achieved through selective breeding is a major objective in finfish aquaculture (Gjedrem & Baranski, 2009), and has been achieved for many aquacultural species including salmonids (Chavanne *et al.*, 2016) and tilapias (Eknath & Hulata, 2009; Gjedrem, 2012; Zak *et al.*, 2014). Many fish species present favourable production life history characteristics (such as high fecundity and existence of broad phenotypic- and genetic variation) commonly exploited in aquacultural domestication. However, husbandry practices (*e.g.* induced spawning, establishment of a founder population and grow-out systems used) and species biology characteristics (*e.g.* group spawner behaviour and response to culturing environments) often present numerous challenges to the establishment of aquacultural breeding programmes. This is largely due to diminutive processes of genetic drift (*e.g.* founders effect) and non-random mating that are amplified in closed populations, such as one subject to a selective breeding

programme. Nevertheless, domestication generally results in the reduction of allelic variability, and could potentially stimulate genetic erosion in cultured populations (Doebley *et al.*, 2006). Conversely, subsequent losses in genetic variation over successive generations is almost unavoidable when establishing a uniform breeding population for a particular production trait. Therefore, whether the overarching goal of an aquacultural programme is to evaluate potential broodstock candidates prior to selection, or produce grow-out animals for commercial production, it is imperative that as much genetic diversity is represented within broodstock cohorts as possible. Furthermore, ample genetic diversity is a prerequisite of any selective breeding programme as this ensures long term sustainability and provides the foundation of continual genetic improvement (Gjerde & Villanueva, 2003; Gjedrem, 2012; Lind *et al.*, 2012).

In the current study, analyses based on general genetic diversity indices indicated that wild individuals of dusky kob harbour moderate levels of genetic diversity. These results are therefore in accordance with similar findings for other sciaenid species, such as red drum (Renshaw *et al.*, 2006; Saillant *et al.*, 2009) and European meagre (Haffray *et al.*, 2012), as well as previous reports for dusky kob (Mirimin *et al.*, 2016; Jenkins, 2018) indicating that individuals sampled from the wild harbour higher level of genetic diversity. Additionally, mean expected heterozygosity for the wild samples was significantly higher than that observed for the CB. This trend was continually observed as the CB cohort display significantly reduced estimates of genetic diversity, however, a significantly higher mean relatedness estimate in comparison to the WS. Although the WS and CB displayed significant overlap (PCoA, Figure 2.2), significant pairwise differentiation (Table 2.2) was detected between these two cohorts, and could explains the significantly reduced effective population size observed in the CB. Therefore, the use of such a curtailed broodstock population already places a major restriction on the genetic variation present at this hatchery. This severely limits future potential genetic improvements achievable, as advantageous genetic variation may already be restricted, even before the start of a potential selective breeding programme. Consequently, the reduced variability observed not only dampens future genetic gains, but together with the elevated relatedness observed between individuals increases the risk of inbreeding, and potentially exacerbate the effects of inbreeding depression. As a matter of fact, significant effects of inbreeding depression have been observed in many finfish species (Kincaid, 1983; Sugama, 2002) including Coho salmon (Gallardo

et al., 2004) and rainbow trout (Kincaid, 1976; Su *et al.*, 1996b), where fitness traits such as fertility, hatchability and number of eggs produced were significantly less over as little as five generations.

Mean expected heterozygosity for the CB was comparable to that observed for the CP, whilst estimates of mean observed heterozygosity deviated significantly between progeny (CP) and progenitors (CB, Figure 2.1). Although the WS and CB harbour elevated levels of genetic diversity compared to the CP, differences between expected and observed heterozygosity were much higher in the progeny cohort. An elevated estimate of observed heterozygosity is to be expected in cultured offspring cohorts following a recent bottleneck event, which is often the case in first-generations of culturing where undomesticated (genetically heterogeneous) broodstock are used to produce large numbers of progeny (Norris *et al.*, 1999; Evans *et al.*, 2004; Hillen *et al.*, 2017). Therefore loss of allelic diversity will occur much faster than loss of heterozygosity (Cornuet & Luikart, 1996; Luikart & Cornuet, 1998) given the fact that low frequency alleles, which have minor impact on heterozygosity estimates, are rapidly lost during first generation of culturing. This is undoubtedly the case within the CP cohort, as significant differences in A_r and PA_r, which constitutes a measure of the number of alleles independent of sample size, were observed between all cohort comparisons. Allelic richness is, therefore, more sensitive than heterozygosity for detecting reduced genetic diversity, which may not be a useful estimate for evaluating genetic variability in first generations of culturing (Hedgecock & Sly, 1990). Nevertheless, the establishment and utilisation of domesticated homogenous breeding populations in selective breeding programmes is expected to display reduced levels of heterozygosity as homogeneity increases (Waples, 1990; Jorde & Ryman, 1995; Blonk *et al.*, 2009). These reduced estimates, together with the occurrence of a recent genetic bottleneck, further supports the loss of genetic diversity, which is similar to estimates observed in other aquacultural species such as brown trout (Aho *et al.*, 2006), European sea bass (Hillen *et al.*, 2017), salmon (Norris *et al.*, 1999) as well as farmed dusky kob (Mirimin *et al.*, 2016; Jenkins, 2018).

The significant reduction in allelic diversity observed in the CP cohort is an expected result given the fact that the breeding population used for spawning represents only a portion of the progenitors analysed, and a fraction of the breeders present on the reported facility. Consequently, the utilisation of such small breeding groups could, therefore, explain the significant genotypic differentiation detected between the CB and

CP cohorts (Table 2.2) as well as significant global genetic differentiation that was detected (Table 2.3, Figure 2.2). This observation is likely due to genetic differences between breeders in the CB, as each resulting progeny cohort (from SPE1 and SPE2) cluster with their respective breeders in the PCoA. This observation further reflects the overall loss of genetic diversity whilst, at the same time, progeny cohorts become more homologues. Differentiation of cultured populations from their progenitors is a commonly observed phenomenon in aquaculture, and has been reported for several other farmed species including carp (Murakaeva *et al.*, 2003), Asian seabass (Senanan *et al.*, 2015) and salmon (Reilly *et al.*, 1999; Withler *et al.*, 2007). However, differentiation of cultured fish species from their wild progenitors raises numerous concerns for the sustainability of proximate wild populations. Escapement of farmed breeds into the wild, and subsequent interaction with native populations is a major concern for many aquacultural sectors. The biological collision resulting from cultured specimens breeding with wild species could not only disrupt the natural biodiversity of wild populations, but also result in subsequent progeny showing reduced fitness characteristics and/or increased predation or competition for natural resources, as has been reported for several aquaculture species including Atlantic salmon (McGinnity *et al.*, 2003; Naylor *et al.*, 2005; Roberge *et al.*, 2006) and European seabass (Toledo Guedes *et al.*, 2009; Leonor Ortega, 2015;). Additionally, given the fact that most marine organisms have high mobility and fecundity, escaped individuals have the potential not only to affect local populations but also spread to neighbouring populations, and potentially on a larger scale, permanently alter the dynamics of fish populations and irreversibly damage the survivability of a species (Naylor *et al.*, 2005).

Another important factor to consider, that can at least account for the levels of differentiation and loss of allelic diversity observed in the present study, is the low participation of broodstock within the respective spawning events. Theoretically, based on the consensus size of the breeding groups utilised by the hatchery, SPE1 and SPE2 could potentially produce two and four families respectively. However, parentage analysis revealed an effective number of breeders of two in both breeding groups (out of three and four respectively), as each breeding group produced only one full-sibling family. Variable broodstock participation is a common occurrence in aquaculture (Sekino *et al.*, 2004; Borrell *et al.*, 2011; Domingos *et al.*, 2014) and has been reported for many aquacultural species such as cod (Bekkevold *et al.*, 2002; Herlin *et al.*, 2008), gilthead and red seabream (Perez-Enriquez *et al.*, 1999; Nugrohoa & Taniguchi, 2004;

Brown *et al.*, 2005; Gold *et al.*, 2010). Generally, when designing mating schemes, the number of female broodstock selected are more than the number of males, as a single male, in practice, should be capable of courting and spawning with more than one female. However, although not utilised by this particular study, female biased mating strategies has reportedly resulted in highly variable female reproductive success in both red drum (Gold *et al.*, 2008; Gold *et al.*, 2010) and dusky kob (Jenkins, 2018). Furthermore, it was suggested that differential spawning chronology amongst females, in addition to other factors influence individual spawning, such as broodstock weight and age (Brown *et al.*, 2005), sexual selection and competition, may have attributed to the low participation rates among females, and could explain the reduced female participation within this study (SPE2 in particular). Additionally, the mass spawning behaviour of this species, together with the relatively small breeding groups utilised by the hatchery (SPE1, SPE2 and similar sized broodstock breeding groups) could have contributed to the observed variation in the participation of broodstock (males in particular), as critical numbers of fish is often required to stimulate natural spawning behaviour in many sciaenid species, similar to observations in gilthead seabream (Gorshkov *et al.*, 1997; Ueng *et al.,* 2007).

On the contrary, male biased mating strategies have seemingly experienced more spawning related adversities than that of females when it comes to successful spawning (Brown *et al.*, 2005; Herlin *et al.*, 2008). In fact, male competition among breeders is a major factor that influences male reproductive success, as has been observed in mass-spawning fish such as gilthead seabream (Brown *et al.*, 2005) and cod (Hutchings *et al.*, 1999; Nordeide & Folstad, 2000; Bekkevold *et al.*, 2002), where male participation has been identified as a common limitation to maximising N_e in marine mass-spawning species (Hedgecock, 1994). Although there were no available records regarding the spawning behaviour in the tanks where SPE1 and SPE2 took place, the lack of participation of the alternative male in SPE2 could be attributed to physiological factors, as this male was smaller in comparison to the dominant male (personal observations). However, such observations were not evident in the breeding group of SPE1. Nevertheless, it is possible that the alternative male in this breeding group did not respond appropriately or in a timely fashion when the female released her eggs, as there was only a single female in this group. Therefore, the reduced participation of broodstock (in both SPE1 and SPE2) as well as the lack of additionally induced SPE's at the time of sampling, majorly constrained the flow of genetic

information and further aided the genetic distinctiveness of the cultured group as there is a rapid increase in common alleles.

The heritability of dusky kob growth traits weight (W) and standard length (sL) were estimated to be 0.40 ±0.25 and 0.39 ±0.25 respectively. Therefore, in comparison to Jenkins (2018), who reported the first estimates of additive genetic variation of growth related traits in dusky kob (W = 0.35 ±0.25; sL = 0.36 ±0.27), the estimates observed in the present study were marginally more precise (slightly lower standard errors) and notably higher than previously observed for this species. Additionally, the moderate heritability estimates observed here are in similar ranges, *i.e.* 0.3 – 0.4, to prior estimates reported for other aquacultural species such as barramundi (Domingos *et al.*, 2013), common carp (Vandeputte *et al.*, 2004; Vandeputte *et al.*, 2008) and silver carp (Gheyas *et al.*, 2009), and higher than estimates observed for red drum (Saillant *et al.*, 2007), Atlantic cod (Gjerde *et al.*, 2004) and white bass (Fuller & Mcentire, 2011). Nevertheless, in contrast to the results observed in the current study, estimates of narrow-sense heritability reported for these earlier studies were evidently more precise (*i.e.* lower standard errors) due to a more robust experimental design (*i.e.* more families and more samples per family and standardised environments). The accuracy and/or bias of heritability estimation is known to be affected by numerous factors such as the number and size of families, the breeding design employed, family rearing approach, type of relatives utilised and the method of analysis applied (Falconer & Mackay, 1996). Therefore, the limited number of families presented in this study (13 families in total), in comparison to previously mentioned aquacultural finfish species (38 - 240 full-sibling families) (Vandeputte *et al.*, 2004; Saillant *et al.*, 2007; Gheyas *et al.*, 2009; Domingos *et al.*, 2013), is notably a limiting factor on the estimation of a more precise estimate. Furthermore, considering the small- and variable sized families reported by Jenkins (2018), variances in phenotypic performance between families are likely to be inflated, and may result in upwardly biased estimates of heritability. Since heritability is estimated as the proportion of the additive genetic variance to the total (environmental and genetic) variance, such components are likely to be less precise as differences between families would be exaggerated. Such occurrences are evident in this study as preliminary analyses (using two full sibling families) indicated estimates of 0.61 ±0.49 and 0.62 ±0.40 for W and sL respectively, which are considerably higher than estimates observed in other finfish species previously reported. Furthermore, similarly to Dominigos *et al.* (2013), families with small contributions (five or fewer assigned

offspring) were subsequently removed from analysis to increase statistical confidence of genetic parameters. However, exclusion of three families (one from Oc_1 and two from PO_2) presented slightly higher (W = 0.46 ±0.29, sL = 0.41 ±0.27), although less precise (larger standard errors) estimates of heritability. Nevertheless, considering the high heritability estimates obtained when utilising data obtained in the present study alone (based on two full-sibling families), and the more precise estimate obtained following the incorporation of data obtained from Jenkins (2018), estimation of a more precise result requires the utilisation of a greater number of larger families. Ultimately, with only 13 families included for analysis, and the imprecise estimates observed, the values obtained here may not be considered with confidence and should be viewed as preliminary.

Although the incorporation of data obtained from Jenkins (2018) yielded more precise estimates of heritability for dusky kob weight and length, the consequent heterogeneous nature of the sample population, obtained from two recirculation- (Oc and PO) and one pond culture facility (Mtz), potentially inflated variances associated with environmental factors likely to have biased estimates of heritability in the present study. In fact, evaluation of the mean phenotypic performance of full-sibling families within each respective hatchery presented evidence supporting the occurrence of significant environmental effects. With regards to samples obtained in the current study (Mtz), each full-sibling family displayed significant differences in phenotypic performance across five and four tanks respectively (Table 2.5). On the contrary, cohort PO_1 also represent a single full-sibling family size graded over three tanks, however, statistical differences in phenotypic performance between these tanks were notably non-significant. Additionally, significant differences in the mean growth rate of two full-sibling family each sampled across four tanks (Oc_1 at at 32 dph and Oc_2 at 393 dph) were also evident. With this in mind, individuals obtained from the PO hatchery displayed the highest growth rate followed by the Oc hatchery (Table 2.5), whilst individuals sampled from Mtz presented the lowest growth rate. Therefore, the reduced phenotypic performance of individuals representing the Mtz hatchery is not surprising considering that Mtz is a pond culture facility where family performance is likely influenced by external environmental conditions. Significant gene (family) by environment (tank) interactions were also evident in this facility, as the mean growth rate of both full-sibling families vary substantially across tanks (Figure 2.5), however, mean growth rate of the SPE1 family was consistently higher. Although these two

families were sampled at different ages (SPE1 at 175 dph and SPE2 at 112 dph), both families are still considered to be in their early juvenile growth phase. Previous studies on dusky kob suggest that growth in this species is linear until individuals reach sexual maturity, after which growth rate plateaus (Griffiths & Hecht, 1995; Griffiths, 1996; Silberschneider *et al.*, 2009; Ferguson *et al.*, 2014). Therefore, it can be argued that individuals sampled from the Mtz hatchery are experiencing a similar growth phase, and that the observable differences in phenotypic performance is likely attributed to genetic differences between families that influence the expression of growth related traits, as has been described by Gjedrem (2000). Nevertheless, the mean phenotypic performance of individuals obtained from this pond culture facility is observantly less in comparison to cohorts of similar ages sampled from the respective recirculation facilities where culturing environment are less variable. However, given the significant tank effects observed in cohorts obtained from the Oc hatchery, non-genetic factors influencing growth traits such as feeding frequency and intensity, type of feed provided, water oxygen levels and stress responses to aggressive behaviour within hatcheries may still persist. Jenkins (2018) reported that the communal rearing of five families (Oc_1) did translate into injuries, mostly amongst slower-growing juveniles. Additionally, the present study observed high genetic and phenotypic correlations between traits weight and length (Table 2.7). These high correlations indicate that, firstly, the genetic improvement of both traits could be achieved by selecting for length, which is practically easier to measure in the field. Secondly, given the fact that genetic correlations between traits reflect the extent to which similar genes are involved in the phenotypic expression of traits being compared (Falconer & Mackay, 1996), the same or closely linked genes are likely involved in the expression of both weight and length. Furthermore, the moderate heritability estimates observed in this study suggest a significant additive genetic component to growth rate in dusky kob, which clearly leaves room for selective breeding for growth, and could be used as a predictor of growth rate during early developmental stages and perhaps to market size.

Another important factor that could potentially explain the inexact heritability estimates observed in this study, is the conceivable occurrence of non-additive (genetic) effects (*e.g.* dominance or epistasis) and/or maternal effects, given that the animal model(s) implemented for estimating variance components assumes all genetic effects to be additive. In fact, maternal effects on offspring growth are more frequent during the early stages of juvenile development and had been reported for several other aquacultural

species such as rainbow trout, sea bass and salmon (Herbinger *et al.*, 1995; Garcia de Leon *et al.*, 1998; Haffray *et al.*, 2012), and tend to dissipate as fish grow older. Moreover, Gjedrem (1983) reported that, for many finfish species, heritability estimates of growth related traits executed on adult individuals are frequently higher, and more precise than in juveniles. Therefore, if this is the case in juvenile dusky kob, maternal effects may have introduced a level of bias in the estimation of heritability as several cohorts in the current study were obtained within one to six months post hatch. Contrary to maternal effects, reports regarding the extent of dominance and epistatic variances in cultured fish is largely unaddressed due to the complex mating designs required to evaluate these effects (Lynch & Walsh, 1998). However, significant dominance and/or epistatic effects on juvenile growth have been observed in several cultured species such as Japanese flounder (Tian *et al.*, 2011), Atlantic salmon (Rye & Mao, 1998) and Chinook salmon (Winkelman & Peterson, 1994), whilst such non-additive effects are reportedly absent and/or minor in juvenile common carp (Vandeputte *et al.*, 2004) and black bream (Winkelman & Peterson, 1994). Even though research efforts regarding the magnitude and significance of non-additive (genetic) effects on fish growth are inconsistent, and often considered to be negligible (Varona & Misztal, 1999), it is not feasible to rule out such effects on growth of dusky kob completely, as significant non-additive effects could potentially bias estimates of heritability (Gjerde, 1986). The presence of non-additive genetic and/or maternal effects were not accounted for in the present study, and investigation into the impact of such effects in dusky gob growth further merits further investigation.

Given the fact that Fulton's conditioning factor (K) is a trait derived from both weight and length, it stands to reason that genes influencing the expression of these two traits are likely to be similar to those prompting the expression of K. Furthermore, considering that estimates of K are directly proportional to W (as K = W/sL), it may be expected that selection for rapid growth, in particular body weight, will lead to indirect selection for K. However, this is not the case in the present study as the underlying genetic correlations between W and K was observably negative which is similar to previous observations in common carp (Vandeputte *et al.*, 2004) and contradictory to several aquacultural species (Nilsson, 1994; Fishback *et al.*, 2002; Martyniuk *et al.*, 2003). Similarly, a negative genetic correlation between length and K is also observed, suggesting that (given the high correlation of W and sL) selecting for increased length should produce heavier, but slimmer fish. Contrary to the moderately high estimates

of heritability for W and sL, estimates of K were not significantly different from zero (Table 2.7), which highlights the low additive genetic and high environmental effects responsible for the total phenotypic variation of this trait, and correspond with similar findings for Asian stocks of barramundi (Wang *et al.*, 2008) and rainbow trout (Gunnes & Gjedrem, 1981). Two major factors could potentially explain the low additive genetic variation attributed to body conformations in cultured dusky kob. Firstly, it has been observed that physiological factors such as lipid stores and protein accretion may alter fish body shape as a reflection of their nutritional intake (Koskela *et al.*, 1998; Kause *et al.*, 2002), and could, therefore, introduce a substantial environmental bias as feeding frequency and intensity may have differed across the farming hatcheries. Secondly, given the fact that growth rate in dusky kob, as in most fish species, plateaus as individuals reach maturity (Griffiths & Hecht, 1995), body mass at a given length can vary throughout an individual's lifetime and as such can produce variable estimates of K depending on the age of the sampled population as has been observed for populations of barramundi (Domingos *et al.*, 2013), red drum (Saillant *et al.*, 2007) as well as common carp (Vandeputte *et al.*, 2008). Therefore, differential growth phases, due to variable age, of the sample population in the present study presented a major limitation to the estimation of a reliable estimate of heritability of K, and could explain the imprecise estimate observed. Furthermore, the low heritability of this trait observed in this study, in addition to the negative genetic correlations observed, would also suggest that expression of K in dusky kob may differ to a greater extent temporally compared with W and sL genes (Falconer & Mackay, 1996), and may, therefore, not be a reliable predictor of fish body weight in juvenile dusky kob.

2.4) Conclusions

Overall, from this study four major conclusions can be drawn. Firstly, results gauging indices of genetic diversity demonstrated that the effects of mass spawning on levels of heterozygosity in cultured dusky kob was significantly less than the impact on allelic richness, as was expected due to the occurrence of a population bottleneck that was produced by the founder event. Secondly, attempts to maximise effective population size through the use of differential breeding schemes and hormonal induction of wild-caught broodstock still resulted in low participation. However, given that the hatchery only performed two successful spawning events at the time of sampling, sampling at a later stage (where additional spawning events may have occurred) would have been

of major benefit to the experimental design of this study. Nevertheless, if the performance of the breeding groups utilised in this study is an indication of performance of the other breeding groups present on the farm, related individuals are likely to be retained as potential broodstock for the next generation of breeders. Thirdly, owing to the lack of a more robust experimental design (*i.e.* few families and variable sizes) and the presence of significant environmental effects within two of the three sampled hatchery facilities (and the possible occurrence of non-additive genetic and/or maternal effects), estimates of h^2 for dusky kob W and sL displayed in general high standard errors. Additionally, environmental effect at the PO hatchery were observably non-significant, therefore, the utilisation of such a controlled rearing environment (*i.e.* recirculation facility) would most likely result in more accurate estimates of heritability for growth related traits. However, regardless of this, it is concluded that additive genetic effects play a significant role in the expression of both these traits. Lastly, given that growth rate in dusky kob plateaus as individual reach maturity, the heterogeneous nature of samples obtained across the three hatcheries may have resulted in inaccurate estimates for K, as the weight-length relationship at any given age is likely to differ. Regardless of this, the moderate heritability estimates observed for W and sL, as well as the negative genetic correlations observed between these traits and K, would suggest that the combination of genes influencing the phenotypic expression of W and sL may be different from those influencing the expression of K. Alternatively, the estimates observed for the above-mentioned traits could also indicate that the genetic interactions influencing the expression of K might differ from those influencing the expression of W and sL. Nevertheless, the moderately to high heritabilities observed in the current study represents the most accurate depiction of heritability of weight and length in dusky kob.

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CHAPTER 3

Association of genetic polymorphisms with growth traits in the dusky kob, identified *via* a candidate gene approach.

Abstract

The genetic improvement of economically valued traits is a major objective in the aquacultural industry. For dusky kob, classical phenotypic-based selective breeding programmes have been the main driver behind the genetic improvement of growth-related traits, whilst the molecular mechanisms underlying the expression of such complex traits are poorly studied. Single Nucleotide Polymorphisms (SNP's) are the most abundant source of heritable genetic variation in any organism's genome and has proven very effective when investigating trait-genotype associations. By employing a candidate gene approach and subsequent association analysis between SNP genotypes and the dichotomous response variable (large *vs* small) in the present study revealed two SNP's, one in the Ladybird homeobox 1 b gene [c.114T > A (*lbx1b*_c.114)] and one in the Tankyrase a gene [c.69T > C (*tnksa*_c.69)], that display statistical association with the trait of interest. This significant association suggests that these markers are either directly involved in the genetic control of the trait or possibly in linkage disequilibrium with a causal variant for growth related traits. The positive association with SNP genotypes and increased growth suggests that the *lbx1b* and *tnksa* could be candidate genes for growth and marker-assisted selection in dusky kob. Furthermore, several SNP's were observed to be family-specific and may have significantly influenced phenotypic performance within families, and more importantly, potentially masked the phenotypic effect of other mutations that may be associated with the trait of interest. However, further validation in larger independent populations from various environmental and genetic backgrounds will be necessary to confirm many pivotal details before these markers can contribute to a marker assisted selection programme for genetic improvement.

3.1) Introduction

Selection for traits associated with fast and efficient growth is a key objective in aquacultural breeding programmes, as improved growth of cultured species can reduce production- and maintenance costs by shortening the rearing cycle, consequently leading to a faster turnover rate (Gjedrem & Baranski, 2009). Traditional phenotypic-based selection methods are commonly used to select for economically important traits, such as increased growth rate. However, such conventional methods are not only expensive and susceptible to environmental factors, the optimisation of subsequent genetic improvements generally require several generations of selection. Alternatively, the application of DNA markers technologies in breeding programmes for aquatic species offer major benefits for achieving genetic improvements and increase productivity of the aquaculture industry. However, DNA marker technology and gene manipulation have yet to significantly influence the aquaculture industry (Hulata, 2001), and has only been applied for a limited number of model or well established industrialised aquatic species (Yue, 2014). Nevertheless, genomic research, especially quantitative trait loci (QTL) mapping in model aquatic species, such as salmonids (McClelland & Naish, 2008; Moen *et al.*, 2008; Rexroad *et al.*, 2008), tilapia (McConnell *et al.*, 2000; Lee *et al.*, 2005) and common carp (Sun & Liang, 2004), have led to the construction of linkage maps, which has accelerated genetic improvements *via* marker-assisted selection (MAS). Moreover, MAS provides a more precise and effective approach for genetic improvement for traits of interest (Dirlewanger *et al.*, 2004; Jena & Mackill, 2008) and requires fewer generations to achieve a breeding goal (Dekkers, 2004; Collard & Mackill, 2008). As a matter of fact, MAS has not only demonstrated accelerated genetic improvements compared to classical selection alone (Davis & DeNise, 1998), but also provide a unique opportunity to select for traits that are often expensive or difficult to measure phenotypically (Davis & Hetzel, 2000).

Single Nucleotide Polymorphisms (SNP's) have great advantages in genetic studies when compared to other markers. They are the most abundant source of heritable genetic variation in any organism's genome and tend to reveal hidden polymorphism not detected with other markers and methods (Liu & Cordes, 2004; Maqsood & Ahmad, 2017). Additionally, SNP's occur in both coding and non-coding regions and have been shown to affect protein function. Thus, certain SNPs may directly influence phenotypic variation among cultured individuals (Beuzen *et al.*, 2000) and can, therefore, be very

effective when investigating trait-genotype associations (Liu, 2007). Furthermore, as SNP markers can identify variation at the single base level, they are ideal for identifying and assessing variation in genes associated with quantitative traits, as such traits are often regulated by small additive effect of many genes which makes the relation between genotype and phenotype difficult to detect (Yano & Sasaki, 1997; Van Ooijen, 1999).

Whilst the application of SNP marker technologies is still relatively new in aquacultural breeding programmes, SNP markers have been identified in many cultured species, such as Atlantic cod (Hubert *et al.*, 2010), Atlantic salmon (Tsai *et al.*, 2015) and Nile tilapia (Xia *et al.*, 2014). Similarly, SNP's have been extensively used to screen candidate genes for growth in a number of aquatic species, including Arctic charr (Tao & Boulding, 2003), Asian seabass (Xu *et al.*, 2006), and shrimps (Glenn *et al.*, 2005). Candidate genes generally refer to genes with known biological function, that either directly or indirectly regulate the developmental processes of the traits of interest. Therefore, candidate gene linked markers are more precisely targeted and can detect variation in genes that relate directly or indirectly to the trait of interest (Lynch & Walsh, 1998; Zhu & Zhao, 2007). In fact, a statistical association between specific genotypes (or alleles) of a candidate gene and the trait of interest is taken as evidence that the candidate gene in question is either directly involved in the genetic control of the trait, or that the marker is sufficiently close to a causal variant(s) so that the two loci are in linkage disequilibrium (Lynch & Walsh, 1998). However, the availability of DNA markers, such as SNP's or microsatellites, for specific species is generally related to the economic value or scientific importance of the target species (Hayes & Andersen, 2005). Therefore, the application of such marker technologies and the discovery of candidate genes in non-model species, such as dusky kob, is limited due to the lack of DNA sequence information available.

Interestingly, several studies have taken advantage of DNA sequence information from related species to identify genetic variation in a target species (Primmer *et al.*, 2002; Aitken *et al.*, 2004; Li *et al.*, 2007; Li & Riethoven, 2010), thereby increasing the number of potential target genes in species with limited DNA sequence information. As a matter of fact, a recent study on dusky kob utilised an exome capture kit designed for the model aquatic species, *Danio rerio* (Zebrafish), to extract and sequence homologues exonic regions in the genome of eight large and eight small dusky kob individuals (Jackson *et al,*. in prep). Furthermore, this study is the first to employ next generation

sequencing (NGS) technologies to obtain DNA sequence information for dusky kob, as well as to identify possible candidate genes associated with growth related traits in this species. This approach presents a promising alternative to the conventional candidate gene approach, as larger group of genes involved in certain physiological traits of interest (Tao & Boulding, 2003; Hancock *et al.*, 2008) can be screened for variation. Additionally, the utilisation of an exome capture approach could potentially increase the likelihood of a successful application of the candidate gene approach by increasing the chances of identifying sites under selection, and more importantly, related to the trait(s) of interest. Therefore, by using a candidate gene approach, the present study aims to confirm and identify additional genetic variants, more specifically SNP's across several candidate genes, that might be correlated to individual growth performance, and potentially explain growth disparities within and between families.

3.2) Materials and methods

3.2.1) Study population and genotyping

Fin clip tissue from broodstock and derived cultured offspring were collected as described in Chapter 2. Furthermore, pedigree reconstruction yielded only two full-sibling families (SPE1 and SPE2), where a subset of 24 individuals (12 large and 12 small based on wet weight) from each full-sibling family were selected (Table 3.1). Fin clip tissue from all broodstock and cultured individuals were preserved in 70% ethanol and stored at room temperature. Genomic DNA extractions was performed on each sample as a single extraction using a standard CTAB DNA extraction protocol (Saghai-Maroof *et al*., 1984). Following extraction, DNA quantity and quality was evaluated using a NanoDrop™ ND 1 000 spectrophotometer (ThermoFisher Scientific) and normalised to a final working concentration of 20 – 30ng/µl.

Table 3.1: Mean phenotypic performance, associated standard deviation and variances for dusky kob growth traits weight (W), standard length (sL) and Fulton's conditioning factor (K) as well as age [days post hatch (dph)] for each respective size cohort for each family.

Family		**SPE1**		**SPE2**	
Age (dph)		**175**		**112**	
Cohort		**Small**	**Large**	**Small**	**Large**
W (g)	**Mean**	12,75	55,00	7,29	28,79
	Standard deviation	±4,05	±20,32	±3,23	±11,85
	Variance	16,43	413,09	10,43	140,42
sL (mm)	**Mean**	87,58	147,08	72,92	118,75
	Standard deviation	±11,43	±,46	±13,08	±16,52
	Variance	130,63	340,63	171,17	272,93
K	**Mean**	1,87	1,67	1,77	1,65
	Standard deviation	±0,30	±0,11	±0,24	±0,08
	Variance	0,09	0,01	0,06	0,01

Candidate genes associated with growth traits (W and sL) in *Argyrosomus japonicus* (dusky kob) were identified in a previous study (Jackson *et al*. in prep) using an exome capture approach. In Jackson *et al.* (in prep.) a solution-based *Danio rerio* (Zebrafish) exome capture kit was used to capture homologous exonic regions in the dusky kob genome of eight large and eight small full-sibling individuals. These exonic regions were subsequently sequenced and aligned to their respective Zebrafish homologues in CLC Bio Genomics Workbench® version 7.0.3 where variation was assessed using the fixed ploidy variant detection tool. Furthermore, genes associated with growth traits were also identified from literature where each respective Zebrafish homologue sequence as well as the relative chromosomal position of each gene in the Zebrafish genome was subsequently identified and extracted from the database of The National Center for Biotechnology Information (NCBI). Therefore, using each Zebrafish homologue, the corresponding dusky kob exonic regions were identified and assessed for variation. A BLAST search was performed to verify sequence identity to the respective Zebrafish homologue followed by primer design for each candidate gene for

dusky kob using the online software package Primer3plus (Untergasser *et al.*, 2007). In the current study, a total of seven candidate genes were selected (Table 3.2) to confirm the SNP's identified with cross-species exome capturing and evaluate the presence of additional genetic polymorphisms in these gene regions.

Table 3.2: The name of each candidate gene as well as their respective chromosomal positions in the Danio rerio *genome is displayed. Additionally, primer names and sequence for each exonic fragment are also displayed along with the annealing temperature and the expected fragment size. Previously identified SNP's for each exonic region also displayed. Shaded primers were used for uni-directional sequencing.*

Gene name	Position in *Danio rerio* genome	Primer name	Fragment size (bp)	Identified SNP's in dusky kob	Primer sequence (5' – 3')	Annealing temperature
Ladybird homeobox 1 b (*lbx1b*)	Chromosome 1 (31532110...31534089)	lbx1b	242	c.114T > A	*F:* TCTGCAGCCTGTAAAACGCT R: ACTCTAACAAGCCGCTGACC	60°C
Fibroblast growth factor 4 (*fgf4*)	Chromosome 7 (54617076 - 54624873)	fgf4	134	c.24C > G c.29G > C c.33 G > C	F: CAGTCCGTCAGAACCGTAGC R: TACCAGCCAACACAACAGCA	60°C
Myogenic differentiation 1 (*myod1*)	Chromosome 25 (31421253 - 31423459)	myod1	223	c.09T > C c.34G > T c.37T > C c.43G > C c.62G > T c.123T > C	F: TCACCATGCCATCAGAGCAG R: CAAGGCCTGCAAGAGGAAGA	60°C
		myod1.1	220	c.125T > C	F: TGCCATCAGAGCAGTTGGAG R: CTCAGCAAAGTCAACGACGC	60°C
		myod1.2	209	c.48C > G	F: TCTTCCTCTTGCAGGCCTTG R: CCTGCCGCCGATGATTTCTA	60°C
Myogenic factor 5 (*myf5*)	Chromosome 4 (21741228 - 21745107)	myf5	304	c.251A > G c.254T > G c.297T > G	F: GGACATTGCCTCCAGTGGG R: CGTCAGAGCAGTTGGACAGT	58°C
Myogenic factor 6 (*myf6*)	Chromosome 4 (21717793 - 21720943)	myf6	220	-	F: TCTGCAAGAGGAAGTCAGCG R: GTCTTCTCCTGCTCGTCCAG	60°C
Tankyrase, TRF1-interacting	Chromosome 21 (19926061 - 20110923)	tnksa	238	c.69T > C c.74C > T c.104G > A	F: CGGAGGTGTCTTCAGCAGAT	58°C

ankyrin-related ADP-ribose polymerase a (*tnksa*)					R: CGCTCGTTGTGATGGTTGTG	
Tubulin, alpha 8 like 2 (*tuba8l2*)	Chromosome 1 (5402484 - 5419116)	tuba8l2	408	c.07G > C c.08A > C c.11G > C c.12A > T c.14T > A c.58G > A c.154G > A	F: AACCTGAACCGCCTCATCAG R: CAGTGGGAGGCTGGTAGTTG	58°C

Polymerase chain reaction (PCR) amplifications were performed in 10µl reaction volume containing 20 - 30ng of template DNA, 1X PCR buffer A, 1.5mM $MgCl_2$, 0.5mM dNTP's, 0.2mM of the forward and reverse primers and 0.5U of KAPA Taq DNA polymerase. PCR conditions were set as follows: 3 min at 95°C; 35 amplification cycles of 95°C for 30 seconds, T_a for 30 seconds, 72°C for 30 seconds; 7min at 72°C (Table 3.2). The resulting DNA fragments were subjected to gel electrophoresis in a 1.5% agarose gel made up with 1X TBE, where subsequent DNA bands were visualised by staining the gel with EtBr at 0.005% of the total gel volume. Where successful amplification occurred, the sequencing reaction was carried out in 10µl reaction volumes, each containing 1X BigDye™ Terminator 3.1 Ready Reaction Mix, 1µl of PCR product and 0.2mM of either primer. Initially, bi-directional sequencing was performed for a set of four individuals in each full-sibling family, followed by uni-directional sequencing with the preferred primer (Table 3.2). PCR cycle sequencing conditions was set as follows: 5 min at 94°C; 35 amplification cycles of 94°C for 10 seconds, 55°C for 10 seconds, 60°C for 4 min and subsequently subjected to Sanger sequencing *via* capillary electrophoresis at the Stellenbosch University Central Analytical Facility (DNA sequencing unit). Following amplification and sequencing as described above, all sequences were visually inspected, edited and aligned with MEGA Version 7.0.26 software (Kumar *et al.*, 2016), using the ClustalW (Thompson *et al.*, 1994) algorithm for multiple alignments. Visual identification of sequence variation in multiple alignments (MEGA v7.0.26 (Kumar *et al.*, 2016), ClustalW (Thompson *et al.*, 1994)) was done and confirmed by investigating individual chromatograms, where a SNP was confirmed if clear double peaks (beyond possible noise) could be identified in heterozygous individuals.

3.2.2) Genetic analysis

As a preliminary assessment of association within the families, a chi-square analysis was done to test for genotypic distortion. Additionally, a Fisher's exact test was also employed to evaluate and verify genotypic distortion within families. Thereafter family datasets were combined, for a cross-family analysis, to create two cohorts consisting of large and small animals respectively. Allele and genotype frequencies for each SNP site were assessed using the online program SNPstats (http://bioinfo.iconcologia.net/snpstats/start.htm) for the large and small cohorts respectively and as a combined population group. Additionally, the SNPstats software

package was also used to evaluate associations between SNP genotypes and the dichotomous response variable (large vs small) by linear or logistic regression depending on the assumed mode of inheritance (the following modes were assessed: co-dominant, dominant, recessive and additive). Tank and age were set as covariates in the regression models. Linkage disequilibrium (using the four gamete rule) was evaluated in HaploView (Barrett *et al*., 2005) where a haplotype association analysis, using default parameters, was performed to evaluate the potential presence of significant haplotypic blocks associated with growth. To further combine linkage and LD evaluations a transmission disequilibrium test was also conducted in HaploView using the four gamete rule (exclude individuals with > 50% missing genotypes; 4th gamete frequency of > 0.01; confidence interval minima for strong LD lower limit: 0.7 and upper limit: 0.98; upper confidence interval maximum for strong recombination: 0.9; informative comparison minimum: 0.95; exclude markers below 0.05 minor allele frequency).

3.3) Results

From a total of nine exonic regions evaluated, one exonic region (*myf6*) did not display any sequence variation in the present study, and is, therefore, in accordance with results from Jackson *et al*. (in prep.), whilst all the SNP's previously identified in *fgf4* were observably monomorphic. Therefore, both *myf6* and *fgf4* were subsequently excluded from further analyses. From the remaining seven exonic regions, 15 SNP's were successfully identified. In region *lbx1b*, the presence of SNP c.114T > A (referred to as *lbx1b*_c.114 from hereon) was confirmed in the current study, whilst in region *myod1*, SNP's c.09T > C, c.37T > C and c.62G > T were observably monomorphic in the families under observation. Regardless of this, SNP's c.34G > T (*myod1*_c.34G), c.43G > C (*myod1*_c.43) and c.123T > C (*myod1*_c.123) were confirmed in this region. Additionally, in regions *myod1.1* and *myod1.2*, SNP c.125T > C (*myod1.1* _c.125) was successfully confirmed while SNP c.48C > G was noticeably monomorphic. However, the presence of a novel SNP [c.166T > C (*myod1.2*_c.166)] was detected in this region. Furthermore, all three SNPs previously identified in region *myf5* were monomorphic in this study, whilst the presence of another novel SNP c.95A > G (*myf5*_c.95) was noted. Regarding region *tnksa*, SNP's c.69T > C (*tnksa*_c.69), c.74C > T (*tnksa*_c.74) and c.104G > A (*tnksa*_c.104) were successfully identified along with three novel SNPs: c.58A > T (*tnksa*_c.58), c.85G > A (*tnksa*_c.85), c.107A > C (*tnksa*_c.107) in this

region, whilst in region *tuba8l2*, only SNPs c.14T > A (*tuba8l2*_ c.14) and c.58G > A (*tuba8l2*_ c.58) were confirmed.

Significant genotypic distortions were detected in both families (Table 3.3). In SPE1, three SNP's (*lbx1b*_c.114, *myod1*_c.123 and *myod1.1* _c.125) displayed significant distortion. Similarly, in SPE2, three SNP's (*lbx1b*_c.114, *tnksa*_c.58 and *tuba8l2*_c.58) followed the same trend. Another SNP (*tnksa*_c.69) displayed significant genotypic distortion in SPE2 when utilising the chi-square method, however, when utilising Fisher's exact test, distortions were observably non-significant in both families. SNP *lbx1b*_c.114 is the only observed SNP that displays significant genotypic distortion in both full-sibling families, whilst the genotypic frequencies of *myod1.1* _c.125 and *myod1*_c.123 in SPE1 and *tuba8l2*_c.58 in SPE2 do not significantly deviate from the expected within the alternative family. Furthermore, *tnksa*_c.58 only displays sequence variation in SPE2 as both parents are heterozygous for this particular SNP, whereas in SPE1, a fixed parental genotype is observed. A similar trend can be observed for *tnksa*_c.104 and *tuba8l2*_c.14 in SPE2 and for *tnksa*_c.107 in SPE1 where sequence variation is observed to be family-specific.

Table 3.3: Observed and expected number of genotypes for each SNP. A chi-squared table (X^2) as well as the degrees of freedom (Df) and corresponding P-values are indicated. Parental genotypes are shaded and significant P-values calculated via a chi-square method and Fisher's exact test (P-values in brackets) are in bold.

	SPE1						SPE2					
SNP	**Genotype**	**Obs**	**Exp**	**X^2**	**Df**	***P*-value**	**Genotype**	**Obs**	**Exp**	**X^2**	**Df**	***P*-value**
*lbx1b*_c.114	T/T	21	12	6.750	1	**< 0.001 (0.011)**	T/T	20	12	5.333	1	**0.001 (0.031)**
	T/A	3	12	6.750			T/A	4	12	5.333		
	A/A	0	0	- 13.500			A/A	0	0	- 10.667		
*myod1*_c.34	G/G	9	12	0.750	1	0.221 (1.000)	G/G	6	12	3.000	1	0.079 (0.351)
	G/T	9	12	0.750			G/T	13	12	0.083		
	T/T	0	0	- 1.500			T/T	0	0	- 3.083		
*myod1*_c.43	G/G	7	6	0.167	2	0.368 (0.591)	G/G	9	6	1.500	2	0.119 (0.276)
	G/C	14	12	0.333			G/C	13	12	0.083		
	C/C	3	6	1.500 2.000			C/C	2	6	2.667 4.250		
*myod1*_c.123	T/T	0	12	12.000	1	**< 0.001 (< 0.001)**	T/T	13	12	0.083	1	0.683 (1.000)
	C/T	24	12	12.000			C/T	11	12	0.083		

	C/C	0	0	- 24.000			C/C	0	0	- 0.167		
myod1.1 _c.125	T/T T/C C/C	3 21 0	12 12 0	6.750 6.750 - 13.500	1	**< 0.001 (0.011)**	T/T T/C C/C	15 9 0	12 12 0	0.750 0.750 - 1.500	1	0.221 (0.561)
myod1.2 _c.166	T/T C/T C/C	6 13 5	6 12 6	- 0.083 0.167 0.250	2	0.882 (0.999)	T/T C/T C/C	0 9 15	0 12 12	- 0.750 0.750 1.500	1	0.221 (0.561)
*myf5*_c.95	G/G A/G A/A	8 8 7	6 12 6	0.667 1.333 0.167 2.167	2	0.338 (0.574)	G/G A/G A/A	0 12 9	0 12 12	- 0.000 0.750 0.750	1	0.386 (0.767)
*tnksa*_c.69	T/T T/C C/C	10 12 2	6 12 6	2.667 0.000 2.667 5.333	2	0.069 (0.254)	T/T T/C C/C	4 18 2	6 12 6	0.667 3.000 2.667 6.333	2	**0.042** (0.158)
*tnksa*_c.104	G/G G/A A/A	24 0 0	- - -	- - - -	1	-	G/G G/A A/A	8 16 0	12 12 0	1.333 1.333 - 2.667	1	0.102 (0.380)
*tnksa*_c.58	A/A A/T T/T	24 0 0	- - -	- - - -	1	-	A/A A/T T/T	13 4 7	6 12 6	8.167 5.333 0.167 13.667	2	**0.001 (0.033)**
*tnksa*_c.74	C/C C/T T/T	12 12 0	12 12 0	0.000 0.000 - 0.000	1	1.000 (1.000)	C/C C/T T/T	14 10 0	12 12 0	0.333 0.333 - 0.667	1	0.414 (0.579)
*tnksa*_c.85	G/G A/G A/A	12 12 0	12 12 0	0.000 0.000 - 0.000	1	1.000 (1.000)	G/G A/G A/A	8 16 0	12 12 0	1.333 1.333 - 2.667	1	0.103 (0.380)
*tnksa*_c.107	A/A A/C C/C	14 10 0	12 12 0	0.333 0.333 - 0.667	1	0.414 (0.579)	A/A A/C C/C	24 0 0	- - -	- - - -	-	-
*tuba8l2*_c.14	T/T A/T A/A	22 0 0	- - -	- - - -	1	-	T/T A/T A/A	16 7 0	12 12 0	1.333 2.083 - 3.417	1	0.065 (0.238)
*tuba8l2*_c.58	G/G A/G A/A	10 12 0	12 12 0	0.333 0.000 - 0.333	1	0.564 (0.777)	G/G A/G A/A	2 21 0	12 12 0	8.333 6.750 - 15.083	1	**< 0.001 (0.003)**

Association analysis between SNP marker genotypes and the dichotomous response variable (large *vs* small) for body weight (W) in 48 individuals revealed only two SNP's that significantly associate with the response across the families (Table 3.4). Marker *lbx1b*_c.114 displays an A allele frequency of 0.07 in the combined population, which is observably much lower than that of the T allele frequency (0.93) (Table 3.4). Furthermore, the minor allele appears exclusively in the small cohort as individuals with a T/A genotype displays significant association with this group ($P < 0.01$), whilst all individuals in the large cohort harbour a T/T genotype. Additionally, in marker *tnksa*_c.69, the C allele frequency (0.40) is lower than that observed for the T allele frequency (0.60). Similarly, in both the large and small cohorts, the C/C genotypic frequency is much lower compared to the T/T genotypic frequency, however, the C/C genotypic frequency is higher in the small cohort. Furthermore, this marker displays significant association under the log-additive mode of inheritance. The haplotype test for associations performed in HaploView did not reveal excess transmissions from parents to offspring for each candidate gene evaluated. Similar results were observed when all SNP's were analysed together.

Table 3.4: Number of individuals genotyped as well as allele and genotypic frequencies for each SNP identified per cohort (large vs small) and as a whole population. The amino acid substitution for each SNP is represented as well as the P-value indicate significant association (in bold), with a value of less than 0.05 indicating significant association.

Locus	Cohort	Successfully genotyped	Genotypic frequencies			Allele frequencies		Amino acid substitution	P-value
*lbx1b*_c.114			**T/T**	**T/A**	**-**	**T**	**A**		
	Large	24	1.00	0	-	1.00	0	Synonymous	**0.0055**
	Small	24	0.71	0.29	-	0.85	0.15		
	Total	48	0.85	0.15	-	0.93	0.07		
*myod1*_c.34			**G/G**	**G/T**	**-**	**G**	**T**	Non-synonymous ***Pro - Arg***	0.15
	Large	16	0.44	0.56	-	0.72	0.28		
	Small	20	0.38	0.62	-	0.69	0.31		
	Total	37	0.41	0.59	-	0.7	0.3		
*myod1*_c.43			**G/G**	**G/C**	**C/C**	**G**	**C**	Non-synonymous ***Pro - Arg***	0.32^{a} 0.94^{b} 0.16^{c} 0.38^{d} 0.61^{e}
	Large	24	0.33	0.54	0.12	0.60	0.40		
	Small	24	0.33	0.58	0.08	0.62	0.38		
	Total	48	0.33	0.56	0.1	0.61	0.39		
*myod1*_c.123			**T/T**	**T/C**	**-**	**T**	**C**		
	Large	24	0.33	0.67	-	0.67	0.33	Synonymous	0.60
	Small	24	0.21	0.79	-	0.60	0.40		
	Total	48	0.27	0.73	-	0.64	0.36		
myod1.1 _c.125			**T/T**	**T/C**	**-**	**T**	**C**		
	Large	24	0.42	0.58	-	0.71	0.29	Synonymous	0.55
	Small	24	0.33	0.67	-	0.67	0.33		
	Total	48	0.38	0.62	-	0.69	0.31		
myod1.2 _c.166			**T/T**	**C/T**	**C/C**	**C**	**T**		0.17^{a} 0.45^{b} 0.10^{c} 0.16^{d}
	Large	24	0.04	0.54	0.42	0.69	0.31	Synonymous	
	Small	24	0.21	0.38	0.42	0.60	0.40		
	Total	48	0.12	0.46	0.42	0.65	0.35		

									1.00[e]
*myf5*_c.95			**G/G**	**A/G**	**A/A**	**A**	**G**	Non-synonymous ***Ala - Thr***	0.86[a]
	Large	23	0.22	0.48	0.30	0.54	0.46		0.58[b]
	Small	21	0.14	0.43	0.43	0.64	0.36		NA[c]
	Total	44	0.18	0.45	0.36	0.59	0.41		0.69[d]
									0.69[e]
*tnksa*_c.69			**T/T**	**T/C**	**C/C**	**T**	**C**	Non-synonymous ***Phe - Ser***	0.06[a]
	Large	24	0.38	0.58	0.04	0.67	0.33		0.07[b]
	Small	24	0.21	0.67	0.12	0.54	0.46		0.10[c]
	Total	48	0.29	0.62	0.08	0.6	0.4		0.56[d]
									0.02[e]
*tnksa*_c.104			**G/G**	**G/A**	**-**	**G**	**A**	Non-synonymous ***Cys - Tyr***	0.75
	Large	24	0.71	0.29	-	0.85	0.15		
	Small	24	0.62	0.38	-	0.81	0.19		
	Total	48	0.67	0.33	-	0.83	0.17		
*tnksa*_c.58			**T/T**	**A/T**	**A/A**	**A**	**T**	Non-synonymous ***Met – Leu***	0.87[a]
	Large	24	0.12	0.08	0.79	0.83	0.17		0.78[b]
	Small	24	0.17	0.08	0.75	0.79	0.21		0.78[c]
	Total	48	0.15	0.08	0.77	0.81	0.19		0.60[d]
									1.00[e]
*tnksa*_c.74			**C/C**	**C/T**	**-**	**C**	**T**	Non-synonymous ***Ser - Phe***	0.94
	Large	24	0.58	0.42	-	0.79	0.21		
	Small	24	0.50	0.50	-	0.75	0.25		
	Total	48	0.54	0.46	-	0.77	0.23		
*tnksa*_c.85			**G/G**	**G/A**	**-**	**G**	**A**	Non-synonymous ***Val - Ile***	0.51
	Large	24	0.50	0.50	-	0.75	0.25		
	Small	24	0.33	0.67	-	0.67	0.33		
	Total	48	0.42	0.58	-	0.71			
*tnksa*_c.107			**A/A**	**A/C**	**-**	**A**	**C**	Non-synonymous	0.34
	Large	24	0.83	0.17	-	0.92	0.08		

	Small	24	0.75	0.27	-	0.88	0.12	***His – Pro***	
	Total	48	0.79	0.21	-	0.9	0.1		
*tuba8l2*_c.14			**T/T**	**T/A**	**-**	**T**	**A**	Non-synonymous ***Lys - Glu***	0.26
	Large	22	0.86	0.14	-	0.93	0.07		
	Small	23	0.83	0.17	-	0.91	0.09		
	Total	45	0.84	0.16	-	0.92	0.08		
*tuba8l2*_c.58			**G/G**	**G/A**	**-**	**G**	**A**	Non-synonymous ***Cys - Tyr***	0.76
	Large	22	0.32	0.68	-	0.66	0.34		
	Small	23	0.22	0.78	-	0.61	0.39		
	Total	45	0.27	0.73	-	0.63	0.37		

Note: Genotypic association was evaluated under five (a-e) modes of inheritance: Codominant ([a]), Dominant ([b]), Recessive ([c]), Over dominant ([d]), Log-additive [multiplicative ([e])].

3.4) Discussion

The genetic improvement of economically valued traits *via* MAS is of major interest in many sectors of the aquacultural industry. However, the molecular mechanisms underlying the expression of complex traits (such as increased growth rate) are poorly studied in finfish aquaculture and has only been undertaken in a few model species. To date, genetic improvement of cultured dusky kob has been relatively slow compared to other aquaculture fish species (McConnell *et al.*, 2000; Sun & Liang, 2004; Lee *et al.*, 2005; McClelland & Naish, 2008; Moen *et al.*, 2008; Rexroad *et al.*, 2008) as traditional phenotypic-based selection methods continues to be the main driver behind the genetic improvement of growth related traits. On the contrary, in comparison to classical selection methods, the construction of linkage maps and subsequent QTL mapping in model species have significantly accelerated genetic improvements *via* MAS (Davis & DeNise, 1998), and continues to demonstrate elevated selection responses of complex traits that are difficult to select for through classical methods of selection (Davis & Hetzel, 2000). However, to the best of our knowledge, no selective breeding programmes have been conducted for dusky kob, and no genetic linkage map has been constructed for this species. Furthermore, the construction of linkage maps usually requires large numbers of variable markers across multiple families (Liu & Cordes, 2004; Yue, 2014), which is a limiting factor for the application of MAS breeding programme in non-model species.

The candidate gene approach is a powerful method to investigate associations of gene polymorphisms with economically important traits in aquaculture. However, there is currently no published literature describing genetic variants (such as SNP's) and possible associations to economically important traits in dusky kob. Alternatively, an exome capture approach presents an effective means of identifying novel candidate genes involved in the expression of both Mendelian and complex traits, such as growth (Tang *et al*., 2016), and could potentially reveal novel signalling pathways involved in the expression of such traits. Therefore, not only can this approach provide new insight into the genetic basis of growth related traits, but the identification of molecular variants associated with such traits could also prove immensely useful in achieving accelerated genetic improvements *via* marker-assisted selection (MAS).

In the present study, across nine exonic regions evaluated, four synonymous (*lbx1b*_c.114, *myod1*_c.123, *myod1.1* _c.125 and *myod1.2* _c.166) and 11

non-synonymous SNP's (*myod1*_c.43, *myod1*_c.34, *myf5*_c.95, *tnksa*_c.69, *tnksa*_c.104, *tnksa*_c.58, *tnksa*_c.74, *tnksa*_c.85, *tnksa*_c.107, *tuba8l2*_c.14, *tuba8l2*_c.58) were successfully validated. However, segregation distortion of SNP marker genotypes across the large and small cohorts in each full-sibling family revealed that the genotypic frequencies of only three markers in SPE1 (*lbx1b*_c.114, *myod1*_c.123 and *myod1.1* _c.125) and four markers in SPE2 (*lbx1b*_c.114, *tnksa*_c.69, *tnksa*_c.58 and *tuba8l2*_c.58) significantly deviate from the expected ratios in the offspring. Therefore, these markers may be associated with the response variable in the present study, as an increased/decreased frequency of a SNP allele or genotype in a particular cohort in case-control studies could potentially indicate elevated/reduced risk of expressing the trait of interest (Lewis, 2002).

Association analysis between genotypes of single SNP's and the response variable (large vs small) for body weight (W) indicated that only two SNP's significantly associate with the response across both families. For *lbx1b*_c.114, the T/T genotype is significantly associated with increased body weight compared to the T/A genotype (Table A3.1). The Ladybird family of homeobox transcription factors (*lbx*) have key roles in neural and muscle development that are relatively conserved in numerous species from *Drosophila* to vertebrates (Lukowski *et al.*, 2011). In Zebrafish, *lbx* genes are constitutively expressed in the dorsal spinal cord, with facultative expression patterns in the hindbrain and appendicular muscles, and are reportedly involved in muscle formation as well as neural specification during embryogenesis (Lukowski *et al.*, 2011). Reportedly, mutations in the Zebrafish *lbx* gene family observably result in axial deformities (Guo *et al.*, 2016). Similarly, in the *Mus musculus* (mouse) genome, the *lbx1* gene (NG_009236.1) codes for a regulatory protein that mediates cell migration of muscle precursors, whilst in humans, polymorphisms in the *lbx1* gene has been linked to the aetiology of idiopathic scoliosis, a condition that causes abnormal curvature of the spine (Grauers *et al.*, 2015; Nada *et al.*, 2018; Jiang *et al.*, 2019). Therefore, the presence of an A allele may have a negative effect on muscle development and thereby negatively impact the expression of growth traits in dusky kob.

Another SNP (*tnksa*_c.69) also showed significant association with the response, however, was only found to be significantly associated under the log-additive mode of inheritance. In case-control association studies where dichotomous response variables are utilised (*e.g.* large *vs* small), a logistic regression model is generally used

(Solé *et al.*, 2006) where additivity is measured as the log-odds of expressing the trait of interest (in this case associating with the large cohort) for a particular genotype (Gauderman, 2002). Therefore, the significance of this SNP under the log-additive model would suggest that the presence of a T allele (T/C and T/T) would have an additive effect to increased body weight compared to a C/C genotype (Table A3.1). Tankyrases (TNKS) are poly-ADP-ribosyltransferase enzymes that catalyse the posttranslational modification of target proteins and directs them toward proteasomal degradation (Cho-Park & Steller, 2013; Wang *et al.*, 2015). However, previous studies have highlighted a connection between TNKS's and metabolic homeostasis. Tankyrases reportedly have important regulatory roles in glucose transport as well as insulin stimulated glucose uptake (Yeh *et al.*, 2007; Guo *et al.*, 2012), where in humans, variants of *tnks1* has been associated with early-onset obesity (Pezzolesi *et al.*, 2004; Scherag *et al.*, 2010). Furthermore, in Zebrafish, *tnks* knockdown mutations result in reduced linear growth of larvae, stimulates insulin genes and glucose transporter proteins, and suppresses gluconeogenic phosphoenolpyruvate carboxykinase 1 genes. These effects suggest that *tnks* knockdown mutations may cause rapid glucose utilisation and reduced gluconeogenesis (Wang *et al.*, 2015).

Interestingly, *lbx1b*_c.114 is a synonymous mutation and no amino acid replacement is expected in the protein products of this genotypes. Consequently, the significant association of this SNP with body weight may indicate that this marker is either directly involved in the genetic regulation of gene expression in some way, or more likely is in linkage disequilibrium (LD) with 'nearby' undetected causal variant(s) for growth related traits (Lynch & Walsh, 1998). In fact, Pre-B cell leukaemia transcription factor (*pbx*) genes, which encode essential cofactors for *hox* proteins (Laurent *et al.*, 2008), have been identified as an important regulator in the expression of *lbx1* in Zebrafish, as the expression of *lbx1* are dependent on *hox* activity (Cooper *et al.*, 2003; Lukowski *et al.*, 2011). Therefore, genetic variants in upstream regulatory pathways may be in functional LD with the SNP observed in *lbx1b* in this study. Alternatively, synonymous (silent) SNP's can potentially alter structural/functional protein properties by affecting alternative splicing, splicing efficiency and messenger RNA turnover (Hunt *et al.*, 2009; Sun *et al.*, 2012; Xu *et al.*, 2013), as the use of rare codons may change the co-translational folding efficiency (Kimchi-Sarfaty *et al.*, 2007; Komar, 2007; Marin, 2008; Kolmsee & Hengge, 2011; Kristofich *et al.*, 2018) of the resulting protein product. As a result, silent SNP's may also influence phenotypic expression of traits of interest and

has been reported in the heat shock protein family and their association with phenotypic traits in animal species (Nikbin *et al.*, 2014), including crustaceans (Zeng *et al.*, 2008). Regardless of the underlying molecular mechanism, *lbx1b* is undoubtedly a strong candidate, given the significant genotypic segregation pattern observed in relation to growth in both full-sibling families (Lewis, 2002) as well as the significant statistical association observed. In contrast, *tnksa*_c.69 is a non-synonymous mutation, and is expected to result in alterations in the proceeding protein products of this genotypes (Kumar *et al.*, 2009). This radical amino acid substitution alters the physicochemical properties of the resulting protein (Weber & Whelan, 2019) and is likely to result in functional differences in the proteins for each respective genotype. Additionally, contrary to *lbx1b*_c.114, this SNP marker was also observed to be significantly associated with growth, albeit under a dominant mode of inheritance, as reported by Jackson *et al*. (in prep.) Therefore, it is likely that this mutation is directly involved in the genetic regulation of growth related traits, as this SNP may influence Tankyrase enzyme activity.

Surprisingly, three additional mutations (*tnksa*_c.74, *myod1*_c.34 and *myod1*_ c.62) were also reported as significant in Jackson *et al*. (in prep.), however, *myod1*_c.62 is observably monomorphic in both full-sibling families under investigation, whilst *tnksa*_c.74 and *myod1*_c.34 did not display significant genotypic segregation distortion nor association with the trait of interest. A major drawback when utilising an exome capture approach to identify candidate genes, is that only the coding regions of the identified candidate genes are represented, and furthermore, whether or not the full coding region for a particular gene is displayed remains unknown. Therefore, it is likely that the SNP's *tnksa*_c.74 and *myod1*_c.34 (Jackson *et al.,* in prep) may have been in LD with undetected causal variant(s) for growth related traits (Lynch & Walsh, 1998), present on different positions in the same gene. Alternatively, both *tnksa*_c.74 and *myod1*_c.34 are non-synonymous mutations resulting in amino acid substitutions. However, although these SNP's may have an effect at the molecular level, they may not significantly affect phenotypic expression for a number of reasons, such as the occurrence of such radical amino acid substitutions in functionally redundant protein domains, the presence of alternative proteins with overlapping function (Labow *et al.*, 1994), alternative molecular pathways with similar effects, the activation of compensating feed-back mechanisms, or simply a minor role for the protein in the expression of the trait of interest. Therefore, the results observed for these markers in

both the current, and previous study (Jackson *et al.,* in prep) suggest that the expression of complex traits, such as growth, may be influenced by genetic differences between families, as has been described by Gjedrem (2000).

Furthermore, genetic differences between families is evident in the current study, as several SNP's display significant genotypic distortions across the large and small cohorts in only one full-sibling family. However, SNP's *myod1*_c.123 and *myod1.1*_c.125 distorting in SPE1 are synonymous mutations, whilst SNP's *tnksa*_c.58 and *tuba8l2*_c.58 distorting in SPE2 are reportedly non-synonymous mutations. Interestingly, SNP's *myod1*_c.123, *myod1.1*_c.125 and *tnksa*_c.58 appear on the same candidate genes with SNP's (*myod1*_c.34, *myod1*_c.62 and *tnksa*_c.74) that were deemed significant by Jackson *et al*. (in prep.). These observations may reflect the occurrence of within-family LD with potential causal variant(s) in the same candidate gene, as linkage phases between markers and QTL may differ from family to family (Dekkers, 2004; Dekkers & van der Werf, 2007), which would explain the lack of apparent genotypic distortions of these SNP's in the alternative family. Furthermore, for several SNP's variation were only observed in one of the families in the study, whilst the alternative family is fixed for the major allele. Four family-specific SNP's were identified where one SNP (*tnksa*_c.107) appears exclusively in SPE1, and three SNP's (*tnksa*_c.58, *tnksa*_c.104 and *tuba8l2*_c.14) appear exclusively in SPE2. However, *tnksa*_c.58 is the only family-specific SNP that displays significant genotypic distortion across the large and small cohorts in SPE2, suggesting that this SNP marker may be involved in the expression of this trait. However, due to the lack of observed variation in multiple families, the effect of these SNP's on the trait of interest could not be accurately assessed. This further supports the occurrence of unique genetic variants between families that may influence the statistical power of associations, and could significantly affect the expression of the trait of interest.

The restricted genetic context provided by the current experimental design (few families) presented a major limitation for assessing the associations of SNP marker genotypes and trait(s) of interest. In actuality, the differential genotypic distortions observed at several SNPs (*myod1*_c.123, *myod1.1* _c.125 and *tuba8l2*_c.58) between families, which is likely due to chance based on the genetic variation of the contributing broodstock, may suggest that the families under observation differ in their response to selection, as these families are likely to express different degrees of variation at various genes. Furthermore, the limited number of families could also explain the occurrence

of monomorphic SNP's, where genotypic variation and significant association were observed in a previous study (Jackson *et al,*. in prep). Therefore, the markers evaluated in the current study, regardless if variation is present, should be further investigated under a broader genetic context (more families with diverse origins) as significant associations may be revealed in a more robust experimental design.

3.5) Conclusions

In conclusion, the identification of novel molecular variants (SNP's) associated with increased growth rate in dusky kob serves as the first step towards the discovery of QTL's for growth related traits. In the present study, five novel SNP's (*myod1.2*_c.166, *myf5*_c.95, *tnksa*_c.58, *tnksa*_c.85 and *tnksa*_c.107) were identified and ten SNP's (*lbx1b*_c.114, *myod1*_c.123, *myod1.1*_c.125, *myod1*_c.43, *myod1*_c.34, *tnksa*_c.69, *tnksa*_c.104, *tnksa*_c.74, *tuba8l2*_c.14 and *tuba8l2*_c.58) were subsequently confirmed. Moreover, assessment of genotypic segregation of SNP marker genotypes revealed that the genotypic frequencies of three markers in SPE1 (*lbx1b*_c.114, *myod1*_c.123 and *myod1.1*_c.125) and four markers in SPE2 (*lbx1b*_c.114, *tnksa*_c.69, *tnksa*_c.58 and *tuba8l2*_c.58) significantly deviate from the expected, and may be associated with the trait of interest (Lewis, 2002). However, only two SNP's (*lbx1b*_c.114 and *tnksa*_c.69) significantly associate with the trait, suggesting either direct genetic influences on the expression of growth traits, or indirect association via LD with proximal QTL's linked to the trait. Interestingly, results from a previous study (Jackson *et al*., in prep) revealed four SNPs (*tnksa*_c.69, *tnksa*_c.74, *myod1*_c.34 and *myod1*_ c.62) that may be associated with increased growth in dusky kob, however, only one SNP (*tnksa*_c.69) displayed significant genotypic distortion and association with growth traits in the current study. Therefore, it is suggested that this non-synonymous mutation is likely directly involved in the genetic regulation of growth related traits, as this SNP may possibly alter ligand binding efficiency and/or catalytic ability of Tankyrase enzymes (Wang & Moult, 2001). Furthermore, SNP's *tnksa*_c.74, *myod1*_c.34 and *myod1*_c.62 did not display any significant genotypic distortions nor association with the trait of interest in the present study. On the contrary, several SNP's in the current study displayed significant genotypic distortions within families, however, given the lack of significant genotypic distortions observed in the alternate family, and that these SNP's are on the same candidate genes (*myod* and *tnksa*) as SNP's reported by Jackson *et al*. (in prep.), it is suggested that these SNP's may be in LD

with causal variant(s) within families, and the extent of their linkage may vary between families. Nevertheless, the positive association with SNP genotypes (*lbx1b*_c.114 and *tnksa*_c.69) and increased growth suggests that *lbx1* and *tnks* could be candidate genes for growth and marker-assisted selection in dusky kob. However, due to the genetically heterogeneous nature of the two families included in the study, several SNP's were observed to be family-specific and may have significantly influenced the statistical power of detecting possible associations across families, as marker informativeness is likely to differ across families with diverse genetic origins. Additionally, not only could the phenotypic effect of these SNP's not be evaluated, the occurrence of family-specific variants could potentially mask the phenotypic effect of other mutations that may be associated with the trait of interest. Further evaluations of these SNP markers are therefore required, and should be repeated in larger independent populations from various environmental and genetic backgrounds to broaden the genetic context of possible associations with growth related traits, and to possibly identify significant haplotypes that may provide insight into factors that influence the dependency among SNP's. Regardless, the results observed in the current study demonstrate the feasibility of employing an exome capture approach to identify possible candidate genes associated with growth traits, and further reflects the robustness of the current experimental design, although recognised limitations, in identifying SNP marker genotype associations with the trait of interest.

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CHAPTER 4

Study conclusions

4.1) Overview

Argyrosomus japonicus (dusky kob) has been identified as an emerging aquaculture species due it its increased popularity as an indigenous line-fish and its overexploited status in the wild. Current production of dusky kob in South Africa relies on the mass spawning of unimproved (wild-caught) broodstock to produce F_1 - offspring for commercial trade. Consequently, considerable efforts have been initiated to retain such individuals with faster growth rates with the specific aim of utilising such candidates as breeders in a selective breeding programme. However, the mass spawning behaviour of this species presents a major challenge to the successful application of selective breeding programme(s), as single-pair mating schemes cannot be easily conducted to evaluate individual family relations and phenotypic performance. Due to favourable life history characteristics (*i.e.* high fecundity and broad phenotypic variation), together with the larger size attained at sexual maturity compared to most fish species, small broodstock populations are generally used to generate large progeny cohorts from which future potential breeding candidates are selected. Understanding how current husbandry practices impact the genetic constitution and phenotypic performance of progeny cohorts is, therefore, of great importance for the effective management and improvement strategies for dusky kob.

Chapter 2 therefore investigates how genetic diversity is represented and maintained within a commercial hatchery of dusky kob by assessing offspring cohorts originating from two mass-spawning events. Furthermore, progeny cohorts were characterised, both in terms of kinship and phenotypic performance, to gain a better understanding of how mass-spawning and other husbandry practices associated with spawning (*e.g.* hormonal induction) and rearing (*e.g.* grading) impact offspring family composition and phenotypic performance. Additionally, pedigree information and phenotypic data from both the current, and a past study (Jenkins, 2018) were combined, in order to estimate the heritability and genetic correlation for dusky kob weight (W), standard length (sL) and Fulton's conditioning factor (K). Thereafter, *Chapter 3* evaluates the presence of

single nucleotide polymorphisms (SNP's) in candidate genes identified *via* a exome capture method in a previous study (Jackson *et al,*.in prep). Several candidate genes were investigated to identify and confirm the presence of SNP's, and to evaluate their association with increased growth in dusky kob.

4.2) Broodstock contributions, genetic diversity and heritability of growth traits in dusky kob

Dusky kob is an indigenous finfish species that naturally spawn in groups in depths of up to 15m in estuarine waters along the east coast of South Africa (Griffiths, 1997). However, in an aquacultural environment, hormonal induction is necessary to overcome the inability to spawn under artificial conditions. Observations in a previous study on dusky kob, by Jenkins (2018), illustrated that hormonal induction of adult broodstock, when utilising female biased mating schemes, does not result in successful seed contribution, and result in highly variable female reproductive success. However, in the present study, two different mating schemes were employed, both of which resulted in variable broodstock partition. In spawning event 1 (SPE1), a male biased mating scheme was used, whilst in SPE2, a non-biased adult sex ratio was utilised. However, these mating schemes did not have an observable effect on broodstock performance in the current study, as only a single male-female pair in both spawning events were found to contribute to the next generation. Evasion of the alternative male in SPE1 may be attributed to differential spawning chronology as only a single female was present in the same tank during the spawn. In SPE2, failure of the alternative male to participate could potentially be due to other factors influence reproductive readiness, such as the physiological condition of broodstock (*i.e.* weight and age) (Brown *et al.*, 2005), as this male was observably smaller in comparison to the dominant male (personal observations) ,whilst nonparticipation of the alternate female in this spawn may be attributed to mate selection and/or differential spawning timings. Nevertheless, male competition among breeders, especially in male biased mating schemes, is a major factor that influences male reproductive success in numerous finfish species, and may explain the low male participation in this study (Brown *et al.*, 2005; Frost *et al.*, 2006; Herlin *et al.*, 2008; Loughnan *et al.*, 2013). Although female biased mating schemes were not employed, female participation in the current study is similar to observation in Jenkins (2018) where only a single female participated in each respective spawning event when the size of the breeding group was reduced (four or five individuals), however, different to the non-biased sex ratio

mating scheme utilised as both males and both females participated in the spawning event in this related study. Therefore, the variable broodstock participations observed across multiple spawning events for this species (Mirimin *et al.*, 2016; Jenkins, 2018), in addition to the observation in the current study, suggests that hormonal induction of adult dusky kob does not assure broodstock participation as differences in gender reproductive performance in commercial mass-spawning species are likely dependant on the mating strategy employed as well as the physiological condition of the breeders involved.

With regards to genetic variability, evaluation of genetic diversity indices indicate that the commercially used broodstock harbour substantially less genetic diversity in comparison to individuals sampled from the wild. Furthermore, this cohort displayed significantly higher relatedness estimates, as well as significantly differentiating from the wild cohort. Therefore, not only does this broodstock cohort places a major restriction on the genetic variation present at this hatchery, future potential genetic improvements are limited as advantageous genetic variation may already be restricted. The resulting progeny cohorts suffered a major reduction in all estimated of genetic diversity, with N_e estimates observably much lower than prior estimates obtained for cultured cohorts of the same species (Mirimin *et al.*, 2016; Jenkins, 2018). This substantial reduction in genetic variability and N_e can in most part be attributed to the founder effect, but also to the relatively low participation of broodstock in both spawning events. Furthermore, the cultured offspring did not experience a reduction in heterozygosity, likely due to the occurrence of a genetic bottleneck produced by the founder effect, however, with the implementation of a selective breeding programme it is anticipated that genetic diversity will decrease as the breeding population becomes more homogenous (Waples, 1990; Jorde & Ryman, 1995; Blonk *et al.*, 2009; Knibb *et al.*, 2014).

The low participation of broodstock as well as the substantially low N_e observed in the current study presented a major setback to the estimation of heritability of growth related traits, as the accuracy and/or bias of such estimates are notably influenced by numerous factors, including the number and size of families employed (Falconer & Mackay, 1996). However, as additional data from four separate spawning events (11 families) were available (Jenkins, 2018), expansion of the resulting dataset and subsequent estimations revealed moderate heritability estimates for dusky kob weight (W) and length (sL). Consequently, the results observed in *Chapter 2* indicate a

significant additive genetic component to W and sL in this species, which could be exploited to achieve genetic improvement of these commercially values traits. Furthermore, the strong positive genetic correlation observed between these traits suggest that similar molecular mechanisms are involved in the expression of both these traits, signifying that selection for sL, which is practically easier to measure, would inadvertently lead to selection for W, and *vice versa*. Furthermore, given the large size of mature adults, measurements of sL and subsequent selection of this trait may be more effective as fish grow older (Gjedrem, 1983). Additionally, Jenkins (2018) reported that phenotypic differences between families, at any given age, were essentially similar, proposing that juvenile growth rate can be used as an early predictor of growth at later developmental stages to market size. On the contrary, heritability of growth related traits in fish is expected to increase with age (Gjedrem, 1983; Su *et al.*, 1996a; Saillant *et al.*, 2006; Wang *et al.*, 2008), and may therefore provide a more appropriate estimate for evaluating potential genetic gains, as offspring growth during early stages of juvenile development are commonly subject to maternal effects which could result in the overestimation of the additive genetic component of the trait of interest. Additionally, the observed heritability estimates are likely influenced by a significant environmental component, as the phenotypic performance of individuals samples from two (out of three) hatcheries were subject to significant environmental effects. Moreover, grading of multiple families also introduces environmental variances between rearing tanks and can lead to an underestimation of heritability estimates (Blonk *et al.*, 2009). Although the phenotypic performance of individuals sampled from recirculation facilities (*i.e.* a more controlled rearing environment) were less variable, and likely to produce a more accurate heritability estimate (by minimising environmental effects), the values observed in the current study may be a more accurate representation of heritability (of W and sL) in dusky kob, as it reflects non-genetic components imposed upon individuals in a practical setting.

In the current study, heritability estimates observer for K were considerably low and not significantly different from zero, indicating that the expression of this trait is attributed to low additive genetic and high environmental components. Furthermore, in accordance with observations in Jenkins (2018), an inverse relationship is observed between K and growth, such that the slowest growing (smaller) individuals display on average the highest K. This inverse relationship is further supported by the negative genetic correlation observed between K and W/sL, and suggests that selective

breeding for faster growth will likely produce heavier, but slimmer fish. Additionally, considering that Fulton's conditioning factor is indicative of body fat accumulation (*i.e.* nutritional status), slower growing individuals appear to be in better physiological condition compared to faster growing individuals. However, when taking into account the low genetic correlations observed between K and traits such as fillet weight and visceral/abdominal fat (Kause *et al.*, 2002), K may not be a reliable indicator of juvenile condition or general "well-being" in dusky kob. Furthermore, if the objective of the breeding programme for this species is to market fillets, a higher K (*i.e.* triangular shape) may have a negative impact on consumer acceptance. However, the observed expression of K, and the negative correlations observed, may be as a result of husbandry practices employed by the hatchery as K has been observed to be highly subject to nutritional intake (Koskela *et al.*, 1998; Kause *et al.*, 2002) and feeding frequency/intensity.

4.3) Genetic polymorphisms and their association with growth traits in dusky kob

Results from *Chapter 2* indicate moderate heritability estimates for weight and length in dusky kob, revealing a significant heritable genetic component to phenotypic expression of growth related traits in this species. Therefore, the results from *Chapter 3* is based on the utilisation of a candidate gene approach to identify molecular components (such as SNP's) associated with increased growth. Until recently, DNA sequence information and relevant molecular studies for dusky kob has been limited to the application of microsatellite marker technologies, however, a recent study by Jackson *et al*. (in prep.) employed a solution based *Danio rerio* (Zebrafish) exome capture kit to capture and sequence homologous exonic regions in the dusky kob genome. Similarly, several studies have taken advantage of sequence information from related species (Primmer *et al.*, 2002; Aitken *et al.*, 2004; Li *et al.*, 2007; Li & Riethoven, 2010) to increase the number of potential target genes in species with limited sequence information. This strategy presents a promising alternative to the conventional candidate gene approach, as larger groups of genes involved in certain physiological traits of interest (Tao & Boulding, 2003; Hancock *et al.*, 2008) can be identified. Therefore, the employment of an exome capture technique has led to the

identification of several candidate genes that are likely involved in the expression of growth related traits in dusky kob.

Preliminary assessment of genotypic segregations patterns, of five novel and ten confirmed SNP's, across the large and small cohorts in two full-sibling families revealed several mutations that displayed significantly distorted frequencies across the two cohorts within each respective family. An increased frequency of a SNP allele or genotype in the large cohort compared to the small indicates that the presence of the SNP allele may elevate the risk of expressing the trait of interest (*i.e.* increased growth) and *vice versa* (Lewis, 2002). However, only two SNP's, one in the Ladybird homeobox 1 b gene (*lbx1b*_c.114) and one in the Tankyrase a gene (*tnksa*_c.69) display statistical association with the trait of interest. Therefore, *lbx1*, which reportedly has important functions in neural and muscle development (Lukowski *et al.*, 2011), and *tnks*, which have key regulatory roles in glucose transport and insulin stimulated glucose uptake (Yeh *et al.*, 2007; Guo *et al.*, 2012), may be considered putative candidate genes for investigating growth related traits in dusky kob. Additionally, a statistical association between specific molecular haplotypes (or alleles) of a candidate gene and the trait of interest is taken as evidence that the gene is either directly involved in the genetic control of the trait, or that the functional polymorphism is sufficiently close to the marker so that the two loci are in linkage disequilibrium (LD) (Lynch & Walsh, 1998). Therefore, *lbx1b*_c.114, which was identified as a non-synonymous mutation, is likely in proximal LD with undetected causal variants(s) in the same gene, or in functional LD with causal genetic variants in essential upstream regulatory pathways. Alternatively, *tnksa*_c.69, which was identified as a non-synonymous mutation, is more likely directly involved in the genetic regulation of the trait. Furthermore, this mutation was also deemed significantly associated with growth in a previous study (Jackson *et al.* in prep) which further highlights the significance of this mutation, and suggests that it may result in functionally significant alterations in the coded enzyme, possibly affecting catalytic ability or substrate binding efficiency.

Significant genotypic distortions observed at multiple SNP's within families indicates that these mutations may also have an effect on the phenotypic expression of growth traits, which is not surprising considering that complex traits are often regulated by small additive effects of many genes (Van Ooijen *et al.*, 1997). However, risk alleles and/or genotypes may be represented in different frequencies across families, and is likely to skew the statistical power of detecting significant associations. Therefore, due

to observable differences between families, likely as a result of a 'population stratification' effect, the expression of complex traits such as growth, may be influenced by genetic differences between family, as has been described by Gjedrem (Gjedrem, 2000). Nevertheless, the results in *Chapter 3* are of relevance in the sense that putative candidate genes are identified, and further investigation into these candidate genes may reveal novel signalling pathways in neural and muscle development, and glucose uptake/transport that influence growth related traits in dusky kob.

4.4) Considerations for the implementation of a selective breeding programme

Results observed in *Chapter 2* highlight a significant additive genetic component to weight and length in dusky kob, whilst analyses in *Chapter 3* reveal molecular components (*i.e.* SNP's) in putative candidate genes that are likely involved in novel signalling pathways altering the expression of growth related traits in this species. Therefore, not only do the findings in both experimental chapters support the successful application of future selective breeding programmes, but current findings also elude to underlying molecular mechanisms (in muscle development and glucose utilisation) significantly influencing phenotypic expression.

The data generated in *Chapter 2*, as well as that of a previous study in dusky kob (Jenkins, 2018), revealed that a single mass-spawning event would not be sufficient to generate adequate numbers of genetically diverse families to establish an efficient selective breeding programme. Several authors have suggested employing a base population with a minimum of 50 unrelated broodstock pairs to limit the loss of genetic diversity, minimise inbreeding and ensure continuous genetic gains (Kincaid, 1983; Bentsen & Olesen, 2002; Sonesson, 2005) through phenotypic-based selective breeding. However, establishment of such a breeding population is not feasible for dusky kob, as the species reaches sexual maturity at a very large size (approximately one meter in length). Alternatively, broodstock can be spawned in multiple isolated smaller breeding groups (three to six individuals) with each generation of cultured dusky kob comprised of offspring originating from multiple and diverse spawns (Robinson *et al.*, 2010). This method presents a promising alternative to large mass-spawning events, as the phenotypic variance, and possible cannibalistic behaviour, among resulting progeny cohorts can be minimised. However, multiple spawns will, therefore, need to be carried out in order to increase the number of unrelated families

required for selective breeding. Furthermore, results from *Chapter 2* illustrate that dusky kob growth rate during early juvenile development can be used as an indicator for growth at later stages to market size. Broodstock candidate selection can, therefore, be performed during the early stages of juvenile development, where faster growing juveniles can be removed from progeny cohorts and primed for spawning from a relatively early age. Rotational spawning and early broodstock candidate selection not only presented a unique opportunity to minimise inbreeding, but also achieve genetic improvement *via* phenotypic-based selection. Regardless, employing a rotational spawning approach will not only increase the generation time, but separate rearing of multiple offspring cohorts is likely to introduce environmental (*i.e.* tank) and physiological (*i.e.* age) variation among cultured offspring (as observed in *Chapter 2*). Consequently, with each consecutive spawn, the percentage of variation attributed to such factors will inevitably become larger, and complicate the estimation of genetic parameters for traits of interest in the breeding population, and potentially mask genetic differences between broodstock candidates and slow genetic improvement.

Although the South African dusky kob industry may not have the capacity to achieve the desired base population size recommended to initiate a selective breeding programme, the industry does meet the infrastructural requirements, and reportedly has numerous suitable breeding candidates (*i.e.* > 30 candidates) (Jenkins, 2018) to greatly increase the size of the breeding population. Therefore, increasing the base population size will allow for greater numbers of genetically unrelated families to be produced. Additionally, as larger breeding populations are often required to stimulate natural spawning behaviour in many sciaenid species (Gorshkov *et al.*, 1997; Ueng *et al.*, 2007; Loughnan *et al.*, 2013; Domingos *et al.*, 2014; Liu *et al.*, 2017), increasing the size of the base population may stimulate more successful broodstock participation and a more even contributions among parents to offspring. Another advantage of conducting a large spawn at the start of a selective breeding programme is that the genetic parameters for a variety of traits and be more accurately determined as non-genetic components or variance (*i.e.* age and tank effects) are regarded as being minimal. Nevertheless, increased growth rate will undoubtable remain the major driver behind selection in fin-fish aquaculture, and considering the moderate heritability observed for these traits in dusky kob, related individuals are still likely to be selected as broodstock candidates to contribute to the next generation. Therefore, a selective breeding programme for this species needs to consider both individual growth

performance as well as genetic relatedness between selected individuals, *e.g.* using walk-back selection. Walk-back selection generally applies DNA profiling as a means of identifying parentage of selected individuals in an attempt to minimise kinship and maximise genetic gains in the absence of pedigree information (Doyle & Herbinger, 1994). Additionally, together with DNA profiling, marker-assisted selection (MAS) presents an opportunity to increase genetic gains even further. Results from *Chapter 3* illustrate favourable genotypes at two candidate genes that are associated with increased growth. Although these findings are still regarded as preliminary, and development of MAS technologies for dusky kob are now considered to be in its infancy, screening potential broodstock candidates for favourable genotypes (or alleles) at candidate genes associated with growth will inevitable aid in the genetic improvement of growth related traits in this species. Furthermore, although the costs associated with DNA profiling and genotyping are relatively high, it can be argued that for species with extremely long generational intervals (such as dusky kob and red drum), the long-term genetic gains and associated benefits (*e.g.* faster production turn-over and larger product) may offset initial investments. Considering the few commercial hatcheries that are in operation in South Africa, one of which commonly distributes seed animals to other hatcheries for grow-out, significant genetic improvements achieved in the nucleus may therefore radicalize improvement of growth traits in this species throughout the industry. Therefore, for this purpose, farmers should separate the improved line from the unselected population to prevent genetic deterioration.

4.5) Shortcomings and perspectives on future undertakings

One clear limitation in the present study is the small sample size (*i.e.* few families) that was utilised. The culturing facility where fin-clip samples were obtained employed a rotational spawning approach, where broodstock individuals were housed in smaller breeding groups of three to six individuals per tank and spawned at different times through the year. However, at the time of sampling, only two (out of five) breeding groups were hormonally induced to spawn. Therefore, sampling at later stages would most likely have resulted in the acquisition of a larger number of samples, and more importantly, an increased number of families being sampled. Regardless of this, hormonal induction of these two breeding groups still resulted in variable broodstock participation, and is, therefore, in accordance with observations in a previous study performed on the same species (Jenkins, 2018), as not all candidates contributed to the offspring cohorts. Therefore, investigation into factors influencing individual

spawning such as sexual selection and competition (Weir *et al.*, 2004; Fessehaye, 2006; Fessehaye *et al.*, 2009), factors influencing reproductive readiness such as sperm quality and competition (Campton, 2004; Wedekind *et al.*, 2007), egg quality and maturation (Watanabe *et al.*, 2005; Bogevik *et al.*, 2012) as well as broodstock weight and maturity (Brown *et al.*, 2005; Loughnan *et al.*, 2013) merits further study. Previous studies have highlighted a strong correlation between egg quality (*e.g.* egg size, number and volume) and larval size and survivability (Su *et al.*, 1997; Vandeputte *et al.*, 2002; Johnson *et al.*, 2010; Johnson *et al.*, 2011). However, results observed in a previous study on dusky kob (Jenkins, 2018) revealed that there may not always be a positive correlation between offspring family size and growth. The size of adult broodstock was not accounted for in the present study, therefore, it would be of interest to investigate how adult breeder size correlates to family size, and the growth of resulting progeny cohorts. Nevertheless, the use of such a curtailed sample population not only failed to represent the true extent of genetic diversity present at the hatchery, but also constrained many aspects genetic analyses.

Preliminary estimates of heritability for dusky kob weight and length were observably inflated due to the overestimation of variance components between the two full-sibling families. However, as pedigree and phenotypic data of several dusky kob families were available from a previous study (Jenkins, 2018), expansion of the dataset and subsequent analysis revealed moderate to high heritability estimates for dusky kob weight and length, demonstrating the presence of additive genetic variation for these traits. However, data was obtained from multiple spawning events spanning over two to three years, therefore, the true heritability at a given age could not be determined. Nevertheless, moderately to high heritability observed in the current study represents the most accurate depiction of heritability of weight and length in dusky kob. Additionally, in order to obtain a reliable estimate for K in dusky kob, many more individuals of similar ages, reared in a common environment, will need to be sampled, as growth phase is likely to vary with age. Alternatively, the results observed in *Chapter 2* would suggest that K may not be a reliable indicator for body conformation is dusky kob. Juvenile shape characteristics may have an impact on market acceptance in a developing or competitive dusky kob industry, therefore, other body conformation indices, apart from K, may have an impact in this species. Body depth (BD) or perhaps body shape (H), which is the ratio between BD and sL, could be a more reliable indicator of body conformation and perhaps juvenile condition. Interestingly, a positive

genetic correlation between fish wet weight and BD (or H) have been reported (Gjedrem & Thodesen, 2005; Domingos *et al.*, 2013;) and the possibility of simultaneous selection of these traits should be further investigated. Regardless of this, the heterogeneous nature of the resulting dataset inflated variances associated with environmental factors, and likely biased estimates of heritability in the present study. In fact, the data generated in *Chapter 2* suggests that offspring cohorts in two (out of three) hatcheries were subjected to significant environmental effects. Therefore, to exploit the full potential of selective breeding programmes for dusky kob, estimation of genotype by environment (G X E) interactions for growth related traits should also be conducted (Dupont-Nivet *et al.*, 2008; Vandeputte & Haffray, 2014; Difford *et al.*, 2017).

Due to the substantially low N_e (1.90) observed in *Chapter 2*, broodstock breeding values could not be accurately determined. Regardless of this, a major drawback of progeny testing (*via* DNA profiling and phenotyping) is that parental breeding values can only be estimated after offspring individuals are large enough to be phenotyped. Alternatively, the estimation of RNA/DNA ratios, which is considered to be an indicator of protein synthesis, has been widely used to predict fish condition and growth (Buckley & Szmant, 2004; Foley *et al.*, 2016). The effectiveness of this method is particularly suited for evaluating larvae, as metabolic activity and cell growth (and thus, RNA regulation) during early juvenile development stages are at its peak (Johnston, 2006). Therefore, similar to MAS, faster growing larvae can be identified before the trait is expressed, and can subsequently be genotyped to reveal faster-growing parents (Marshall & Morgan, 2011; Domingos *et al.*, 2013). Additionally, this technique has also proven useful for predicting growth of adult fish, though mostly with fish of similar age (Bulow, 1970; Smith & Buckley, 2003). To a larger extent, this method can also be used for population comparisons across multiple locations (De Raedemaecker *et al.*, 2012; Vinagre *et al.*, 2008), which will allow hatchery managers and farmers to source broodstock from specific geographic locations. However, if such methods were to be employed, estimates of N_e throughout species ranges, between subpopulations, and more importantly individual relatedness should be considered.

The small sample size (*i.e.* few families) utilised in *Chapter 3* is notably not ideal for assessing SNP genotype association with increased growth. Evaluation of genotypic segregation patterns and genotypic (or allelic) frequencies associated with the trait of interest across similar age families would have strengthened association analyses, and

possibly revealed additional polymorphisms in other candidate genes where effects were masked in the current study. Additionally, although the exome capture technique presented major advantages in the identification of candidate genes involved in the expression of growth traits, this approach does present some limitations. One clear limitation of this technique is that only the coding regions of a particular gene is captured and sequenced. Therefore, promoter regions, located near the transcription start site in the 5' upstream region, and intronic regions, located within the gene of interest, are not displayed. Furthermore, whether or not the full coding region is displayed remains unclear. As a result, causal variants occurring in these regions are likely to be missed, and LD between such variants and other detected SNP's could lead to false positives. Nevertheless, utilising an exome capture approach could lead to the identification of candidate genes, and the subsequent identification of molecular variants (albeit through LD), and should be further investigated. To date, the study by Jackson *et al*. (in prep.) is the only study to employ a next generation sequencing (NGS) approach to generate and utilise sequencing information of dusky kob. Therefore, employing a whole genome sequencing approach to generate the first ever reference genome sequence for dusky kob, to allow for the investigation into the generic architecture, construction of linkage maps and the identification and subsequent mapping of QTL's is the logical next step towards the eventual application of MAS technologies for this species.

4.6) Concluding remarks

Overall, this study represents the most comprehensive survey of genetic variation in farmed populations of dusky kob and South African marine finfish in general. The results observed in *Chapter 2* suggests that, firstly, mass-spawning production practices did not impact genic heterozygosity, which can be attributed to a detected bottleneck likely produced by the founder effect. Secondly, the substantial increase in genetic relatedness observed in cultured populations will most likely lead to excessive inbreeding and significant losses of genetic diversity in later generations of selective breeding. Thirdly, given the moderate to high heritabilities observed for dusky kob weight and length, selection for growth rate would further accelerate the effects of inbreeding, and will likely lead to inbreeding depression. Therefore, whilst growth traits will likely remain the major phenotypic criterion for selection, and group spawns continued to be implemented, a prospective selective breeding programme will need

to consider individual relatedness in order to minimise inbreeding. Additionally, the results observed in *Chapter 3* revealed that two candidate genes significantly influence expression of this trait. Therefore, in addition to evaluating parentage, individuals can be screened to identify favourable genotypes at SNP markers in such candidate genes to aid in the genetic improvement of growth related traits even further. With this in mind, molecular genetics is expected to play a crucial role in the development of aquaculture breeding programmes, and aid in the genetic improvement of commercially valued traits *via* marker-assisted selection.

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Appendix A

Supplementary information for Chapter 2

Table A2.1: Genetic diversity indices across three populations for each microsatellite marker, expressed as mean number of alleles (A_n), allelic richness (A_r), effective number of alleles (A_e), private allelic richness (PA_r), expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e) and fixation index (F).

Table A2.2: Mean and median phenotypic performance, associated standard deviation and variances for dusky kob growth traits weight (W), standard length (sL) and Fulton's conditioning factor (K). Additionally, the covariation and correlation between each respective trait is also indicated. Each sampling cohort is indicated along with the hatchery of origin, the number of tanks sampled for each cohort, the age of the cohort (in days post hatch) as well as the number of individuals sampled per tank.

T**able A2.3**: Mean and median growth rate, associated standard deviation and variances for dusky kob growth traits weight (W), standard length (sL) and Fulton's conditioning factor (K). Additionally, the covariation and correlation between each respective trait is also indicated. Each sampling cohort is indicated along with the hatchery of origin, the number of tanks sampled for each cohort, the age of the cohort (in days post hatch) as well as the number of individuals sampled per tank.

Table A2.1: Genetic diversity indices across three populations for each microsatellite marker, expressed as mean number of alleles (A_n), allelic richness (A_r), effective number of alleles (A_e), private allelic richness (PA_r), expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e) and fixation index (F).

	AJAP06	***UBA05***	***UBA06***	***UBA42***	***AJAP12***	***AJAP14***	***AJAP37***	***UBA40***	***AJAP05***	***UBA50***	***UBA854***	***UBA853***
Cultured progeny (CP, n = 368)												
A_n	5.000	3.000	4.000	6.000	6.000	4.000	5.000	6.000	4.000	5.000	3.000	7.000
A_e	3.248	2.989	3.512	3.060	5.287	3.856	3.083	3.215	3.369	4.097	2.540	6.570
H_o	0.735	0.853	0.745	0.703	0.992	0.756	0.749	0.717	0.826	0.837	0.678	0.934
H_e	0.692	0.665	0.715	0.673	0.811	0.741	0.676	0.689	0.703	0.756	0.606	0.848
uH_e	0.693	0.666	0.716	0.674	0.812	0.742	0.677	0.690	0.704	0.757	0.607	0.849
A_r	4.977	3.000	3.994	5.205	5.982	4.000	4.091	5.730	3.999	4.959	2.997	6.964
PA_r	0.000	0.000	0.000	0.103	0.003	0.000	0.011	0.112	0.000	0.003	0.000	0.053
F_{IS}	-0.062	-0.282	-0.042	-0.044	-0.223	-0.021	-0.109	-0.040	-0.175	-0.107	-0.117	-0.102
Commercial broodstock (CB, n = 23)												
A_n	7.000	6.000	6.000	6.000	7.000	7.000	5.000	7.000	6.000	6.000	4.000	7.000
A_e	5.568	4.301	4.283	2.665	4.483	5.426	2.023	3.875	3.245	3.162	2.446	4.054
H_o	0.739	0.913	0.870	0.609	0.522	0.783	0.609	0.783	0.826	0.450	0.650	0.652
H_e	0.820	0.767	0.767	0.625	0.777	0.816	0.506	0.742	0.692	0.684	0.591	0.753
uH_e	0.839	0.785	0.784	0.639	0.794	0.834	0.517	0.758	0.707	0.701	0.606	0.770
A_r	6.999	5.870	5.984	5.855	6.739	6.868	4.855	6.609	5.867	6.000	4.000	6.737
PA_r	0.198	0.167	0.130	0.359	0.359	1.878	0.061	0.268	0.091	1.016	1.000	0.009
F_{IS}	0.099	-0.190	-0.134	0.026	0.328	0.041	-0.204	-0.055	-0.194	0.342	-0.099	0.134
Wild samples (WS, n = 34)												
A_n	7.000	8.000	9.000	10.000	12.000	14.000	6.000	8.000	12.000	9.000	7.000	12.000
A_e	3.461	6.568	6.943	3.778	6.820	7.387	2.083	4.587	8.000	6.780	3.451	6.440
H_o	0.706	0.882	0.794	0.765	0.794	0.853	0.559	0.824	0.882	0.765	0.735	0.941
H_e	0.711	0.848	0.856	0.735	0.853	0.865	0.520	0.782	0.875	0.853	0.710	0.845
uH_e	0.722	0.860	0.869	0.746	0.866	0.878	0.528	0.794	0.888	0.865	0.721	0.857

A_r	6.332	7.826	8.799	8.176	10.458	12.100	5.537	7.230	11.071	8.514	5.945	10.338
PA_r	0.001	2.127	3.011	2.688	4.163	7.122	0.835	1.532	5.167	3.547	2.945	3.947
F_{IS}	0.007	-0.041	0.072	-0.040	0.069	0.014	-0.075	-0.053	-0.008	0.103	-0.035	-0.114
PIC	0.707	0.718	0.745	0.645	0.789	0.778	0.523	0.703	0.719	0.733	0.569	0.790
F_{IS}	0.020	-0.161	-0.030	-0.021	0.055	0.012	-0.127	-0.050	-0.116	0.105	-0.081	-0.033
F_{IT}	0.059	-0.110	-0.001	-0.009	0.074	0.040	-0.081	-0.016	-0.060	0.164	-0.043	0.004
F_{ST}	0.040	0.044	0.028	0.012	0.021	0.029	0.041	0.032	0.051	0.066	0.035	0.036

Table A2.2: Mean and median phenotypic performance, associated standard deviation and variances for dusky kob growth traits weight (W), standard length (sL) and Fulton's conditioning factor (K). Additionally, the covariation and correlation between each respective trait is also indicated. Each sampling cohort is indicated along with the hatchery of origin, the number of tanks sampled for each cohort, the age of the cohort (in days post hatch) as well as the number of individuals sampled per tank.

					Traits											
Hatchery	***Cohorts***	***Age***	***Tanks***	***N***	**Weight (g)**				**Standard Length (mm)**				**K**			
					Median	***Mean***	***s.d.***	***Variance***	***Median***	***Mean***	***s.d.***	***Variance***	***Median***	***Mean***	***s.d.***	***Variance***
Oc	Oc_1	32	1	28	0.496	0.707	±0.465	0.217	31.100	31.964	±6.894	47.532	1.929	1.927	±0.496	0.246
			2	31	0.351	0.543	±0.430	0.185	25.700	27.403	±7.371	54.334	2.158	2.195	±0.411	0.169
			3	34	0.300	0.425	±0.291	0.085	24.400	26.509	±5.963	35.563	1.940	2.016	±0.322	0.103
			4	10	0.164	0.178	±0.052	0.003	20.450	20.750	±1.712	2.929	1.972	1.950	±0.157	0.025
		152	5	100	41.500	42.170	±12.823	164.430	137.000	139.230	±15.749	248.017	1.532	1.523	±0.144	0.021
		796	6	100	490.000	503.650	±168.545	28407.503	325.000	326.200	±41.585	1729.354	1.404	1.412	±0.142	0.020
	Oc_2	393	7	19	240.000	238.684	±68.977	4757.895	245.000	251.842	±28.830	831.140	1.428	1.471	±0.207	0.043
			8	21	245.000	222.143	±84.344	7113.929	240.000	243.333	±31.358	983.333	1.456	1.473	±0.141	0.020
			9	22	332.500	344.773	±121.307	14715.422	280.000	281.136	±32.875	1080.790	1.494	1.492	±0.126	0.016
			10	36	347.500	355.833	±147.844	21857.857	282.500	284.583	±40.327	1626.250	1.450	1.466	±0.122	0.015
PO	PO_1	484	11	25	415.000	412.360	±144.573	20901.490	330.000	326.400	±38.851	1509.417	1.157	1.140	±0.093	0.009
			12	50	390.000	416.740	±162.264	26329.707	325.000	330.400	±38.503	1482.490	1.150	1.119	±0.167	0.028
			13	25	380.000	419.400	±102.474	10500.833	330.000	334.400	±26.509	702.750	1.081	1.107	±0.103	0.011
	PO_2	121	14	102	20.000	23.176	±10.333	106.761	119.000	119.255	±17.161	294.489	1.274	1.286	±0.117	0.014
Mtz	Mtz	112	15	33	4.000	4.242	±1.464	2.143	59.000	58.485	±6.699	44.883	1.993	2.052	±0.213	0.045
			16	62	10.000	9.831	±1.797	3.229	80.000	79.790	± 5.176	26.791	1.900	1.919	±0.139	0.019
			17	70	12.750	14.843	±7.389	54.604	86.500	90.600	±14.230	202.504	1.938	1.907	±0.186	0.034
		175	18	74	11.500	11.924	±4.305	18.529	88.000	86.892	±11.297	127.632	1.751	1.756	±0.206	0.043
			19	55	24.500	25.000	±5.742	32.972	115.000	114.436	±10.094	101.880	1.653	1.661	±0.210	0.044
			20	49	46.500	50.357	±12.281	150.833	143.000	144.082	±10.981	120.577	1.658	1.661	±0.147	0.022

Hatchery	*Cohorts*	*Age*	*Tanks*	*N*	Weight / sLength		Weight / K		sLength / K	
					Covariance	Correlation	Covariance	Correlation	Covariance	Correlation
Oc	Oc_1	32	1	28	3.052	0.951	0.026	0.112	-0.464	-0.136
			2	31	3.097	0.977	0.004	0.025	-0.340	-0.112
			3	34	1.697	0.978	-0.006	-0.059	-0.357	-0.186
			4	10	0.084	0.950	0.004	0.512	0.062	0.230
		152	5	100	187.036	0.926	0.053	0.029	-0.697	-0.308
		796	6	100	6803.404	0.971	-7.416	-0.310	-2.943	-0.499
	Oc_2	393	7	19	1880.336	0.946	-3.306	-0.231	-3.100	-0.519
			8	21	2551.250	0.965	2.670	0.225	-0.019	-0.004
			9	22	3858.604	0.968	3.521	0.231	0.205	0.049
			10	36	5774.643	0.969	-0.137	-0.008	-0.975	-0.198
PO	PO_1	484	11	25	5486.350	0.977	2.761	0.205	0.060	0.016
			12	50	5491.841	0.879	7.675	0.283	-1.108	-0.172
			13	25	2526.292	0.930	1.260	0.120	-0.659	-0.242
	PO_2	121	14	102	170.875	0.964	0.271	0.224	0.040	0.020
Mtz	Mtz	112^a 175^b	15	33	9.348	0.953	0.044	0.140	-0.191	-0.134
			16	62	8.677	0.933	0.019	0.075	-0.200	-0.278
			17	70	101.965	0.970	-0.539	-0.393	-1.440	-0.545
			18	74	46.474	0.956	-0.093	-0.105	-0.794	-0.341
			19	55	48.870	0.843	-0.066	-0.054	-1.215	-0.572
			20	49	126.689	0.939	0.268	0.149	-0.297	-0.184

Table A2.3: Mean and median growth rate, associated standard deviation and variances for dusky kob growth traits weight (W/dph), standard length (sL/dph) and Fulton's conditioning factor (K/dph). Additionally, the covariation and correlation between each respective trait is also indicated. Each sampling cohort is indicated along with the hatchery of origin, the number of tanks sampled for each cohort, the age of the cohort (in days post hatch) as well as the number of individuals sampled per tank.

					Traits											
Hatchery	***Cohorts***	***Age***	***Tanks***	***N***	**Weight (g/dph)**				**Standard Length (mm/dph)**				**K/dph**			
					Median	***Mean***	***s.d.***	***Variance***	***Median***	***Mean***	***s.d.***	***Variance***	***Median***	***Mean***	***s.d.***	***Variance***
Oc	Oc_1	32	1	28	0.016	0.022	0.015	0.972	0.999	0.215	0.060	0.060	0.016	0.016	0.022	0.015
			2	31	0.011	0.017	0.013	0.803	0.856	0.230	0.067	0.069	0.013	0.011	0.017	0.013
			3	34	0.009	0.013	0.009	0.739	0.804	0.180	0.059	0.061	0.010	0.009	0.013	0.009
			4	10	0.005	0.005	0.002	0.620	0.629	0.052	0.060	0.059	0.005	0.005	0.005	0.002
		152	5	100	0.273	0.277	0.084	0.901	0.916	0.104	0.010	0.010	0.001	0.273	0.277	0.084
		796	6	100	0.616	0.633	0.212	0.408	0.410	0.052	0.002	0.002	0.000	0.616	0.633	0.212
	Oc_2	393	7	19	0.611	0.607	0.176	0.623	0.641	0.073	0.004	0.004	0.001	0.611	0.607	0.176
			8	21	0.623	0.565	0.215	0.611	0.619	0.080	0.004	0.004	0.000	0.623	0.565	0.215
			9	22	0.846	0.877	0.309	0.712	0.715	0.084	0.004	0.004	0.000	0.846	0.877	0.309
			10	36	0.884	0.905	0.376	0.719	0.724	0.103	0.004	0.004	0.000	0.884	0.905	0.376
PO	PO_1	484	11	25	0.857	0.852	0.299	0.682	0.674	0.080	0.002	0.002	0.000	0.857	0.852	0.299
			12	50	0.806	0.861	0.335	0.671	0.683	0.080	0.002	0.002	0.000	0.806	0.861	0.335
			13	25	0.785	0.867	0.212	0.682	0.691	0.055	0.002	0.002	0.000	0.785	0.867	0.212
	PO_2	121	14	102	0.165	0.192	0.085	0.983	0.986	0.142	0.011	0.011	0.001	0.165	0.192	0.085
Mtz	Mtz	112[a] 175[b]	15	33	0.036	0.038	0.013	0.527	0.522	0.060	0.018	0.018	0.002	0.036	0.038	0.013
			16	62	0.089	0.088	0.016	0.714	0.712	0.046	0.017	0.017	0.001	0.089	0.088	0.016
			17	70	0.073	0.094	0.071	0.494	0.555	0.183	0.011	0.011	0.002	0.073	0.094	0.071
			18	74	0.098	0.104	0.040	0.772	0.757	0.120	0.016	0.015	0.002	0.098	0.104	0.040
			19	55	0.140	0.143	0.033	0.657	0.654	0.058	0.009	0.009	0.001	0.140	0.143	0.033
			20	49	0.269	0.291	0.072	0.817	0.833	0.087	0.009	0.010	0.001	0.269	0.291	0.072

Hatchery	*Cohorts*	*Age*	*Tanks*	*N*	Weight / sLength		Weight / K		sLength / K	
					Covariance	Covariance	Correlation	Covariance	Correlation	Covariance
Oc	Oc_1	32	1	28	0.003	0.951	0.000	0.112	0.000	-0.136
			2	31	0.003	0.977	0.000	0.025	0.000	-0.112
			3	34	0.002	0.978	0.000	-0.064	0.000	-0.194
			4	10	0.000	0.950	0.000	0.512	0.000	0.230
		152	5	100	0.008	0.926	0.000	0.029	0.000	-0.308
		796	6	100	0.011	0.971	0.000	-0.310	0.000	-0.499
	Oc_2	393	7	19	0.012	0.946	0.000	-0.231	0.000	-0.519
			8	21	0.017	0.965	0.000	0.225	0.000	-0.004
			9	22	0.025	0.968	0.000	0.231	0.000	0.049
			10	36	0.037	0.969	0.000	-0.008	0.000	-0.198
PO	PO_1	484	11	25	0.023	0.977	0.000	0.205	0.000	0.016
			12	50	0.023	0.879	0.000	0.283	0.000	-0.172
			13	25	0.011	0.930	0.000	0.120	0.000	-0.242
	PO_2	121	14	102	0.012	0.964	0.000	0.224	0.000	0.020
Mtz	Mtz	112^a 175^b	15	33	0.001	0.953	0.000	0.140	0.000	-0.134
			16	62	0.001	0.933	0.000	0.075	0.000	-0.278
			17	70	0.012	0.962	0.000	0.497	0.000	0.573
			18	74	0.004	0.919	0.000	0.119	0.000	0.201
			19	55	0.002	0.843	0.000	-0.054	0.000	-0.572
			20	49	0.005	0.818	0.000	0.264	0.000	0.369

Appendix B

Supplementary information for Chapter 3

Table A3.1: Number of individuals genotypes as well as mean weight (in grams) for all genotypes at each respective SNP marker per family.

Table A3.1: Number of individuals genotypes as well as mean weight (in grams) for all genotypes at each respective SNP marker per family.

Locus	Cohort	Successfully genotyped	Genotypic frequencies		
			T/T	**T/A**	**-**
*lbx1b*_c.114	SPE1	24	37.50 ±5.61	8.50 ±0.87	-
	SPE2	24	20.50 ±3.11	5.75 ±1.45	-
			G/G	**G/T**	**-**
*myod1*_c.34	SPE1	18	29.61 ±9.04	32.94 ±8.64	-
	SPE2	19	13.92 ±3.47	17.82 ±4.24	-
			G/G	**G/C**	**C/C**
*myod1*_c.43	SPE1	24	28.50 ±6.32	36.68 ±8.31	33.33 ±12.02
	SPE2	24	15.89 ±4.52	20.23±4.04	13.50 ±11.50
			T/T	**T/C**	**-**
*myod1*_c.123	SPE1	24	-	-	-
	SPE2	24	19.06 ±3.18	16.84 ±5.07	-
			T/T	**T/C**	**-**
myod1.1 _c.125	SPE1	24	35.17 ±9.13	33.69 ±5.96	-
	SPE2	24	17.15 ±3.04	19.52 ±5.85	-
			T/T	**C/T**	**C/C**
myod1.2 _c.166	SPE1	24	25.50 ±13.70	33.69 ±6.55	44.40 ±10.31
	SPE2	24	-	20.61 ±4.07	16.50 ±3.87
			G/G	**A/G**	**A/A**
*myf5*_c.95	SPE1	23	36.62 ±8.70	24.44 ±7.68	44.93 ±11.64
	SPE2	21	-	18.38 ±3.41	17.5 ±4.97
			T/T	**T/C**	**C/C**
*tnksa*_c.69	SPE1	24	47.10 ±9.05	26.08 ± 6.04	14.50 ±3.00
	SPE2	24	13.62 ±3.75	18.04 ±3.07	26.85 ±23.85
			G/G	**G/A**	**-**
*tnksa*_c.104	SPE1	24	-	-	-
	SPE2	24	25.40 ±7.09	14.36 ±2.01	-
			T/T	**A/T**	**A/A**
*tnksa*_c.58	SPE1	24	-	-	-
	SPE2	24	14.76 ±3.08	12.00 ±3.71	21.67 ±4.72
			C/C	**C/T**	**-**
*tnksa*_c.74	SPE1	24	42.71 ±8.12	25.04 ±6.07	-
	SPE2	24	16.36 ±3.47	20.40 ±4.88	-
			G/G	**G/A**	**-**
*tnksa*_c.85	SPE1	24	41.04 ±8.52	26.71 ±5.92	-
	SPE2	24	22.84 ±6.49	15.64 ±2.74	-
			A/A	**A/C**	**-**
*tnksa*_c.107	SPE1	24	34.79 ±6.64	32.60 ±9.06	-
	SPE2	24	-	-	-
			T/T	**T/A**	**-**
*tuba8l2*_c.14	SPE1	22	-	-	-
	SPE2	23	18.84 ±3.55	15.71 ±5.68	-
			G/G	**G/A**	**-**
*tuba8l2*_c.58	SPE1	22	38.40 ±6.56	30.38 ±8.74	-
	SPE2	23	10.75 ±1.25	18.57 ±3.20	-