GENETIC STRUCTURE OF CRIMSON JOBFISH, *Pristipomoides filamentosus* (VALENCIENNES, 1830) POPULATION OF THE SOUTHWEST INDIAN OCEAN

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Declaration

This thesis is my original work and has not been presented for the award of a degree in any other university or for any other award.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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Dedication

I dedicate this research to my beloved husband for his moral support and to my parents who taught me that even the largest task can be accomplished if it is done one step at a time.

Abstract

Crimson jobfish, *Pristipomoides filamentosus* is a commercially important tropical snapper. Their aggressive nature and relatively large size makes them more vulnerable to fishing gears. In addition it is vulnerable to overfishing due to low rates of growth and recruitment, high natural mortality and prolongation of the attainment of sexual maturity. *P. filamentosus* in South West Indian Ocean has shown signs of decreased abundance of yields and catch per unit effort beyond sustainable levels. This highlights the need for more directed and stringent fisheries management. Therefore if the genetic population structure of a species is known, the distribution of subpopulations in mixed fisheries can be estimated and this will ensure long term management of fish stocks. In the present study, genetic connectivity and population structure of *P. filamentosus* in SWIO was studied in order to clarify whether its populations are genetically distinct or admixed. The entire research work was conducted from July 2012-April 2013.Samples were collected from Kenya, Tanzania, Madagascar, Comoros, Seychelles, Mauritius and South Africa from the artisanal and commercial fishers of the respective countries. DNA was extracted using purelink kit, amplification was done by 15 hyper-variable nuclear microsatellites and mtDNA cytochrome *b* markers, statistical softwares used for genotyped data were Genemapper, GeneAlex, Powermarker and Darwin and Arlequin, Bioedit, CLC main work bench and network for sequenced data. Results indicated significant and moderate (mtDNAF_{ST}=0.062; microsatellites F_{ST}=0.100) genetic differentiation of *P. filamentosus* in Southwest Indian Ocean. Three distinct populations were detected across the region (K=3), it was also revealed that populations are expanding (D=-1.5387). It is recommended that countries sharing the same population of *P. filamentosus* should enforce coordination and cooperation in the management of this species to enhance sustainable harvesting.

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List of Acronyms

ABI	Applied Biosystems
AMOVA	Analysis of molecular Variance
BecA	Bio Science eastern and central Africa
bP	Base pair
ddH_20	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
EAC	East Africa Coastal Current
ESU	Ecological Significant Unit
FAO	Food Agricultural Organization
Hd	Haplotype diversity
IUCN	International Union for Conservation of Nature
L_m	Length at first maturity
Mt	Mitochondrial
N	Number
Na	Number of alleles
NE	North East
NH	Number of haplotypes
NJ	Neighbour joining
Nm	Gene flow
Pfil	*Pristipomoidesfilamentosus*
PCR	Polymerase Chain Reaction
QIA	Qiagen
RNA	Ribonucleic acid
rpm	Rotation per minute
SE	South East
SSR	Simple sequence repeat
SWIO	Southwest Indian Ocean

Tm	Melting temperature
WIO	Western Indian Ocean

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CHAPTER ONE

INTRODUCTION

1.1: Background

1.1.1: Description of *Pristipomoides filamentosus*

Pristipomoides filamentosus has an elongated body and its inter orbital space is flat but with a slightly protruding lower jaw; both jaws have an outer row of conical canine teeth and an inner band of villiform teeth (Froese and Pauly, 2011). Canines at front of lower jaw are not greatly enlarged and vomerine tooth are patched triangular but the tongue has no teeth. Dorsal and anal fins have both spines and soft rays, dorsal fins have 10 spines and 12 soft rays, anal fins has 3 spines and 8 soft rays, their bases have no scales and their last soft ray is extended in to short filaments(Froese and Pauly, 2011). Pectoral fins have 15 or 16 rays and are long reaching the level of anus and the caudal fin is forked. Its body has relatively small scales, it is estimated that there are around 60-65 scales in the lateral line, which are arranged in a backward manner parallel to the lateral line (Froese and Pauly, 2011). The body colour of this species varies, colour of back and sides varies from brownish to lavender or reddish purple, the snout and inter-orbital space has yellow lines and blue spots and the dorsal and caudal fins are light blue or lavender with reddish orange margins (Froese and Pauly, 2011) this is as shown in plate 1.

Plate 1: Crimson jobfish*, Pristipomoides filamentosus* (Source: Keoki Stender, 1992).

1.1.2: Life history and classification

The snapper family, Lutjanidae, belongs to the order Perciformes, which is the largest order of vertebrates with 148 families and nearly 9,300 species. The Perciformes is a large group of spiny-rayed fishes that are common in tropical and subtropical seas, and are usually found in coastal areas; however, it also includes a few families restricted to fresh water (Nelson, 1994). The family Lutjanidae includes four subfamilies: Etelinae, Apsilinae, Paradichthyinae and Lutjaninae that together encompass 107 species (Iwatsuki *et al.*, 1993, Moura and Lindeman, 2007). The largest is the subfamily Lutjaninae with three monotypic genera (Hoplopagrus, Ocyurus, and Rhomboplites), the two genera Macolor and Pinjalo with two species each, and the genus Lutjanus with 66 species. Three smaller subfamilies include the Paradicichthyinae with two monotypic genera (*Symphorus* and *Symphorichthys)*, the Etelinae with five genera (*Aphareus, Aprion, Etelis, Pristipomoides* and *Rhandallichthys*) and 18 species, and the Apsilinae with four genera (*Apsilus, Lipocheilus, Paracesio* and *Parapristipomoides*) and 10 species (Allen, 1985). The genus *pristipomoides*consists of 11 species (*P. aquilonaris, P. argyrogrammicus, P. auricilla, P. filamentosus, P. flavipinnis, P. freemani, P. macrophthalmus, P. multidens, P. sieboldii, P. typus, P. zonatus*) (Anderson, 1986).

Summary on the classification of *P. filamentosus*

Kingdom:	Animalia
Phylum:	Chordata
Class:	Actinopterygii
Order:	Perciformes
Family:	Lutjanidae
Genus :	*Pristipomoides*
Species:	*Pristipomoides filamentosus*

1.1.3: Biology of *P. filamentosus*

Adults and large juveniles of the deep-water snappers and groupers tend to be caught over high relief features at depths of 100-500m, indicating a preference of this type of habitat. *Pristipomoides filamentosus* is found in the depth of 30–360 m (Moffitt, 1993). They mainly feed on fish, cephalopods, benthic invertebrates and pelagic gastropods and urochordates (Allen, 1985). Brodziak *et al* (2011) in an aging study suggests that this species matures at around 3 years of age in life (Grimes, 1987). They can live for 44 years (Andrew *et al*., 2012), reaching a maximum length of 100 cm (Anderson, 1986) and weight of 9kg (Manooch, 1987; Randall, 2007). Growth of *P. filamentosus* is moderate, estimated to range from 0.15-0.21 per year (Ralston and Miyamoto, 1983; Moffitt and Parrish, 1996). Studies by Kikkawa (1983) have shown that spawning season of *P. filamentosus* to be June through December in the wild but in general, peak spawning of bottom fish including *P. filamentosus* generally occurs from July to

September in Hawaiian waters (Haight *et al*., 1993). Females can produce up to 1 million eggs (Kikkawa, 1983) which is moderately high fecund.

1.1.4: Distribution

Pristipomoides filamentosus is widely spread in the tropical Indo-Pacific Ocean from East Africa to Hawaii and Tahiti, North to Southern Japan, South to Eastern Australia and Lord Howe Island in Southwest Indian Ocean (Mees, 1993). This species is widely distributed across the Western Pacific to Indian Oceans (Plate 2) with populations ranging latitudinal from Japan to Australia and longitudinally from East Africa to Hawaii (Froese and Pauly, 2011). It is discontinuously distributed in the Western Indian Ocean (WIO) and has been recorded from Madagascar, Reunion, the east coast of Africa, the west coast of India, and the Chagos archipelago (Allen, 1985).

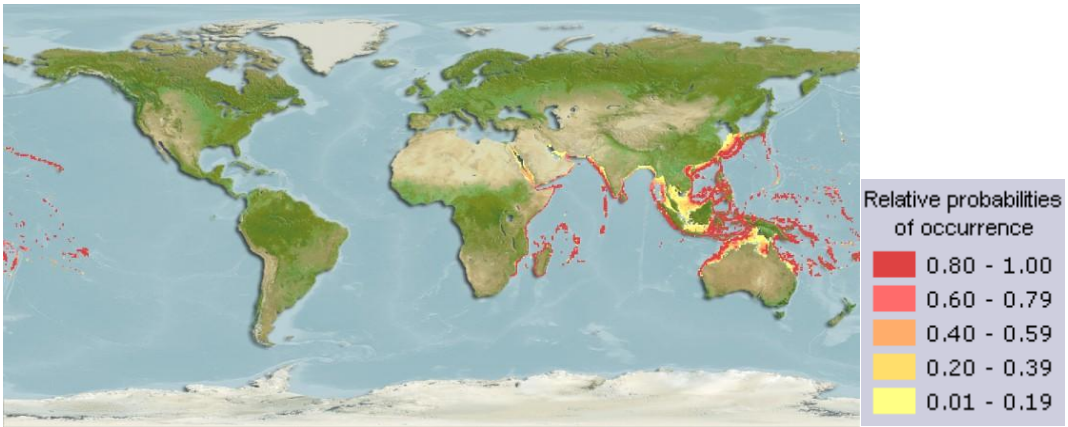

Plate 2: Global distribution of *P. filamentosus*, (Source: Global biodiversity Information facility database, 2013).

1.1.5: Oceanographic description of SWIO

Southwest Indian Ocean (SWIO) occurs on the western part of the Indian Ocean, there are several current systems that affect biodiversity and structuring of populations in SWIO. The Agulhas Current strongly influences the marine environment and biota of the SWIO region, and particularly along the eastern and southern coasts of South Africa and the Agulhas Bank. The current originates in the area between 25°S (southern Mozambique) and 30°S (Durban, South Africa) and flows in a south westerly direction along the coast, roughly steered by the edge of the continental shelf. The current moves further offshore at latitude of approximately 36°S, following the contours of the Agulhas Bank, it retroflex to form the Agulhas return current which flows eastwards along the edge of the subtropical Convergence (Lutjeharms and Van ballegooyen, 1988)

Two main seasons, North-East (NE) monsoon and South-East (SE) monsoon influence physical and oceanographic conditions in Kenya and Tanzania. During the SE monsoon period (April to October) prevailing winds drive the East Africa Coastal current (EAC) north along the coast to form the Somali Current off the horn of Africa. The strength of the monsoon winds decline during the NE monsoon period (November to March), the EAC slows down and the Somali current reverses its direction to flow southwards. Its confluence with the EAC off northern Kenya then flows offshore to form the Equatorial Counter current.

An inshore northwards current seems to be present along most of the Mozambican coast, probably as a result of the presence of the cyclonic eddies. These currents are influenced by flux of the equatorial current and by trade winds, and they are relatively steady and moderate to strong.

The western seaboard of Madagascar is characterized by a zone of turbulence where current direction and strength is highly variable. The turbulence is driven by changes in the wind regime, tidal amplitude, the relief of the seabed, and the configurations of the

opposing continental and island coastlines. Apart from the large-scale oceanographic features of the region as described above, medium and smaller scale physical and oceanographic characteristics (i.e. bays, estuaries, mud banks, reefs and bottom topography) influence the distribution and abundance of resources in the region.

1.1.6: Factors that determine genetic structures of marine fish populations

Larval dispersal

Early life history studies indicate that *P. filamentosus* can remain planktonic at a large size (37–70 mm TL) with a pelagic duration lasting 60–180 days (Moffitt and Parrish, 1996). The length of the early pelagic phase and the ability of some *P. filamentosus* to move a great distance indicate that this species may be more dispersive than shallow-water reef associated species. Tagging studies indicate that the majority of adults exhibit restricted movement (0–22 km) while some travel great distances (>400 km) and are able to cross deep-water channels (Kobayashi, 2008). Highly dispersive species may genetically homogenize populations, as immigrants originate in widely different selective environments. In contrast geographically restricted gene flow enhances differentiation among populations, as the relatively few immigrants arriving from nearby population tend to resemble residents genetically (Endler, 1973).

Oceanic currents

Oceanic currents can have various influences on the genetic structure of marine populations. They can be responsible for the dispersion of planktonic larvae, acting as gene-exchange corridors or alternatively, can constitute extrinsic and invisible physical barriers to gene flow (Palumbi, 1994). Along the East African coastline, three main current systems influencing the coast can affect the dispersal potential of planktonic larvae (Lutjeharms, 2006). These includes (1) the warm Agulhas Current, which flows

southwards from Mozambique along the eastern coast of South Africa; (2) the Mozambique Current, through the Mozambique Channel, with a contribution coming from east of Madagascar, the East Madagascar Current; and (3) the Equatorial Convergence, which at the south coast of Tanzania splits, proceeding northwards and southwards along the Tanzanian and Mozambican coastlines. The Agulhas Current in particular has a marked influence on the distribution of a number of species in the Southwest Indian and South Atlantic Oceans (Lutjeharms, 2006). First, certain species may have evolved adaptations to the Agulhas Current system, using it as a mean of transportation during particular stages of their life cycles; second, the current may at random carry organisms such as larvae within its waters (Lutjeharms, 2006).

Oceanic physical barriers

Physical barriers may reduce or prevent gene flow. At large scales, a phylogeographical structure has been shown to exist as a result of the historical separation of different ocean basins and persistent oceanographically constraints. For example, many marine fish exhibit strong genetic differentiation between the Atlantic and the Mediterranean Sea (Bargelloni *et al.,* 2003). Other well-known phylogeographical barriers include the separation between the Gulf of Mexico and the Western Atlantic (Gold and Richardson, 1998; Blandon *et al.,* 2001), the Indian Ocean and the Western Pacific separated by the Torres Strait (Chenoweth *et al.,* 1998) and within the Pacific Ocean, the Eastern Pacific barrier (Lessios and Robertson, 2006) and the disjunction between Gulf of California and Pacific populations (Bernardi *et al*., 2003). At smaller scales, the topography of the environment and its heterogeneity may also act as an efficient barrier to dispersal at different life-history stages (Sarvas and Fevolden, 2005). Despite the existence of such physical barriers, several studies have observed contrasting patterns of spatial genetic variation in different species living across the same geographical barriers (Bargelloni *et*

al., 2003), suggesting that additional variables are important in determining population structure.

1.2: Problem statement

Demersal fisheries are a very important component of global capture fisheries. Due to the excellent quality of the meat and high demand, it makes them some of the most appreciated species in the market today. *Pristipomoides filamentosus* in particular is a highly valued food and game fish for example in Maheplateu, Seychelles (FIRMS, 2009). Landings of snappers are of significant volume and economic value; about10.7 million dollars were landed by Florida commercial fishermen. According to FAO data for "Other marine fishes" for 2003,itconstitutes the largest category of reported landings by SWIO countries (~200000 metric tonnes).Records showed that the commercial capture of Crimson jobfish sharply dropped to 4,400 tonnes in 2009 from 25,300 tonnes the previous year (FIRMS,2009). *Pristipomoides filamentosus* is vulnerable to overfishing firstly due to the concentration of the stock in a narrow depth band making it an easy target by the fishermen (Mees, 1993).Secondly this fish is long lived with a maximum age of 44years(Andrews**,**2012) and slow growth rate(*K*0.15-0.25 yr^{-1}) (Brodziak *et al.*, 2011),it reaches sexual maturity at approximately 50% of their total length; (L_m=52cm)and assessment age report at maturity for *P. filamentosus* is approximately 3.5 years(Grimes, 1987).Furthermore, this species poses concern about the status on its conservation of Nature and it is considered at risk of extinction (IUCN 2006).

1.3: Justification

Most marine species show shallow population structure on a broader geographic scale (Quattro *et al.*, 2002). Though the lack of physical barriers in the ocean allows a great

degree of mixing between fish from different locations, behavioural limits to dispersal are among the various factors responsible for population subdivision in marine species (McLean *et al.,* 1999). Some studies have shown that while direct methods such as fishery investigations and tagging studies have the advantage of providing a contemporary estimate of the population on an ecological time scale, population genetic analyses can provide insights into historical population demography (Slatkin, 1994). Moreover, population genetic structure can be used to examine the spatial components of phylogeographical lineages and the evolutionary process of geographically related populations (Avise, 2000).

In the present study, mitochondrial DNA cytochrome *b*and hyper-variable nuclear microsatellites were used to determine the structure of *P.filamentosus* in SWIO. Mitochondrial DNA (mtDNA), owing to its fast mutation rate, maternal inheritance, small effective population size than a nuclear genome, reduced cases of recombination and low cases of selection has made it a useful marker for population and phylogenetic analyses (Avise *et al*., 1987). Microsatellites markers are useful in this study because they have a high mutation and heterozygosity rate and so tend to be highly variable (polymorphic) in a genetic population study. The two markers are very popular in population genetic studies; they can answer questions of phylogeny and population structure in fish, identification of stocks and analysis of mixed fishery. Furthermore analysis based on molecular markers has proved to be a strong method of identifying genetic differentiation among population and structuring (King *et al.,* 2001).

Since fisheries management is mainly concerned with reduction of genetic resources of fish as part of a larger global concern for the genetic resources of the biosphere, molecular

genetics research should be strongly supported, as it is vital to the long-term management of fisheries resources (Park and Moran, 1995). For example it is not clear whether populations of *P. filamentosus* within the SWIO are a single local population or composed of several meta-populations. Clearly, if it is a single local population, appropriate joint management of the fisheries in SWIO region would be required and if distinct then the subpopulations should be managed effectively to prevent extinction. Furthermore, in East Africa very little is known on the stock structure, distribution or movements of this species. Moreover, data on population biology and on commercial catches are inadequate. Such data are crucial for stock assessment and management. For the above mentioned reasons genetic population structure of *P. filamentosus* in the SWIO region was examined. When the genetic population structure of a species is known, the distribution of subpopulations in mixed fisheries can be estimated (Utter, 1991). This will enable us to have scientific evidence on whether the species are of the same or different populations within the region, hence lead to new management initiatives.

1.4: Objectives

1.4.1: General objective

The study aims at describing the genetic diversity and population structure of Crimson jobfish, *P. filamentosus* using mtDNA and microsatellites markers in order to ensure improved species management practices of its populations in the Southwest Indian Ocean (SWIO).

1.4.2. Specific Objectives

1. To determine the genetic population structure of Crimson jobfish, *P. filamentosus* in SWIO.

2. To find out whether populations of Crimson jobfish, *P. filamentosus* from SWIO are genetically distinct or admixed (genetic connectivity).

1.5: Hypothesis

H_0: There is no significant difference in the genetic structure of the *P. filamentosus* populations in SWIO.

H_0: There is no genetic connectivity among the *P. filamentosus* in SWIO.

CHAPTER TWO

LITERATURE REVIEW

2.1: Importance of Genetic process in marine fish populations

Population genetics can be defined as the science of how genetic variation is distributed among species, populations and individuals. Fundamentally it is concerned with how the evolutionary forces of mutation, natural selection, random genetic drift, non-random mating and migration affect the distribution of genetic variability. Patterns of genetic diversity or variation among populations can provide clues to the populations' life histories and the degree of evolutionary isolation. Genetic differences are expressed as differences in the quantity and quality of alleles, genes, chromosomes, and gene arrangements on the chromosomes that are present within and among constituent populations (Williamson, 2001).

Principally, genetic composition in nature changes continuously as a response to environmental change. In a long term, genetic changes will lead to two consequences: survival adaptation and extinction. In a shorter term, genetic changes affect populations characteristics and demography (Avise, 2004). The four major micro evolutionary processes that could change population genetic characteristics, include mutation, genetic drift, gene flow and natural selection (Allendorf and Luikart, 2007).

Mutation is the ultimate source of genetic variation in populations. There are two major types of mutations: point (gene) mutations and chromosomal mutations. A point mutation is a change in one nucleotide or several nucleotides in a single gene. The change could be due to base pair substitutions, insertion or deletion. Chromosomal mutation is a change in the number of chromosome or gene arrangement in chromosomes (Hallerman *et al.,* 2003). In the maternally inherited haploid DNA such as mtDNA where recombination

does not occur, most of genetic variation comes from mutation events, particularly point mutations thus creating or deleting nucleotide(s) in mtDNA sequences (Avise, 2004).

Genetic drift is the changes in allele frequency in a population in successive generations due to a random process. The magnitude of genetic drift in a population depends on the levels of deviation from an ideal population such as unequal number of male and female breeders, variance in family size, and different number of parents in successive generations (Hallerman *et al.,* 2003). The outcome of genetic drift cannot be predicted because of the random process. Effect of genetic drift, however, can be estimated through simulation and the result highly depends on population size (Ne). The impacts of genetic drift are more obvious in small populations. Two major impacts on the genetic composition of small populations are change of allele frequency and loss of genetic variation(Allendorf and Luikart, 2007). Theoretically, mtDNA has fourfold lower population size than nuclear DNA this making it more susceptible to genetic drift effects than nuclear DNA (Avise, 2004).

Gene flow (or migration) is any movement of alleles from one population to another. Those alleles recombine with local alleles through sexual reproduction. Genetic interactions between two or more populations through gene flow will increase or maintain genetic variability within a population, but will decrease genetic distinctiveness among populations (Ayre and Hughes, 2004). In mitochondrial DNA, gene flow can be indicated by haplotypes shared between two or more genetically related populations (Avise, 2004). There are two major factors governing gene flow in a natural population: intrinsic and extrinsic factors. Intrinsic factors cover the role of biological aspects of the species such as reproductive system (e.g. asexual reproduction, auto-gamy, out-crossing, and ploidy), behaviour and dispersal (e.g. gametic or zygotic dispersal, gender

differences and breeding behaviour) and historical processes, such as historical events in populations. Extrinsic factors include physical barriers and environmental factors for example, light, temperature, which also determines the survival of a particular species (Lowe *et al.*, 2004). Gene flow can also be as a result of human activities such as artificial culture including restocking, marine ranching and fish escaped from culture system.

Natural selection is the process in which favourable heritable traits become more common and unfavourable heritable traits become less common in successive generations due to differential survival or reproduction of phenotypes in a population (Haliburton, 2004). Genetic processes such as mutation, genetic drift and gene flow can cause change overtime, but natural selection is the primary process of adaptive evolution. Since phenotypes are highly associated with genotypes, unequal probability of alleles survive or reproduce the future generation will determine their allele frequency in a population (Allendorf and Luikart, 2007).

2.2: Molecular markers for population genetic studies

2.2.1: MtDNA

This is non-nuclear, located within organelles in the cytoplasm called mitochondria. Unlike nuclear DNA, it is held in a varying number of loops containing lower numbers of base pairs. MtDNA does contain genes, which code for proteins, usually those involved in cellular respiration. The major features of mtDNA are:(1) in general maternally inherited a haploid single molecule; (2) the entire genome is transcribed as a unit; (3) not subject to recombination and provides homologous markers; (4) mainly

selectively neutral and occurs in multiple copies in each cell; (5) replication is continuous, unidirectional and symmetrical without any apparent editing or repair mechanism; and (6) optimal size, with no introns present (Billington, 2003).

Since mtDNA does not undergo recombination at reproduction, it is passed from one generation to another largely unchanged for many generations. This is particularly valuable for use in phylogenetic or lineage studies. Application of mtDNA in animals, including fishes has some major problems as well. Major disadvantage is the low level polymorphism in some species and populations (Park and Moran, 1995). The recent demonstration of the presence of mitochondrial pseudo-genes which often result from the accumulation of multiple mutations within a gene whose product is not required for the survival of the organism. These pseudo genes occur in the nuclear genome of a wide range of organisms which is unwanted reality (Zhang and Hewitt, 2003) hence this has weakened the effectiveness of using mtDNA in population genetic studies. In addition mtDNA represents only a single locus which is like looking through a single window of evolution that reflects at best only the maternal lineage (Skibinski *et al.*, 1994) which could well differ from that overall of populations or species. Therefore, the inference we make on species/population history is likely to be highly biased and the need for independent genomic molecular markers to support mtDNA analysis is clear. Second, the effective population size of mtDNA in a nuclear autosomal sequences; that means mtDNA lineages have a much faster lineage sorting rate and higher allele extinction rate (Zhang and Hewitt, 2003).

Despite the problems mentioned above, mtDNA has a number of applications in fisheries biology, management and aquaculture. In the past 15 years mtDNA has

attracted a lot of attention in many species, especially for population and evolutionary studies (Avise, 1994). It has become a very popular marker and dominated genetic studies designed to answer questions of phylogeny and population structure in fish for more than a decade. MtDNA studies can particularly contribute to identification of stocks and analysis of mixed fishery, provide information on hybridization and introgression between fish species, serve as a genetic marker in forensics analysis and provide critical information for use in the conservation and rehabilitation programmes (Billington, 2003).

2.2.2: Microsatellites

A microsatellite is a simple DNA sequence that is repeated several times at various points in an organism's DNA. Such repeats are highly variable enabling that location (polymorphic locus or loci) to be tagged or used as a marker. They have a high mutation rate and so tend to be highly variable (polymorphic) in a population. However, microsatellites are not necessarily species specific and the same microsatellite sequence may be found in closely related species (Feral, 2002).

Microsatellite markers have a number of advantages over other molecular markers and have gradually replaced allozymes and mtDNA. Microsatellite loci are typically short, making them easy to amplify by polymerase chain reaction(PCR), and the amplified products can subsequently be analysed on either "manual" sequencing gels or automated sequencing. The much higher variability in microsatellites results in increased power for a number of applications including diagnostics and forensics (Luikart and England, 1999). Moreover, there is potential for significant increases in the number of samples that can be genotyped in a day using automated fluorescent sequencers.

Microsatellites have been used in a number of analyses including biomedical diagnostics and in forensics both for human and wildlife cases (Evett and Weir, 1998).In a biological/evolutionary context microsatellite, are useful markers for parentage analysis and can also be used to address questions concerning degree of relatedness of individuals or groups. Microsatellites serve to evaluate inbreeding levels (F_{IS}) enabling analysis of genetic structure of subpopulations and populations by calculation of *F*-statistics and genetic distances. They can be used to assess demographic history (for example reveal evidence of population bottleneck events), to assess effective population size (N_e) and to assess the magnitude and direction of geneflow between populations. Microsatellites provide data suitable for phylogeographic studies that seek to explain biogeographic and genetic histories of the floras and faunas of large-scale regions. They are also useful for fine-scale phylogenies up to the level of closely related species (Selkoe and Toonen,2006).

Despite the advantages of microsatellite markers they are not without constraints. One of the main problems is the presence of "null alleles" (O'Reilly and Wright, 1995; Pemberton *et al.*, 1995; Jarne and Lagoda, 1996). Null alleles occur when mutations take place in the primer binding regions of the microsatellite locus, that is not in the microsatellite DNA itself. The presence of null alleles at a locus is a concern particularly in individual based analyses such as relatedness estimation and assignment tests so most researchers prefer to discard loci exhibiting null alleles (Hansen, 2003). Even though microsatellites have already proven to be powerful single locus markers for a variety of genetic studies,(Queller *et al.*, 1993) the development of species-specific primers for PCR amplification of alleles can be expensive thus primers developed to amplify markers in one species may amplify the homologous markers in related species as well (Morris *et al.*, 1996).

Another disadvantage of microsatellite alleles is that amplification of an allele via PCR often generates a ladder of bands (1 or 2 base pairs (bp) apart) when resolved on the standard denaturing polyacrylamide gels. These accessory bands (also known as stutter or shadow bands) are thought to be due to slipped-strands impairing during PCR (Tautz, 1989) or incomplete denaturation of amplification products (O'Reilly and Wright, 1995). The practical outcome of PCR stutter is that it may cause problems scoring alleles. However, trinucleotide and tetranucleotide microsatellite typically exhibit little or no stuttering

2.3: Review of genetic population structure of the family Lutjanidae

Studies on genetic population structure of shallow-water reef organisms is still developing in many oceanic features, though there are few studies that have examined these same processes in deep-water species across the Indo-Pacific Ocean. Of particular interest to our study, are the demersal snappers (subfamily Etelinae) found between 100–400 m on the continental shelves and islands throughout the tropical Indo-Pacific. The following is a review of population structure of some reef fishes including both shallow waters and deep waters species of the family Lutjanidae:

In northwest Australian waters Johnson *et al (*1993) reported little genetic subdivision measured by allozyme loci in four finfish species, including *Lutjanus sebae,* two species of lethrinids and one serranid species. Bagley *et al* (1999) used allelic variation at seven di-nucleotide microsatellite loci to analyse population structure in *Rhomboplites aurorubens* indicating that there was a single population of this species on the south-eastern coast of the United States, including the Gulf of Mexico. Similarly in the Gulf of

Mexico Camper *et al* (1993) studied mtDNA haplotype frequencies of three populations of *Lutjanus campechanus* and found no population genetic structure.

Allozyme studies of six populations of *P. filamentosus* in the Hawaiian Archipelago indicated similar allozyme frequencies suggesting a single fisheries stock (Shaklee and Samollow, 1984). In contrast, another study of the mtDNA and allozymes of two snapper species in Indonesia (*Lutjanus erythropterus and Lutjanus malabaricus*), which have similar distribution with *Pristipomoides multidens* in the East Indies triangle, revealed significant population structure. However, analysis of *Lutjanus argentimaculatus* also within the same range revealed little population genetic structure based on microsatellite and mtDNA markers (Ovenden and Street, 2003). Hence this confirms the need of studying genetic population structure of each species by each case.

Several recent studies have been carried out to determine the genetic structure of *P. filamentosus* and other closely related species. Gaither *et al* (2011) carried out a study in the Indo-Pacific region to determine the genetic structure of *P. filamentosus* across the region using cytochrome*b* region of mtDNA and 11 microsatellite loci. This study detected low but significant population structure across the range of the species. However, when only the Hawaiian populations were considered overall population structure was not significant.

Ovenden *et al* (2004) conducted a study on a related species, *P. multidens* which is a large, long-lived, fecund snapper species distributed throughout the East Indies and Indo Pacific tested for genetic discontinuities in population structure. In this study, genetic variation in the control or D-loop region of the mitochondrial genome assayed using restriction fragment length polymorphism and direct sequencing showed pronounced genetic

population subdivision among central and eastern Indonesian populations of this species. This included differentiation of geographically close populations.

CHAPTER THREE

MATERIALS AND METHODS

3.1: Sampling sites-description of sampling sites

Fig 1: Sampling sites of *P. filamentosus* in SWIO during the study period.

Table 1: Geographical coordinates and sample size of *P. filamentosus* collected from SWIO.

Sampling area	Geographical coordinates	Sample size
Kenya	S 4° 34';E 39° 24'	30
Tanzania	S 6° 52'; E 39°	27
Comoros	S 4° 41';E 55° 27'	28
Madagascar	S44° 20';E16°43'	34
Seychelles	S 4° 41'; E 55° 27'	31
Mauritius	20°32';19°59'	22
South Africa	S 29^0 2'; 32^0 45'	26

3.2: Sample collection and preparation

198 specimens of Crimson jobfish were collected from Southwest Indian Ocean (Fig 1). Table 1 below summarizes the sampling sites and sample size in each locality.

Samples were collected from the fish markets/commercial fishers from Kenya, Tanzania, Madagascar, Comoros, Seychelles, South Africa and Mauritius. Whole fish specimens were frozen and transferred to the laboratory. Fin clips of 2cm obtained from the pectoral fin were taken and preserved in 1.5 ml micro centrifuge tubes containing absolute ethanol (96%) for subsequent analysis. These tissue samples were taken to the

International Livestock Research Institute, Bioscience eastern and central Africa (BecA-ILRI Hub), in Nairobi for molecular analysis.

3.3: DNA extraction, quantification and normalization

3.2.1: DNA extraction protocol

Purelink^{TM} genomic DNA extraction kit (Invitrogen, USA) was used to extract genomic DNA from the ethanol preserved fin clips; this was done in accordance to the manufacturer's protocol (www. lifetechnologies .com) with slight modification (details as below) to yield good quantity and quality of DNA.

Approximately 25mg of macerated finclip was placed into a sterile 1.5ml micro centrifuge tube to which 180µl Genomic Digestion Buffer and 20µl Proteinase K was added. The buffer was used in order to break down the cell membranes and expose DNA while Proteinase K was added to remove protein contaminants from the mixture while ensuring that the tissue is completely immersed in the buffer mix. The tissue and buffer mix was incubated in a water bath at 55°C with occasional vortexing(every 20minutes) for 4 hours until lysis was complete.

In order to remove particulate materials, the lysate was then centrifuged at 13,000 rounds per minute (rpm) for 3 minutes at room temperature. The sample was then transferred in to a sterile 1.5ml micro centrifuge tube to which 20µlRNaseA was added, mixed by brief vortexing and incubated at room temperature for 2 minutes. This enzyme was added in order to remove Ribonucleic acid (RNA). Genomic lysis binding buffer of 200µlwas added in order to bind the DNA together, it was mixed by vortexing and then 200µlabsolute ethanol added to the lysate, followed by thorough mixing for 5 seconds vortexing in order to enhance binding of the DNA. The lysate, which by this point was

approximately 640 µl was transferred to the spin column which is used to suspend the DNA fragments. The column was centrifuged at 13,000 rpm in a microcentrifuge for 1minuteat room temperature and the collection tubes containing the supernatant through discarded while the spin column containing DNA was placed into a new collection tube. 500 µl Wash Buffer 1 was added to the column followed by spinning at 13,000 rpm at room temperature for 1 minute. This cleaning stage is important as it removed ethanol soluble impurities. The cleaning process was repeated using 500 µl Wash Buffer 2 to ensure all the remnant impurities are removed. The flow through was discarded but the collection tube returned, then centrifuged again for another 1 minute at 13000rpm. This step is necessary to remove excess ethanol from the column because any remnant ethanol inhibits further DNA assessment processes. The columns were transferred into a sterile 1.5ml microcentrifuge tube to which 50µlof warm (55ºC). Double distilled water (ddH_2O) was added and left at room temperature for 2minutes. The columns were spinned at 14,000 rpm for 1 minute to elute the DNA, ddH_2O was then added at the centre of the column in order to ensure complete dissolution of the DNA particles. A second elution was carried out using a separate tube with 25 µl ddH_2O so as to obtain any DNA remnants. After the extraction was complete, 1 µl of the DNA was analysed on a Nanodrop spectophotometer (Thermo scientific, 2008) in order to verify the quality and quantity of DNA extracted and thereafter stored at -20ºC.

3.4: mtDNA amplification and Sequencing

Prior to DNA amplification, the DNA extract was normalised by double distilled water (ddH_2O) to10ng/µl this was necessary to ensure uniformity of results.

Polymerase chain reaction (PCR) was used to amplify mitochondrial cytochrome *b* using primers H15020-F (Meyer, 1994) and L15573-R (Taberlet *et al.*, 1992). PCR was performed in 20µlmicrocentrifuge tubes of AccuPower® premix (Bioneer, Korea), 3µl of

template DNA and master mix which contained 0.5mM of each the forward and reverse primers;0.4µl of $MgCL_2$ and distilled water to top up the solution to 20µl (Table 2). The AccuPower premix contains deoxy-nucleotide triphosphate (dNTPs),250µM reaction buffer, 10mMof tris-amino methane hydrochloride (Tris-HCL), 30mMof KCL and 1.5mM of Magnesium chloride ($Mgcl_2$), a tracking dye, and a stabilizer. The premix already contained $MgCl_2$, though more was added to boost the reaction.

Table 2: Master Mix prepared for amplification of mtDNA cytochrome*b* markers for *P. filamentosus* samples at the BecA-ilri Hub, Nairobi.

Reagents	**Concentration**	**Volume of 1reaction**
Primers F R	10 pmol 10 pmol	0.5 0.5
$Mgcl_2$	25 Mm	0.4 Mm
Template DNA	20 ng/µl	2 mM
ddH2O		16.6
Total		**20 mM**

Polymerase chain reaction was performed in a thermal cycler using the following settings: initial denaturation at 95^0C for 5 minutes followed by 35 cycles of: denaturation at 94^0C for 30 seconds; annealing at55^0C for 30 seconds, extension of 72^0C for 45 seconds and a final extension of 72^0C for 15 minutes.

The success of amplification was confirmed by gel electrophoresis. 2µl of PCR product was loaded in 2% agorose gel for 35 minutes at 5V/cm. The amplicots were purified using QIAquick PCR Purification kit (Qiagen, USA) according to the following protocol:

Procedure for DNA purification

Five volumes of Buffer PBI (guanidine hydrochloride and isopropanol) was addedto 1 volume of the PCR sample and mixed (For example, 500µl of Buffer PBI added to 100µl PCR sample).The QIAquick(brand name of spin column tubes provided in the Qiagen purification kit) spin column was placed in the 2ml collection tube provided and the sample was applied to the QIAquick column and centrifuged for 30–60 seconds in order to bind DNA. The flow-through was discarded and the QIAquick column placed back into the same tube, the collection tubes were re-used to reduce plastic waste. Washing of DNA was done by adding 0.75 ml Buffer PE (Ethanol and Tris buffer) to the QIAquick column and centrifuged for 30–60 seconds. The flow-through was discarded and the QIAquick column placed back in the same tube and centrifuged again for an additional 1 minute, then QIAquick column was placed in a new1.5 ml microcentrifuge tube. To elute DNA, 50µlof dd$H_2O$was added to the centre of the QIAquick membrane and left to stand for 1 minute so as to increase concentration of DNA and then centrifuged for 1 minute.

The purified PCR products were sequenced using ABI 3370 Genetic analyzer (Applied Biosystems) at BecA-ILRIHub. The resulting sequences were checked against their respective chromatograms for accuracy, conflicts were resolved manually using CLC main workbench software which is a software package for analysing sequence data and for bioinformatics(CLC bio A/S Science, Denmark). The sequences were further assembled and consensus exported in FASTA (text-based format for representing nucleotide sequences in which nucleotides or amino acids are represented using single-letter codes) format and computational misalignments were corrected using Clustalx2 (multiple sequence alignment program for DNA or protein) (Larkin *et al.*, 2007).

3.5: Microsatellite amplification and genotyping

Fifteen (15) microsatellite primers designed by Gaither *et al* (2010) were used to amplify all the 198 DNA samples. Polymerase chain reaction was initially done according to protocols of Gaither *et al* (2010)with modifications for optimization of each primer by subjecting them to a range of 12 temperatures (53ºC-64ºC) using gradient PCR in order to identify the optimal annealing temperature for each marker. PCR was thereafter carried out using this optimal temperature using a master mix as specified in Table 3 below.

Table 3: Master mix for PCR for amplification of microsatellites markers

Components	**Stock**	**Final concentration**	**X1 Volume (µl)**
PCR Buffer with $MgCl_2$	10X	1x	1
dNTPs	2 mM	0.16 mM	0.8
Forward primer	2 µM	0.2 µM	1
Reverse primer	2 µM	0.2 µM	1
DreamTaq	5 U	0.02 U	0.04
Sterile H_20			3.16
Template DNA		30 ng	3

PCR was performed in thermal cycler using the following setting: Initial denaturation of 94^0C for 5 minutes, 40 cycles of denaturation at 94^0C for 30 seconds, Annealing of 55^0-61^0c for 1 min (appendix 3), extension of 72^0C for 1 minute and final extension of 72^0C for 20 minutes**.** DNA amplification was confirmed by 2% gel electrophoresis. The PCR products were thereafter analysed on an ABI 3730 Genetic Analyzer (Biosystem) at BecA-ILRI. Results for genotyped data were scored manually using Gene Mapper software v4.1 (Applied Biosystem). Electropherograms that were clear and fall within the size range

were scored. A maximum of two alleles were scored, this was based on the diploid nature of fish.

3.6: DATA ANALYSIS

3.6.1: Genotyped data

Summary statistics of each marker was analysed by Power marker software version 3.25 (Liuand Muse, 2005). Statistics determined were number of observed alleles, gene diversity, heterozygosity, polymorphism information content, major allele frequency and data availability. This analysis was important to verify the quality of microsatellite markers used.

GenAlEx software (Peakall and Smouse, 2012) was used to determine genetic variation among all samples; Analysis performed were: Analysis of Molecular Variance (AMOVA)(Excoffier *et al*.,1992) based on co dominant data; Population differentiation which was tested between all population pairs and among all populations at each locus and over all loci; observed and expected heterozygosity and number of private alleles. Genetic distance was also done on Nei Unbiased distance to show the geographical relationships of samples from different localities ((Nei and Kumar, 2000).

Population structure of *P. filamentosus* was determined by Bayesian clustering analysis of STRUCTURE v2.3 (Pritchard *et al.*,2000).An admixture model was used, it was run at 100, 000 steps and a burn in of 50, 0000, 5 replicates was done with K ranging from 1-9. STRUCTURE HARVESTER0.6.1 (Evanno *et al.*, 2005) was used to determine

which K best fits the data and then Distruct 1.1 (Noah, 2007) was used to generate the graphical display of structure.

Darwin v5 (Perrier and Jacquemoud-Collet, 2006) was used to construct a neighbour joining (NJ) tree to show how samples from different localities clustered together. 1000 permutations of booth straps were selected and the tree presented was labelled according to the colour codes of sampling sites. Principle coordinate analysis was also done to elaborate more on how samples from different localities clustered together.

3.6.2: Sequenced data

From the alignment of mtDNA cytochrome*b* region, FASTA files were subjected to Haplotype Collapser and Converter option in FABOX v.1.35 (Villesen, 2007)to identify unique haplotypes and convert the FASTA files to Arlequin format (Excoffier *et al.,* 2005) to be used for further statistical analyses. Genetic variation, determined by Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992), was performed by apportioning hierarchical levels of among populations and among individuals. Genetic differentiation was determined by F_{ST}, values of Wright (1951) and pairwise F_{ST} matrix for sampling regions. A test of neutrality performed by both Tajima's (1989a) *D* and Fu's (1997) were calculated to distinguish between a DNA sequence evolving randomly ("neutrally") and one evolving under a non-random process, including directional selection or balancing selection, demographic expansion or contraction, genetic hitchhiking or introgression (Tajima, 1989b).

Final editing of aligned sequences was done using BioEdit (Hall, 1999) and file saved in Phylip format to be uploaded into DnaSP software (Librado and Rozas, 2009). Diversity indices, which included nucleotide diversity (pi), number of polymorphic sites (s) and

number of pairwise differences (*k*) between individuals in the samples was determined. DnaSP v.5 was used to present a single representative of haplotypes data, which was generated and converted to Network software. A median- joining network (Bandelt *et al.*, 1999) was constructed in Network v.4.6 to depict and examine the genealogical relationships among haplotypes and determine potential evolutionary paths of the samples.

CHAPTER FOUR

RESULTS

4.1: DNA extraction, quantity and quality

DNA quantity and quality for most of the samples extracted enough for subsequent laboratory procedures. The quantity was measured by the concentration with values above 10ng/µl considered adequate for amplification while quality was measured by the

(260nm/280nm) and (280 nm/260nm) ratio, values of 1.8-1.9 and2.0-2.1 respectively were obtained, this values showed that DNA extracted had less impurities hence fit for subsequent analysis. Values of quantity and quality for our study are presented in appendix 1.

4.2: Microsatellites amplification, gel electrophoresis and allele scoring

Amplification of microsatellite markers was done using annealing temperatures of 55°C-61°C (Appendix 2).Amplification of PCR products as confirmed by the 2% agorose gel electrophoresis is shown in Fig 2. It shows correct amplicots as determined by the expected band size obtained which had a range of 100-300 base pairs (bp) for all markers (Appendix 3). Genotyped data scored revealed high level of polymorphism and heterozygosity in most of the markers (Fig. 3)

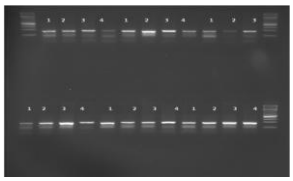

Fig 2: Electrophoresis of PCR products of *P. filamentosus* samples ran on a 2 % agorose gel.

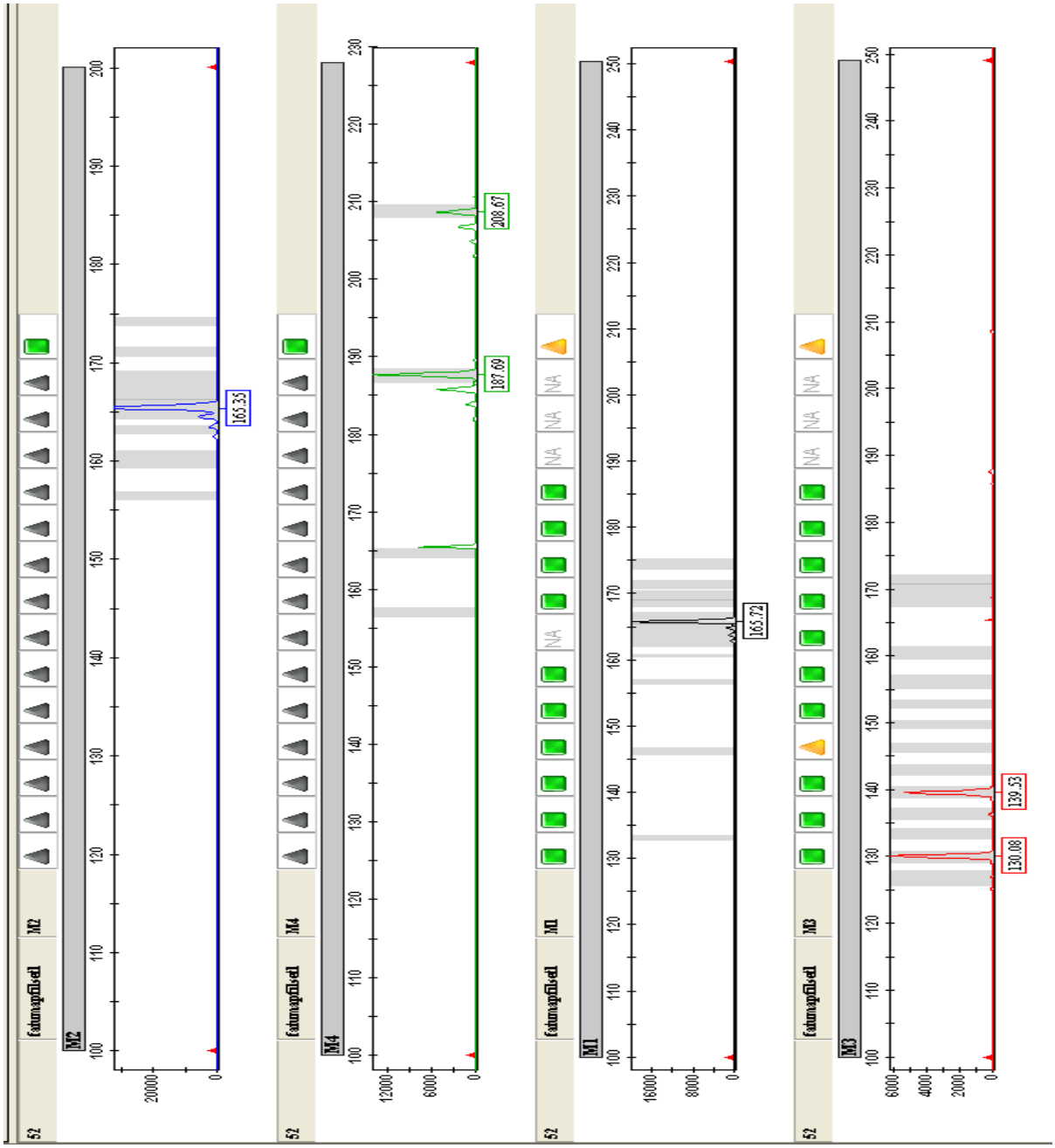

Fig 3: Electropherograms retrieved from Genemapper software showing polymorphism and sizes of different alleles of *P. filamentosus* samples (sample 52) genotyped by microsatellite markers (M1, M2, M3& M4) during the study.

4.3: Microsatellites data analysis

Genotyped data analyses were carried out in order to describe allelic frequency (p), number of alleles (K), gene diversity (D), heterozygosity (H) and percentage of polymorphism (PI)(Table 3). Out of the 15 markers initially selected, only 12 were useful for these analyses, the other three markers had poor amplification results in majority of the samples and were thus removed from the analyses. The lowest number of allele was observed in markerPfi1.6B2thathada total of 6 alleles while the highest number of allele was observed in Pfi2.9C with 26 alleles. There was a mean of 17.9167 alleles in all markers. Heterozygosity was seen in all the markers and the lowest was observed inPfi1.10D with 0.1835, the highest being Pfi2.9C with 0.7231 and a mean of 0.4515. Polymorphism information content of each marker was moderately high; the lowest polymorphism content was in marker Pfi1.6B2 with 0.4756 and the highest polymorphic one was marker Pfi2.9C with 0.8992 and an average of 0.7108.

Table 4: Statistics summary of microsatellites primers used on *P. filamentosus* from SWIO during the study.

Marker	Major allele frequency	Allele No	Gene Diversity	Heterozygosity	PIC
Pfi1.6B2	0.6347	6	0.5304	0.2953	0.4756
Pfi1.6B3	0.6843	7	0.5066	0.2828	0.4827
Pfi1.7E	0.2081	13	0.8473	0.5145	0.8294
Pfi1.9C	0.3626	23	0.8065	0.6758	0.7877
Pfi1.10D	0.5538	9	0.6384	0.1835	0.6036
Pfi2.2E	0.5813	20	0.6287	0.3813	0.6055
Pfi2.8A	0.6183	24	0.6064	0.457	0.5976
Pfi2.8E	0.4056	22	0.7631	0.6389	0.7367
Pfi2.9C	0.1564	26	0.9064	0.7231	0.8992
Pfi4A	0.3056	25	0.8571	0.5278	0.8459
Pfi1.3A	0.3503	19	0.7928	0.322	0.7678
Pfi1.5C	0.1988	21	0.9051	0.4161	0.8983
Mean	**0.4216**	**17.9167**	**0.7324**	**0.4515**	**0.7108**

4.3.1: Diversity index

Gene diversity index provides information on the mean number of alleles (Na), mean number of private alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), fixation index (F) and their standard errors (SE). Observed heterozygosity (H_o) was higher than the expected heterozygosity (He) in all the sampling sites, the lowest number

of private alleles was seen in samples collected from South Africa while the highest was from Comoros (Table 5).

Table 5: Gene diversity index of *P. filamentosus* obtained from each sampling locality during the study (Na=Number of alleles, Ne=Number of effective alleles, H_o=Observed heteroxygote, H_e= Expected heterozygote, F= fixation index)

Locality	Na	Ne	Ho	He	F
Seychelles	7.333 ± 1.018	3.176± 0.466	0.481± 0.065	0.596± 0.06	0.185± 0.067
Madagascar	8.753± 0.942	3.799± 0.556	0.443± 0.055	0.658± 0.054	0.328± 0.057
Tanzania	7.500± 0.764	3.342± 0.491	0.398± 0.064	0.612± 0.059	0.351± 0.078
Kenya	8.417± 1.111	4.105± 0.668	0.391± 0.055	0.693± 0.039	0.420± 0.084
Comoros	8.500± 0.764	4.341± 0.595	0.379± 0.053	0.729± 0.03	0.472± 0.077
Mauritius	6.917± 0.925	3.682± 0.479	0.544± 0.08	0.661± 0.051	0.167± 0.104
South Africa	6.333± 0.655	3.172± 0.302	0.504± 0.082	0.637± 0.048	0.192± 0.111
Total	**7.679± 0.339**	**3.659± 0.195**	**0.449± 0.025**	**0.655± 0.019**	**0.302± 0.033**

4.3.2: Genetic differentiation

There was significant genetic differentiation (P=0.001) among population of *P. filamentosus* in SWIO (Fig 4, Appendix 4). Analysis of molecular variance showed differentiation of 10% among population, 37% among individuals and 53% within all the individuals of *P. filamentosus* in SWIO.

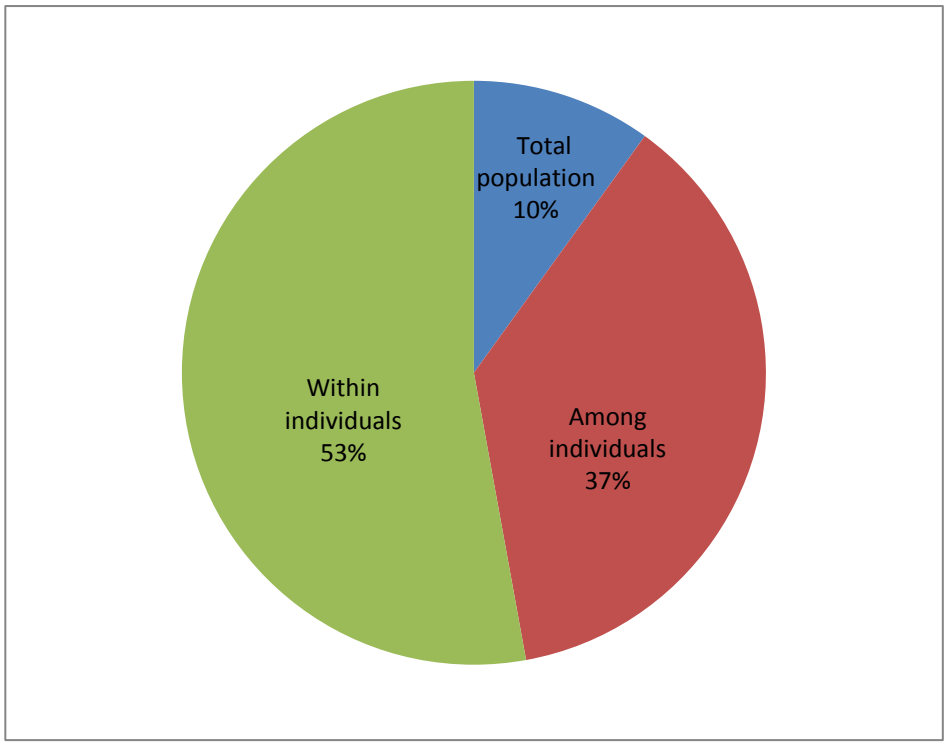

Fig 4: Analysis of molecular variance of *P. filamentosus* samples in all the localities studied: Pops = populations.

F_{ST} value of 0.100co-efficient of genetic differentiation showed moderate differentiation among the samples. Inbreeding level, which is represented by the abbreviation F_{IS} was moderate with a value of 0.413. There was low gene flow (Nm) of 2.262 occurring among all the individuals (Appendix 4).

Genetic distance of the samples in the seven sites is presented in Table 6.It was found

out that samples collected from Seychelles and Comoros were genetically separated (0.644) while South Africa- Mauritius and Seychelles- Madagascar samples were genetically closely related, both sets having a value of 0.067.

Table 6: Pairwise population matrix of Nei unbiased genetic distance of *P. filamentosus* samples in SWIO during the study period.

Sey	Mad	Tan	Ken	Com	Mau	S.Africa	
0							Seychelles
0.067	0						Madagascar
0.145	0.083	0					Tanzania
0.302	0.184	0.152	0				Kenya
0.644	0.482	0.428	0.265	0			Comoros
0.312	0.287	0.203	0.353	0.523	0		Mauritius
0.378	0.347	0.212	0.343	0.499	**0.067**	0	South Africa

4.3.3: Population structure

Bayesian clustering analysis of STRUCTURE 2.3.3 revealed three (3) main clusters of *P. filamentosus* populations occurring in SWIO (Fig 5).*K*, which determines the number of populations was maximum at 3 as confirmed by the Evvano graph (Fig 6). The populations consisted of three groups namely: Mauritius-South Africa samples, Kenya-Tanzania-Comoros-Madagascar samples and Seychelles-Madagascar-Tanzania-Kenya samples.

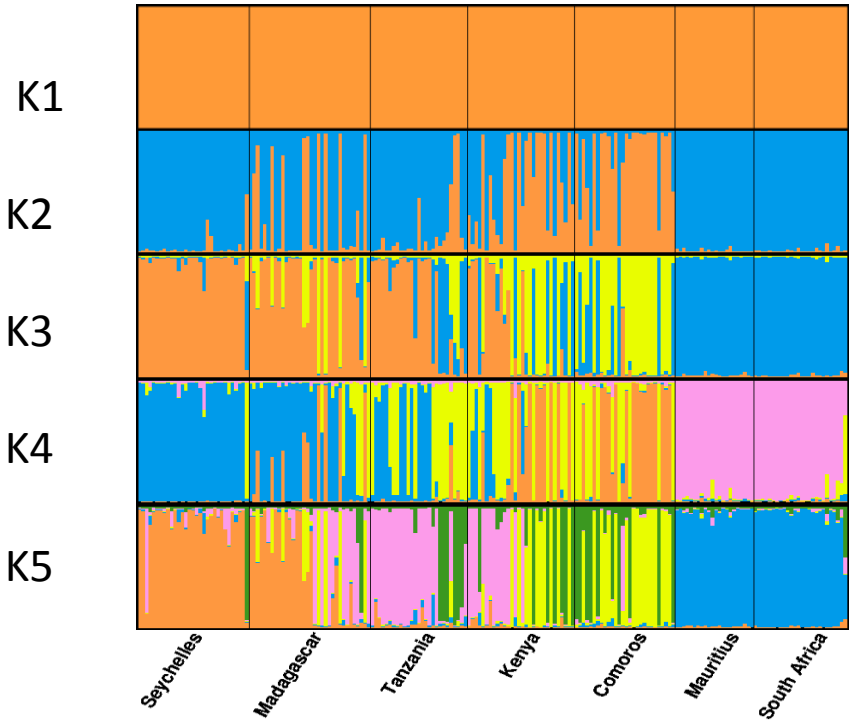

Fig 5: Bayesian clustering analysis of STRUCTURE showing K1-K5, the right K is seen at K3, which shows three populations of *P. filamentosus* in SWIO region.

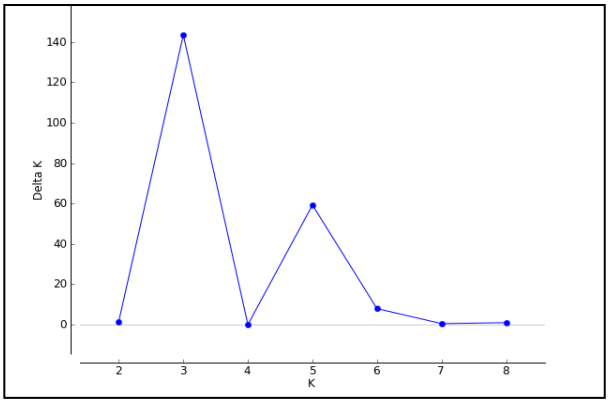

Fig 6: Evanno graph showing the true number of clusters (K) of *P. filamentosus* from SWIOas 3.

Cluster analysis

Cluster analysis done by Principle Coordinate Analysis and presented by means of a neighbour joining (NJ) tree showed how samples from different populations clustered together; it also explained the three clusters of populations obtained from the structure analysis. Samples from South Africa and Mauritius were clustered together and distinct from the rest of the samples; Samples from Kenya are spread out in all the clusters (Fig 7). Some of the Comoros samples appeared within the South Africa-Mauritius cluster but most of them clustered together with Tanzania, Madagascar and Kenya samples. There was another cluster of samples from Seychelles, Tanzania, Madagascar and Kenya, which appeared in all clusters. Similar clusters were congruent with NJ tree (Fig 8).

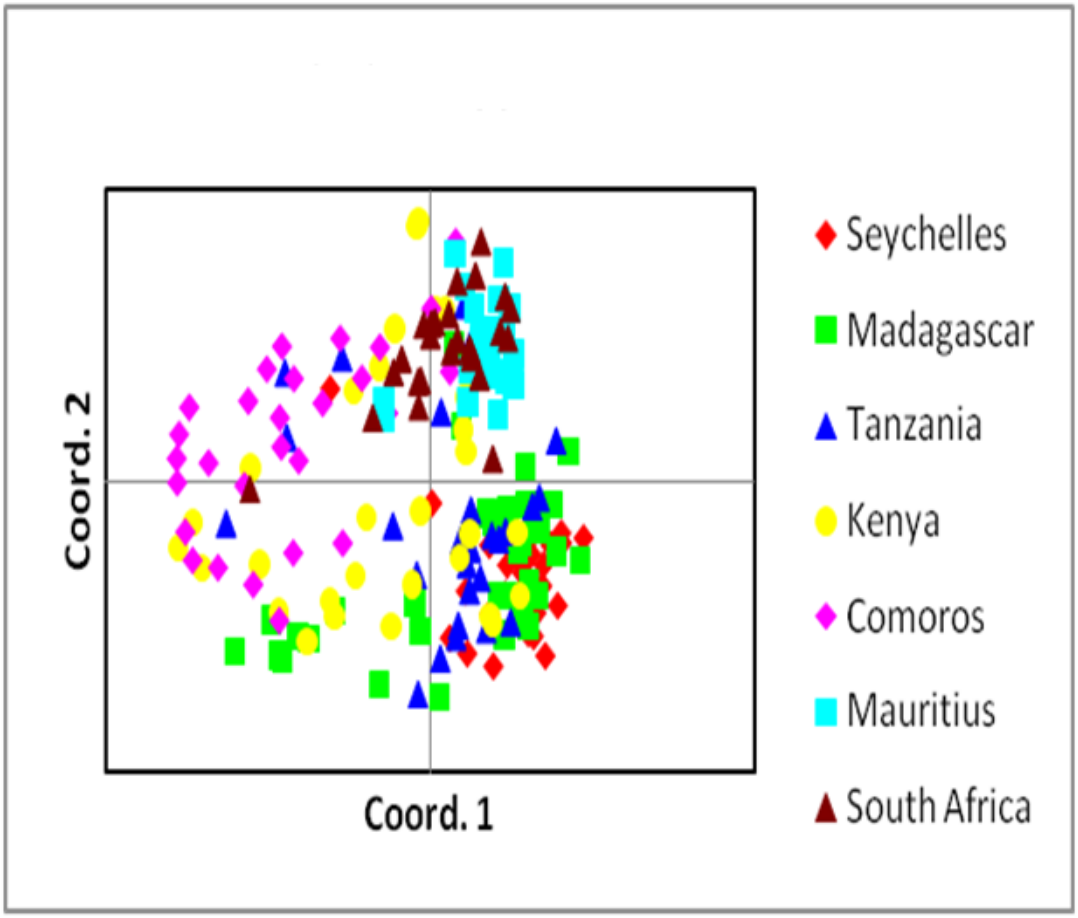

Fig 7: Principle Coordinate analysis of GeneAlex 6.5 showing how samples of *P. filamentosus* from SWIO clustered together.

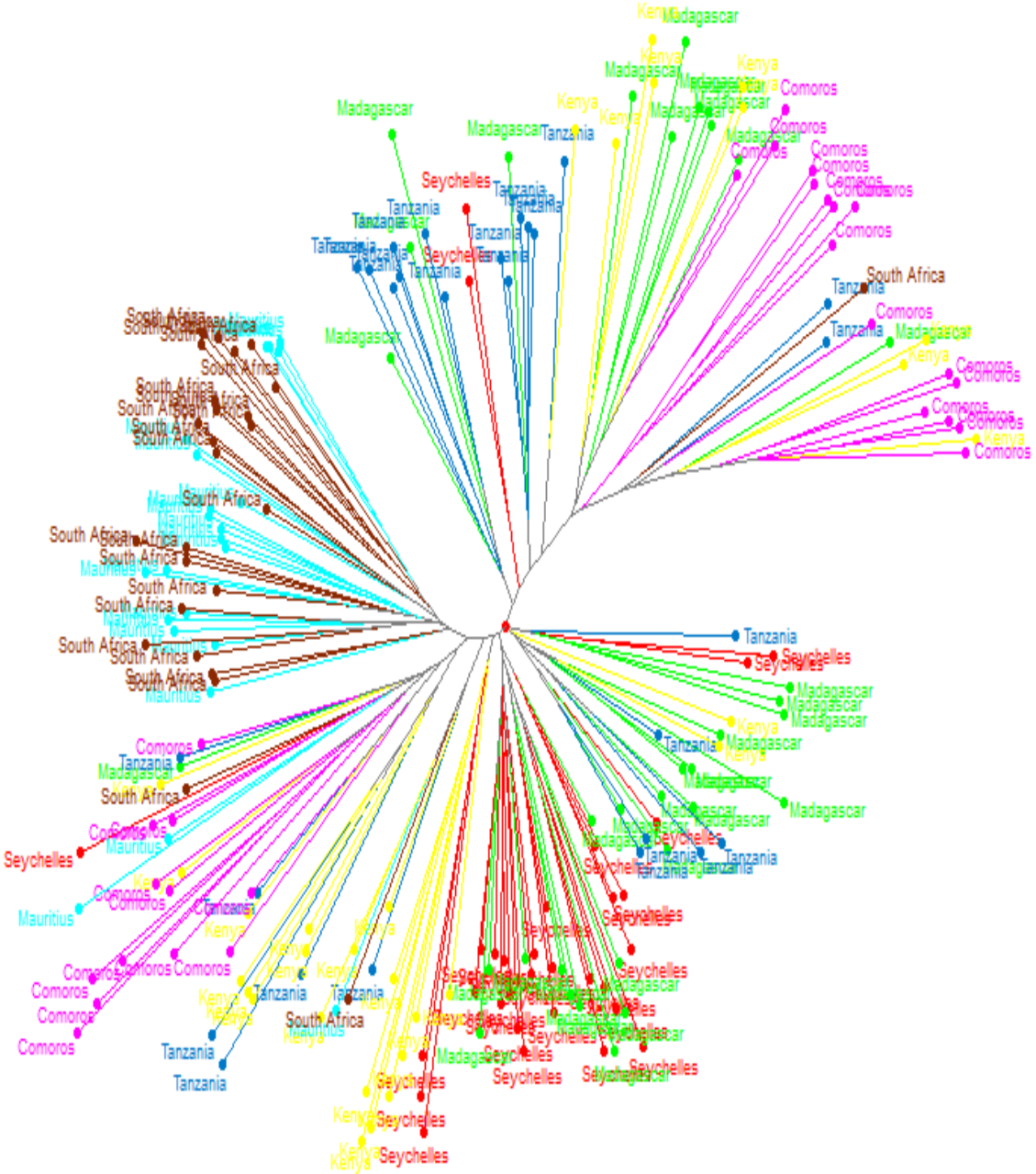

Fig 8: Neighbour joining tree of Darwin v5 showing how samples of *P. filamemtosus* from different localities of SWIO grouped together.

4.4: Mt DNA Sequences analysis

4.4.1: Gene diversity index

The number of individuals (N), number of haplotypes (NH), haplotype diversity (Hd), nucleotide diversity (pi)for each population is presented in Table 7. The cytochrome *b* sequences generated resolved to a final alignment of 506bp from which127polymorphic sites were observed and a total of twenty eight (28) unique haplotypes were obtained. Out of these 7 were shared among populations from different regions while 20were singletons or unique. The Kenya population had the highest number of haplotypes, which were shared and none of which were unique. Overall nucleotide diversity in *P. filamentosus* was P = 0.02358 while the corresponding haplotype diversity (Hd) was 0.6584±0.053.

Table 7: Genetic diversity index of mtDNA cytochrome *b* of *P. filamentosus* samples from SWIO.

Locality	**NH**	**Hd**	**Pi**
Comoros	4	0.0642	0.063
Kenya	10	0.68775	0.04247
Tanzania	6	0.68182	0.00203
Madagascar	5	0.61818	0.04094
Seychelles	6	0.54167	0.00177
Mauritius	7	0.81699	0.02081
South Africa	5	0.5263	0.00121
Total	**28**	**0.65844**	**0.02358**

4.4.2: Genetic differentiation

Genetic differentiation among populations was examined using $F_{ST,}$ which resulted to 0.0629 this reflected a moderate differentiation (Table 8). Estimates of genetic differentiation were presented as a pair wise difference matrix in Table 9.

Table 8: Genetic differentiation of *P. filamentosus* samples from mtDNA analysis obtained during the study.

Source of variation	df	Sum of square	Variance component	Percentage of variation
Among populations	6	50.045	0.2869	6.29
Within population	94	401.499	4.27127	93.71
Total	100	451.545	4.55817	

Table 9: Pairwise genetic differentiation matrix showing comparison of genetic distance of *P. filamentosus* in SWIO. Mad = Madagascar, Sey=Seychelles

	Kenya	Comoros	Mad	Mauritius	S.Africa	Sey	Tanzania
Kenya	0.0000						
Comoros	0.22124	0.0000					
Mad	0.00234	0.32202	0.0000				
Mauritius	0.05162	0.61403	0.0043	0.0000			
S.Africa	0.09018	0.75066	0.04809	0.01136	0.0000		
Sey	0.07591	0.71274	0.02748	0.01135	0.01028	0.0000	
Tanzania	0.05633	0.65221	0.00466	0.00826	0.0142	0.00987	0.0000

4.4.3: Neutrality test

TajimasD test for most of the sites were negative values, with an overall value of -1.5387. The Fu's and Fs test was significant (P< 0); -9.7×10^{37}) (Table 10). This indicates that the population had recently begun to expand and that mutations that occurred were unlikely to be lost. Fu's simulations suggest that Fs is a more sensitive indicator of population expansion and genetic hitchhiking than Tajimas D test. Genetic hitchhiking is the process by which an allele may increase in frequency by virtue of being linked to a gene that is positively selected (Barton, 2000).

Table 10: Exact and P values of FUs and Tajmas D test of *P. filamentosus* in each locality

Comoros	**Tajmas D test**	**Fu'sFs test**
Kenya	-0.6692± 0.278	-7.96238±0.007
Tanzania	-1.8942±0.005	-19.98721±0.095
Comoros	-0.8159±0.227	-0.19509±0.283
Seychelles	-2.0621±0.012	-3.4×10^{38} ±0.000
South Africa	-1.4098±0.076	-3.4×10^{38} ±0.000
Mauritius	-2.1785±0.003	-12.0213±0.000
Madagascar	-1.7411±0.014	-15.10444±0.087
Mean	**-1.5387±0.088**	**-9.7×10^{37} ±0.041**

4.4.4: Median joining network

Median joining network shows 28 haplotypes obtained from the populations analysed by mtDNA cytochrome *b*, it further explains how those haplotypes are connected to each

other (Figure 9). Each circle represents one haplotype and the size of the circle is proportional to the number of individuals within that particular haplotype while colours represent the populations (Fig 9). There is one big haplotype, which is shared among all samples from the study sites and several singletons from all the geographical sites except from Tanzania and Comoros. Samples from Kenya had the most number of shared haplotypes compared to the rest of the localities

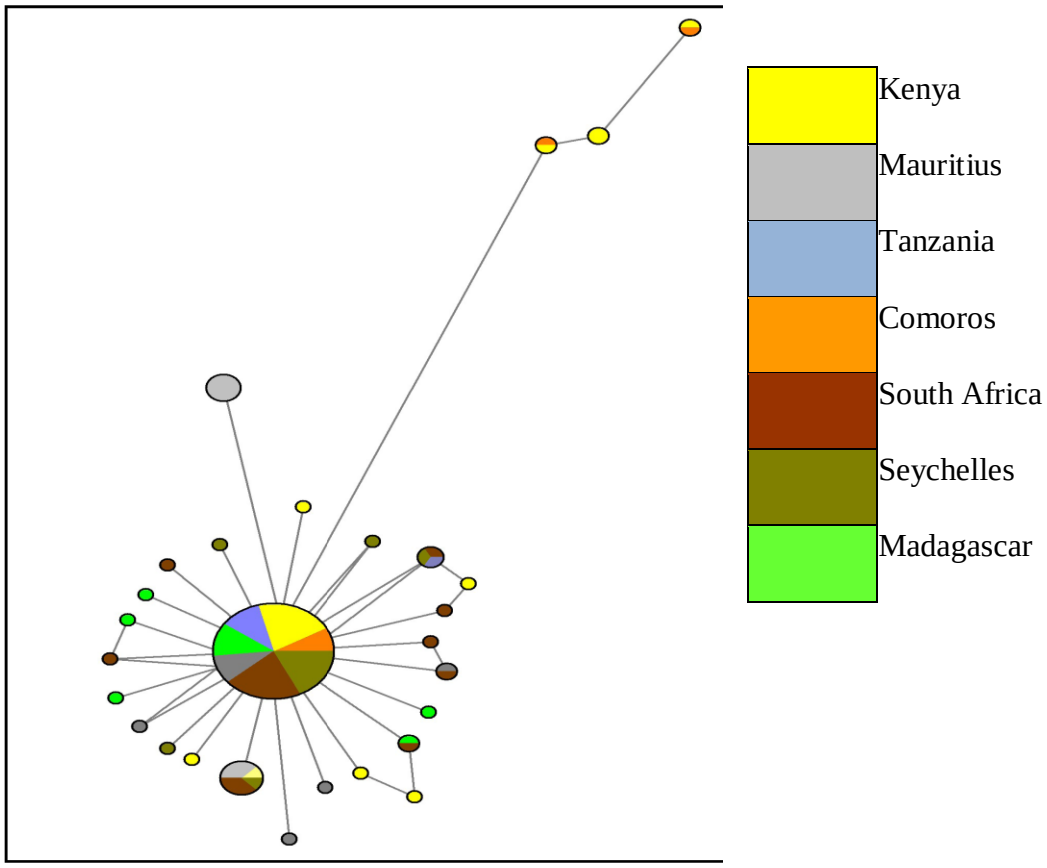

Fig 9: Median-joining network depicting genealogical relationships among the 106 samples of mitochondrial DNA cytochrome*b* among *P. filamentosus* in SWIO.

CHAPTER FIVE

DISCUSSION

5.1: Diversity index

The mean number of alleles observed in genotyped data was 17.916. This was slightly higher (14.888) than the ones obtained by Gaither *et al* (2011) in a study of *P. filamentosus* using the same primers on populations from Indo-Pacific Ocean. This study showed that the markers used were informative for diversity study. Diversity index statistics supported the arguments about the utility and accuracy of these markers to provide the correct information.

5.2: Genetic differentiation

In this study, a moderate genetic differentiation of *P. filamentosus* was observed, revealed by both microsatellite and mitochondrial DNA cytochrome*b* markers (F_{ST}, 0.1000and F_{ST}=0.1394) respectively. Pair wise genetic distance showed a significant distance between Seychelles and Comoros populations which follow the pattern observed in a similar study on parrot fish *Scarus ghobban* (Visram *et al.*,2010) and the mangrove crab *Neosarmatium meinerti* (Ragionieri *et al.,* 2010) in SWIO. In these studies, populations from Seychelles appeared phylogeographically isolated from East African localities. However, Dorenbosch (2006) found little genetic differentiation among populations of *Lutjanus fulviflamma* in Kenya, Tanzania and Comoros. While In the present study of *P. filamentosus* the populations of Kenya, Tanzania and Comoros show little differentiation and cluster together as one population.

5.3: Population structure

There was a significant difference of population structure among *P. filamentosus* in SWIO. The three populations consisted of clusters of Kenya-Tanzania-Comoros-Madagascar, Seychelles-Madagascar-Tanzania-Kenya and Mauritius-South Africa populations.

From the mtDNA analyses, populations of *P. filamentosus* appear to be expanding from a small population, which is explained by the high haplotypes diversity, negative values of Fu's and Fs test and illustrated in the median joining network. Other studies confirm or conform to these results including the study on population structure of the genus *Pristipomoides* by Gaither *et al* (2010) in the Indo- Pacific region which showed a low but significant population structure in *P. filamentosus*. However, when only the Hawaiian populations were considered overall population structure was not significant so Hawaii was treated as a single population.

Ovenden *et al* (2004) studied *P. multidenes,* a species closely related to*P. filamentosus,* across the Indo-west Pacific using mitochondrial control region. Their study showed a significant population structure. In another study of two snapper species in Indonesia (*Lutjanus erythropterus* and *Lutjanus malabaricus*), significant population structure was also observed on mtDNA and allozymes (Ovenden *et al.,* 2004).

Significant population structure has also been shown in *Lutjanus kasmira* and *Lutjanus fulvus*inthe Indo Pacific barrier (Gaither *et al.*, 2010). This highly significant population structure in this species was attributed primarily to the phylogenetic distinctiveness of their Marquesas Islands populations.

Some authors including Shaklee and Salini (1985) and Chenoweth & Hughes (2003), however, have reported that pronounced genetic population structure is a relatively uncommon state for marine finfish populations (Shaklee and Bentzen, 1998) unless they are in-shore or estuarine dependent. This has been shown in microsatellite and mtDNA analyses of *Lutjanus argentimaculatus* in the indo-west Pacific, which have resulted insignificant but little genetic population structure (Ovenden and Street, 2003).

Muths *et al* (2012) conducted a study on *L. Kasmira* on populations from East Africa, Madagascar, Seychelles, Maldives and Mozambique Channel on mtDNA cytochrome *b* markers. In contrast to this study, there was no significant population structure of this species in that region.

5.4: Genetic connectivity

In the present study, there was an indication of genetic connectivity although not pronounced. Structure analysis showed groupings of samples from Kenya-Tanzania-Comoros, South Africa-Mauritius and Seychelles-Madagascar which appeared as two separate populations. Overall there was no homogeneity of *P. filamentosus* in Southwest Indian Ocean. This is also revealed by the median joining network where various populations are seen to share the same haplotypes. Genetic structure between populations is likely to be correlated with geographic distance resulting in isolation by distance: populations close to each other have a stronger genetic connectivity than populations situated far from each other. In contrast to this study, *L. kasmira* in the WIO was reported to have high level of genetic homogeneity (Muths *et al.,* 2012). The same was observed by several studies conducted on marine fishes in the WIO and

demonstrated high levels of marine connectivity between their localities (Ridgway *et al.*, 2001, Ragionieri *et al.*, 2010 and Visram *et al.*, 2010).

Although *P. filamentosus* is considered to be highly dispersive a hypothesis proved by Gaither *et al* (2010), little genetic connectivity and significant population structure in SWIO was observed. Population structure could have resulted to restricted movements of *P. filamentosus*. Tagging studies have indicated that the majority of adults exhibit restricted movement of 0 to22 km, while some travel great distances of up to400 km and are able to cross deep-water channels (Kobayashi, 2008).

Early life history studies indicate that *P. filamentosus* can remain planktonic at a large size (37–70 mm TL) with a pelagic duration lasting 60–180 days (Moffitt and Parrish, 1996). The length of the early pelagic phase and the ability of some *P. filamentosus* to move great distances as adults, indicate that this species may be more dispersive than shallow-water reef associated species.

Despite the high dispersal rate of *P. filamentosus* there are factors that could have led to genetic differentiation and structuring of populations in SWIO; larval exchange between populations, which appear to be low, and most larvae remaining near their natal areas is one such explanation. Gene flow between populations is therefore limited and results in genetic differentiation of populations (closed populations).

Oceanic currents are a possible source of the significant genetic differentiation observed in *P. filamentosus*. Palumbi (1994) suggested that ocean currents have different effects on the genetic structure of marine populations, which may be responsible for the

dispersion of planktonic larvae, acting as gene-exchange corridors or alternatively act as extrinsic and invisible physical barriers to gene flow. The ocean current systems of the SWIO region are complex in the tropical part of the SWIO region. This is so because the South Equatorial Current (SEC), which flows from east to west in the Indian Ocean, divides into two main circulation components when it reaches the eastern coast of Africa. The southern component includes the Mozambique Current (MC) and the North Madagascar Current (NMC) whilst the East African Coastal Current (EACC) comprises the northern component (Fratini *et al.*, 2010).

These currents are expected to have separated populations of Tanzania and Kenya from those of Mozambique and South Africa (Fratini *et al.*, 2010), and thus explain the distinction observed between the Kenya-Tanzania-Comoros and those from South Africa and Mauritius. The SEC acts as likely mechanism of larval transportation from islands to the east African mainland whereas the EACC transports the larvae along the coast in a northerly direction (Visram *et al.*, 2010).

Further south in the SWIO the main process is the Agulhas Current (AC) action. It flows very close to the shelf edge at the northern part of its flow route compared to the south. The AC forms from the combined action of other currents. The Mozambique current joins up with the southern branch of SEC, the South East Madagascar Current (SEMC) that leads to the AC flowing southwards (Quartly and Srokosz, 2004). This current system operating in the region might be involved in connecting or separating populations of marine species. Agulhas current due to its nature is expected to link populations of South Africa and Mauritius. From the study, populations of Madagascar

and Seychelles appear to be genetically connected by the South Equatorial current that flows westwards.

The Western Indian Ocean (WIO) forms a coherent subdivision of the tropical Indo-Pacific (Sheppard, 2000), and thus represents an important biogeographic region of tropical seas. In spite of this, Kenya, Mozambique, Somalia, South Africa, Tanzania, Comoros, Madagascar, Mauritius, Reunion, and the Seychelles have shown major signs of environmental degradation, as well as declines in natural resources and biodiversity (Berg *et al*., 2002). This assumption is based on the generalization that marine species with long larval phases are thought to disperse further, have higher gene flow, larger geographic ranges, and lower levels of genetic differentiation among populations (Féral, 2002). However, the present genetic research appears to challenge this long-held view of 'openness' in marine systems because genetic pools of widely distributed species are rarely homogenous from one end of their distribution to the other (Reeb and Avise, 1990; Hilbish, 1996; Ayre and Hughes, 2004).

It is also revealed that genetic differentiation among populations in the Western Indian Ocean was higher than those in the Pacific, indicating that the populations in the Pacific appear more connected than their counterparts in WIO (Benzie, 1999).Tentatively there is a divide in the Indian Ocean on structure of species. A study on the starfish *Linckia laevigata* showed a single WIO population from South Africa as distinct from populations of Western Australia (Eastern Indian Ocean) (Williams and Benzie, 1998), providing a suggestion of a divide of populations in the Indian Ocean.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1: CONCLUSION

Significant population structure of *P. filamentosus* was observed in SWIO; three clusters of populations appear to be occurring in Mauritius–South Africa, Kenya-Tanzania-Madagascar-ComorosandSeychelles-Madagascar-Tanzania-Kenya. Ocean currents could be the prime factors of population differentiation in SWIO. The populations of *P. filamentosus* in SWIO are expanding from one central position to several others. It is remarkable that a species with high depth preference and of highly dispersive nature could have such significant population structure and genetic differentiation. The assumption on the generalization that marine species with long larval phases are thought to disperse further, have higher gene flow, larger geographic ranges, and lower levels of genetic differentiation among populations has once again being challenged.

6.2: RECOMMENDATION

Due to the discovery of three distinct genetic populations in the localities studied, it is clear that *P. filamentosus* needs separate management units based on the three populations observed. The fact that these three populations are shared between countries there is a need for regional co-operation in the management and conservation measures of this species. For this there is need for co-operation and co-ordination among the countries and the development of joint fisheries management plans.

There is also a need for further development of this work with more sampling in numbers and localities in order to establish the presence of ecological significant units (ESUs) or ecological management units, which would best describe the group of clusters of populations assumed to be distinct. In the present study, the theory of ESUs was not used due to the limited number of populations.

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APPENDICES

Appendix I: Nano-drop reading of DNA extracted and normalized during the study

Sample ID	**Nucleic Acid Conc.ng/ul)**	**260/280**	**260/230**	**DNA TO PICK**	**dd water to add**
ken8	107	1.86	2	9.3	90.7
ken 10	31.9	1.86	1.75	31.3	68.7

ken 21	257.2	1.89	2.28	3.9	96.1
ken 19	116.1	1.84	1.74	8.6	91.4
ken 2	69.1	1.87	1.9	14.5	85.5
ken 22	167.1	1.86	2.12	6.0	94.0
ken 11	14.8	1.91	1.46	67.6	32.4
ken 5	24.4	1.83	1.7	41.0	59.0
ken 3	37.6	1.98	2.04	26.6	73.4
ken 12	32.6	1.95	1.91	30.7	69.3
ken 14	52.6	1.92	2.17	19.0	81.0
ken 4	96.6	1.94	2.28	10.4	89.6
ken 1	122	1.86	1.98	8.2	91.8
ken 16	157	1.91	2.2	6.4	93.6
ken 6	130.5	1.91	2.13	7.7	92.3
ken6	258.9	2.06	2.23	3.9	96.1
ken25	96.6	1.63	0.93	10.4	89.6
ken27	86.6	2.04	2.26	11.5	88.5
Sample ID	**Nucleic Acid Conc.ng/ul)**	**260/280**	**260/230**	**DNA TO PICK**	**dd water to add**
ken15	351.4	2.08	2.34	2.8	97.2
ken24	48.7	2.1	2.13	20.5	79.5
ken28	173.1	2.13	2.35	5.8	94.2
ken28	218.8	2.09	2.35	4.6	95.4
ken18	243.7	2.05	2.28	4.1	95.9

ken20	31.7	2.04	1.85	31.5	68.5
ken26	32.7	1.95	1.87	30.6	69.4
ken33	84.1	2	1.71	11.9	88.1
ken29	93.1	1.99	2.32	10.7	89.3
ken31	83.5	1.97	2.01	12.0	88.0
ken7	353.7	2.05	2.29	2.8	97.2
ken23	125.9	2.06	2.32	7.9	92.1

Sample ID	Nucleic Acid Conc.(ng/ul)	260/280	260/230	DNA to pick	dd Water to add
tz2	126.9	1.68	1.09	7.9	92.1
tz21	151.8	1.52	0.85	6.6	93.4
tz24	133.8	1.6	0.96	7.5	92.5
tz26	171	1.61	1.03	5.8	94.2
tz25	117	1.57	0.75	8.5	91.5
tz30	123.4	1.46	0.69	8.1	91.9
tz12	169.5	1.51	0.84	5.9	94.1
tz11	160.1	1.54	0.94	6.2	93.8
tz22	318.6	1.76	1.29	3.1	96.9
tz19	157.3	1.53	0.91	6.4	93.6
tz9	90	1.77	1.32	11.1	88.9
tz20	92.6	1.8	1.72	10.8	89.2
tz3	131.4	1.64	1.21	7.6	92.4
tz1	4.9	1.55	0.55	204.1	-104.1
tz10	10.1	1.54	0.64	99.0	1.0
tz14	8.4	1.77	0.59	119.0	-19.0
tz17	422.8	0.49	0.5	2.4	97.6
tz16	90.6	1.48	0.76	11.0	89.0
tz4	52.7	1.9	2.02	19.0	81.0
tz8	41.3	1.81	1.13	24.2	75.8
tz12	33.6	1.48	0.74	29.8	70.2

Sample ID	**Nucleic Acid Conc.ng/ul)**	**260/280**	**260/230**	**DNA TO PICK**	**dd water to add**
tz28	50	1.52	0.86	20.0	80.0
tz27	128.4	1.84	1.95	7.8	92.2
tz23	17.9	1.9	1.31	55.9	44.1
tz9	23.4	1.65	1.14	42.7	57.3

Sample ID	Nucleic Acid Conc. (ng/ul)	260/280	260/230	DNA to pick	dd water to add
sey13	279.8	1.85	2.28	3.6	96.4
sey4	217.1	1.86	2.42	4.6	95.4
sey6	115.1	1.85	2.1	8.7	91.3
sey34	402.3	1.86	2.37	2.5	97.5
sey42	366.1	1.84	2.21	2.7	97.3
sey17	167.9	1.89	2.57	6.0	94.0
sey14	102.7	1.84	2.32	9.7	90.3
sey1	55.8	1.93	2.27	17.9	82.1
sey9	114.9	1.92	2.58	8.7	91.3
sey10	224	1.87	2.24	4.5	95.5
sey11	353.4	1.85	2.25	2.8	97.2
sey3	342.9	1.86	2.4	2.9	97.1
sey5	109	1.88	2.3	9.2	90.8
sey16	120.2	1.9	2.58	8.3	91.7
sey7	91.9	1.96	2.49	10.9	89.1
sey2	259.4	1.86	2.34	3.9	96.1
sey12	302.4	1.86	2.43	3.3	96.7
sey 32	34	1.85	1.71	29.4	70.6
sey 27	180.6	1.89	2.12	5.5	94.5
sey 29	111.8	1.86	1.96	8.9	91.1
sey 28	54.1	1.95	2.12	18.5	81.5

Sample ID	**Nucleic Acid Conc.ng/ul)**	**260/280**	**260/230**	**DNA TO PICK**	**dd water to add**
sey 15	71.4	1.83	1.77	14.0	86.0
sey 24	-6.5	1.72	-7.33	-153.8	253.8
sey 19	144.5	1.92	2.22	6.9	93.1
sey18	28.3	1.82	1.59	35.3	64.7
sey 35	85.3	1.86	1.46	11.7	88.3
sey 30	28.7	1.91	1.5	34.8	65.2
sey 22	67.8	1.86	2.06	14.7	85.3
sey 33	30.2	1.89	1.7	33.1	66.9
sey 8	249.6	1.86	2	4.0	96.0
sey 26	206	1.91	2.33	4.9	95.1
sey 21	80.8	1.77	1.21	12.4	87.6
sey 20	16.1	1.76	1.15	62.1	37.9
sey 23	75.3	1.88	1.88	13.3	86.7

Sample ID	Nucleic Acid Conc.(ng/ul)	260/280	260/230	DNA to pick	dd water to add
com21	20.1	1.47	0.78	49.8	50.2
com22	4.7	1.15	0.3	212.8	-112.8
com25	10.1	1.31	0.49	99.0	1.0
com14	13.9	1.44	0.53	71.9	28.1
com27	10.7	1.31	0.56	93.5	6.5
com24	6.3	1.08	0.39	158.7	-58.7
com15	8.4	1.37	0.73	119.0	-19.0
com31	8.2	1.25	0.51	122.0	-22.0
com3	24	1.51	0.91	41.7	58.3
com30	32.1	1.49	0.83	31.2	68.8
com19	19.7	1.53	0.87	50.8	49.2
com26	7	1.1	0.35	142.9	-42.9
com9	9.8	1.17	0.73	102.0	-2.0
com7	13.5	1.3	0.77	74.1	25.9
com10	4.6	1.25	-1.51	217.4	-117.4
com12	7.4	1.32	0.78	135.1	-35.1
com20	25	1.47	0.81	40.0	60.0
com22	14.7	1.01	0.36	68.0	32.0
com13	11.3	1.12	0.29	88.5	11.5
com5	11.4	1.18	0.49	87.7	12.3
com6	8.6	1.3	0.59	116.3	-16.3
com23	15.5	1	0.26	64.5	35.5

Sample ID	**Nucleic Acid Conc.ng/ul)**	**260/280**	**260/230**	**DNA TO PICK**	**dd water to add**
com8	9.2	1.33	2.65	108.7	-8.7
com1	31.5	1.48	0.87	31.7	68.3
com4	33.5	1.47	0.83	29.9	70.1
com16	9.2	1.09	0.44	108.7	-8.7
com18	12.5	1.04	0.28	80.0	20.0
com2	18.4	1.16	0.52	54.3	45.7
com17	28.3	1.46	0.85	35.3	64.7
Com8	13.9	1.01	0.37	71.9	28.1

Sample ID	Nucleic Acid Conc. (ng/ul)	260/280	260/230	DNA to pick	dd water to add
MAD90	318.1	1.88	2.38	3.1	96.9
MAD20	185	1.86	2.41	5.4	94.6
MAD1	304.8	1.86	2.31	3.3	96.7
MAD33	256.9	1.85	2.33	3.9	96.1
MAD7	461.7	1.86	2.13	2.2	97.8
MAD17	195.9	1.91	2.41	5.1	94.9
MAD32	598.6	1.85	2.35	1.7	98.3
MAD14	80.9	1.86	2.83	12.4	87.6
MAD5	375	1.87	2.44	2.7	97.3
MAD9	343.7	1.88	2.16	2.9	97.1
MAD50	189.7	1.92	2.17	5.3	94.7
MAD3	175.1	1.87	2.33	5.7	94.3
MAD18	105.5	1.87	2.58	9.5	90.5
MAD40	186.7	1.86	2.47	5.4	94.6
MAD10	232.9	1.87	2.37	4.3	95.7
MAD26	128.6	1.84	2.04	7.8	92.2
MAD24	276.3	1.86	2.23	3.6	96.4
MAD70	216.8	1.85	2.38	4.6	95.4
MAD19	560.1	1.86	2.22	1.8	98.2
MAD11	299.8	1.83	2.03	3.3	96.7
MAD29	251.9	1.87	2.21	4.0	96.0
MAD3	230.8	1.83	1.95	4.3	95.7

Sample ID	**Nucleic Acid Conc.ng/ul)**	**260/280**	**260/230**	**DNA TO PICK**	**dd water to add**
MAD2	137.5	1.85	2.02	7.3	92.7
MAD16	268.7	1.85	2.06	3.7	96.3
MAD13	179	1.86	2.09	5.6	94.4
MAD15	136.5	1.85	1.93	7.3	92.7
MAD23	275.3	1.86	2.04	3.6	96.4
MAD27	327	1.86	2.1	3.1	96.9
MAD6	297.5	1.85	2.2	3.4	96.6
MAD31	609	1.85	2.24	1.6	98.4
MAD21	526.5	1.86	2.26	1.9	98.1
MAD30	318.8	1.86	2.18	3.1	96.9
MAD22	468.3	1.85	2.19	2.1	97.9
MAD8	803.2	1.85	2.3	1.2	98.8
MAD4	152	1.87	1.53	6.6	93.4
MAD12	696.5	1.85	2.23	1.4	98.6

Sample ID	Nucleic acid conc. ng/ul	260/280	260/230	DNA to pick	dd Water to add
Mau13	381.42	1.86	2.4	2.62	97.38
Mau12	562.35	1.81	2.39	1.78	98.22
Mau19	515.88	1.82	2.4	1.94	98.06
Mau22	210.18	1.88	2.29	4.76	95.24
Mau5	317.22	1.83	2.16	3.15	96.85
Mau21	350.83	1.81	2.49	2.85	97.15
Mau1	332.02	1.76	2.07	3.01	96.99
Mau20	357.52	1.85	2.34	2.80	97.20
Mau4	333.74	1.85	2.14	3.00	97.00
Mau7	373.38	1.85	2.33	2.68	97.32
Mau2	292.9	1.87	2.41	3.41	96.59
Mau9	492.68	1.84	2.43	2.03	97.97
Mau14	354.64	1.86	2.42	2.82	97.18
Mau17	204.04	1.84	2.3	4.90	95.10
Mau6	523.16	1.84	2.46	1.91	98.09
Mau10	328.29	1.86	2.33	3.05	96.95
Mau8	536.05	1.83	2.43	1.87	98.13
Mau18	516.94	1.82	2.38	1.93	98.07
Mau15	382.41	1.85	2.37	2.61	97.39
Mau3	417.62	1.84	2.39	2.39	97.61
Mau11	309.09	1.84	2.19	3.24	96.76

Sample ID	Nucleic acid conc. ng/ul	260/280	260/230	DNA TO PICK	dd Water to add
SA18	227.82	1.79	1.91	4.39	95.61
SA17	44.14	1.51	1.4	22.66	77.34
SA25	20.04	1.19	1.04	49.90	50.10
SA16	27.37	1.39	1.2	36.54	63.46
SA1	32.7	1.74	1.61	30.58	69.42
SA4	182.93	1.77	2.05	5.47	94.53
SA27	21.37	1.76	1.31	46.79	53.21
SA5	126.6	1.88	2.12	7.90	92.10
SA25	60.73	1.8	1.57	16.47	83.53
SA24	18.18	1.16	0.81	55.01	44.99
SA14	27.97	1.66	1.48	35.75	64.25
SA15	77.77	1.73	1.65	12.86	87.14
SA7	50.23	1.82	1.8	19.91	80.09
SA2	60.58	1.77	1.48	16.51	83.49
SA21	14.84	1.61	0.95	67.39	32.61
SA20	12.79	1.6	1.19	78.19	21.81
SA8	196.22	1.81	1.98	5.10	94.90
SA13	13.18	1.46	0.79	75.87	24.13
SA3	75.49	1.68	1.64	13.25	86.75
SA12	356.53	1.84	2.3	2.80	97.20
SA10	221.21	1.79	1.97	4.52	95.48

Sample ID	**Nucleic Acid Conc.ng/ul)**	**260/280**	**260/230**	**DNA TO PICK**	**dd water to add**
SA26	56.49	1.62	1.44	17.70	82.30
SA11	30.34	1.39	1.29	32.96	67.04
SA19	68.08	1.79	1.69	14.69	85.31
SA9	54.9	1.67	1.3	18.21	81.79
SA23	252.87	1.8	2.08	3.95	96.05
SA9	24.8	1.33	0.94	40.32	59.68

Appendix II: Optimised annealing temperatures of microsatellites primers used during the study.

Locus name	**Forward**	**Reverse**	**Annealing temp (°C)**
Pfi1.3A	GTCTCTCTTCATCCCACATTCC	AGTAAAGCAACACGTCATCCCT	55
Pfi1.5C	AATCAAGCATCTGCTCCACAT	ACCATGATTACGCCAAGCTATT	55
Pfi1.6B2	CATGGTGGAGTGGAGCTATACA	ATTAAAGCCCCAAGAAGAGAGG	59
Pfi1.6B3	CATGGTGGAGTGGAGCTATACA	ATTAAAGCCCCAAGAAGAGAGG	59
Pfi1.7E	GGTATTCCCTCCAAGAAGACCT	ACTCTGAGGACTGAGGGGAAG	59
Pfi1.9C	TCAGCTTCTGGTACAGCAAGAG	GCGTGTTTGAAATTTGATGAGA	59
Pfi1.10D	CATGTAAATGGTGCAGAAATACG	TGTATGTGTGTGTGTAAGGAGGC	59
Pfi2.1D	AAGAAGACTAAGGCGGTGTGAG	TGTGACCCTGCAGAGGATAAG	61
Pfi2.2E	GAAACTAACACATCACGAGCCA	TTTTACCTTGATAGTCCGGCAT	61
Pfi2.8A	AATGTCAGCTGGGATA	CCGCGGGGTCTTAAAA	58

	GACTCC	GTAT	
Pfi2.8E	AGTGAACTGCAGCCAGATGTTA	ATTCTGCTGAAACCATCCATTC	58
Pfi2.9C	GAGGAAGGCCAGTGAGAAGTT	CAAGAGCATATCAACCAACCAA	55
Pfi2.12F	TCCTGTGTTCCTCTCTCTCTCC	TCAGGTGGTCAGAGTTGGTAAA	58
Pfi2D	TCTCTTTACTCACCAGCACCAA	TGAATGGAACAGTAGCAATGATG	59
Pfi4A	GGTCACCTGTGTGAAAGTTCTG	TGTTAGTGGTTGTTCTTGCCAC	58

Appendix III: Expected band sizes of microsatellites primers

Size range (bp)	**Code**
219–305	M15
162–189	M16
191–194	M1
170–190	M2
147–178	M3
172–192	M4
127–161	M5
111–160	M7
235–271	M8
216–236	M9
257–273	M10
174–210	M11
217–275	M12
191–197	M13
218–274	M14

Appendix IV: F statistics of *P. filamentosus* samples analysed by microsatellite markers during the study

F-Statistics	**Value**	**P(rand >= data**
F_{ST}	0.100	0.001
F_{IS}	0.413	0.001
F_{IT}	0.471	0.001
Nm	2.262	