POPULATION GENETIC STRUCTURE OF THE WHITE TEATFISH, *HOLOTHURIA FUSCOGILVA*, IN SOUTH-COAST, KENYA

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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Fisheries of Pwani University

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DECLARATION

This thesis is my original work and has not been presented in any other University or any other Award.

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DEDICATION

I would like to dedicate this thesis to my family for being my pillar and for offering emotional support.

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ABSTRACT

The white teatfish, *Holothuria fuscogilva*, is widely distributed in coastal areas, around coral reefs and sea grasses in the Indo-Pacific region. In Kenya, the species is distributed in areas of shallow reefs, with higher landings reported from the Gazi-Vanga-Shimoni seascape. The species exhibits density-dependent reproduction with broadcast spawning augmented by low recruitment rates, making it vulnerable to overexploitation. Sea cucumbers are excellent species for regulating water quality through the recycling of nutrients in sediments. Therefore, their ecological importance cannot be overstated. The present study investigated the genetic structure, patterns of effective population size, and effects of fishing pressure on *H. fuscogilva* populations from Gazi, Shimoni and Vanga sites in south-coast Kenya, using mitochondrial cytochrome oxidase I (COI). A total of 60 individuals, 25 from Gazi, 27 from Shimoni and 8 from Vanga were sampled with 47 individuals yielding high-quality sequences producing 22 haplotypes with high haplotype diversity but low nucleotide diversity (h = 0.922, π = 0.008). Samples from the northernmost site, Gazi had higher genetic diversity (h = 0.933) compared with the Vanga and Shimoni populations. In addition, *H. fuscogilva* along the south coast of Kenya exhibited a unique clade when compared with individuals from other geographical locations outside the region. The genetic structure was analyzed using Analysis of Molecular Variance (AMOVA) with the highest proportion of variation evident within the populations than, among the populations. The fixation index F_{ST} showed a lack of structure among *H. fuscogilva* (F_{ST} = 0.01186, p.value = 0.29069).To visualize past population dynamics and patterns of effective population sizes of *H. fuscogilva* populations, Mismatch distribution analyses revealed a past population expansion event, while Bayesian skyline plots indicated a gradual demographic growth due to an increase in effective population sizes. The *H. fuscogilva* populations in the three study sites along Kenya's south coast appear to have genetic connectivity, but no significant genetic structure. The genetic structure estimates and intra-population genetic diversity depict a robust population that can regenerate lost individuals after a fishing event. There is occurrence of population expansion evident from new haplotypes in population and increase in effective population sizes. The Intra-population genetic diversity regenerates the lost individuals after fishing pressure. *H. fuscogilva* distinction from other species around the world emphasizes the importance of conservation at the local and regional level. Future studies should then be conducted in other areas of the Kenyan coast to provide comprehensive countrywide data critical in formulation of sound harvest strategies and conservation management policies for the *H. fuscogilva* species along the Kenya coast.

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ABBREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
BLAST-N	Basic Local Alignment Search Tool-Nucleotide
CITES	Convention on International Trade of Endangered Species of Wild Flora and Fauna.
COI	Cytochrome Oxidase subunit I
DNA	Deoxyribonucleic Ribonucleic Acid
EACC	East African Coastal Current
mtDNA	Mitochondrial DNA
NCBI	National Center for Biotechnology Information
N_e	Effective population size
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
SNPs	Single Nucleotide Polymorphisms
WIO	Western Indian Ocean

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Sea cucumbers are marine animals that belong to the Phylum Echinodermata and Class Holothuroidea. There are over 1400 sea cucumber species globally, with approximately 140 species reported in the Western Indian Ocean (UNEP-WIOMSA, 2015). They have elongated bodies with no apparent skeleton (Lewerissa et al., 2021). They are keystone species that are linked to various ecosystem roles, including improvement of sediment quality through burrowing, improvement of water chemistry through feeding on detritus, and involvement in nutrient cycling (Mmbaga, 2013; Purcell et al., 2016). In addition, they contribute to increased biodiversity by transporting organic matter to higher trophic levels and providing a habitat for a variety of parasites and symbionts (Conand, 2017).White teatfish, *Holothuria fuscogilva,* Cherbonnier 1980 is one of the many sea cucumbers found in the world. It is a tropical sea cucumber found on seagrass meadows and coral reef slopes in the Indo-Pacific region (Leopardas, 2021). It has protruding white teats on its oval-shaped body, giving it the name "white teatfish" (Purcell et al., 2013).

Globally, sea cucumber fishery is an important source of livelihood and income to many coastal communities throughout the Indo-Pacific region, including the isolated coastal villages of south-coast Kenya and the Lamu archipelago (Conand, 2008; Ochiewo et al., 2010; Purcell et al., 2013). However, the fishery no longer yield substantial benefits to the coastal communities that once derived from the small-scale fisheries due to declining catches and the presence of exploitive middlemen (Conand, 2008; Ochiewo et al., 2010). Furthermore, the rising consumer demand in Asia continues to push the over-exploitation of these vulnerable species globally (Purcell et al., 2014; Eriksson et al., 2015). The most targeted are the members of the genus Holothuria, with *Holothuria scabra* and *Holothuria fuscogilva* (Purcell et al., 2018), with average market prices for sea

cucumber imported to Hong Kong recorded at 80USD/kg (Louw and Burgener, 2020). Inadequate management practices endanger their long-term viability (Eriksson et al., 2012), with intense fishing having a long-term influence on both abundance and diversity (Anderson et al., 2011; Mmbaga, 2013, Hasan, 2019). Consequently, the ability of the sea cucumber populations to tolerate higher fishing pressure, may hinder their adaptation to environmental and anthropogenic changes (Anderson et al., 2011; Friedman et al., 2011). Furthermore, overfishing of sea cucumber populations is exacerbated by their sedentary nature and the ease with which they can be collected from inshore habitats (Eriksson et al., 2012). Deeper refugia areas are also widely exploited by divers using better boats and SCUBA gear, taking advantage of improved access to international markets (Erikkson et al., 2012). The sea cucumber species exploited in south-coast Kenya include *Stichopus hermani, Holothuria scabra, Holothuria fuscogilva* and *Thelonata anax* with exports majorly destined for south-east Asia (Ochiewo et al., 2010; Purcell et al., 2013; Conand et al., 2022).

In south-coast Kenya, most of the sea cucumber fishery is multispecies (Ochiewo et al., 2010; Muthiga and Conand, 2014). In addition, the rising demand for sea cucumber as observed by Ochiewo et al., (2010) led to fishermen targeting juveniles to increase revenues that potentially would negatively impact their populations. Continual exploitation of sea cucumbers has been linked to loss of genetic diversity due to many individuals being removed leading to genetic structuring among the sea cucumbers (Marty et al., 2014; González-Wangüemert et al., 2015). Moreover, the population of *H. fuscogilva,* given its high market value, is diminishing in many coastal areas due to severe fishing pressure (CITES, 2019).

Population genetics investigates how organisms change over time in response to their environment (Upling, 2020). The evolution of marine invertebrates has been studied using population genetics, while also pinpointing their population structure (Thorpe et al., 2000;

Upling, 2020). Additionally, molecular markers have been utilized in fisheries studies to identify the genetic diversity and structure of animals (Chauhan and Rajiv, 2010; Dudgeon et al., 2012). Mitochondrial DNA (mtDNA) markers particularly due to their numerous gene copies to amplify (Beer, 2014) have often been used in population genetic studies. These markers, which include cytochrome oxidase I (COI), reveal genetic diversity and structure among populations (Beer, 2014). The population genetic structure of heavily exploited holothurians using COI has been studied at various spatial scales (Rodrigues et al., 2015; Valente et al., 2015; Soliman et al., 2016). However, there have been few studies on the *H. fuscogilva* population genetic structure at the regional level and none defined yet at the local level. This study therefore seeks to understand population genetic structure of *H. fuscogilva* in south-coast Kenya.

1.2 Problem statement

Threatened species, including those classified as 'vulnerable,' are frequently associated with small and declining effective population sizes (Ne) (Frankham, 2003). Inbreeding and genetic diversity loss among such small populations have increasingly become more prevalent (Abdul-Muneer, 2014; Neaves et al., 2015). Consequently, the ability of organisms to adjust to environmental changes is affected by the loss of genetic variation and effective population size over time (Abdul-Muneer, 2014). Moreover, the declining effective population size of a species has repeatedly been connected to habitat degradation and extreme fishing pressure (Abdul-Muneer, 2014; Mkare et al., 2017). In marine ecosystems, changes due to evolution result in changes to the population structure among species and subsequently make them genetically distinct populations (Mkare et al., 2017). These changes to the marine environment can also be aggravated by fishing and other anthropogenic activities.

Sea cucumber fisheries in Kenya are dominated by *H. fuscogilva*, with juveniles also being caught to meet Asian market demand (Conand, 2008; Ochiewo et al., 2010). Therefore, there is a possibility that the current population may be inbred and lack genetic diversity due to fishing pressure. In addition, given the high demand at the international markets *H. fuscogilva* was listed in Appendix II of the Convention on International Trade of Endangered Species of Wild Flora and Fauna (CITES, 2019) as means of regulating its trade. Therefore, it is necessary to monitor and reveal important information on the genetic variation and structure of the species to aid in conservation efforts amid the continual risk of fishing pressure in the small-scale cucumber fisheries of coastal Kenya.

1.3 Justification

Global studies on vulnerable populations like *H. fuscogilva* have highlighted extensive reductions in their population owing to fishing pressure, affecting both their genetic diversity and effective population sizes (Abdul-Muneer, 2014; Fernández-García, 2017). Therefore, *H. fuscogilva* as a commercially exploited species requires that it's genetic diversity and effective population size be investigated. The findings of the study can show whether, *H. fuscogilva* are being affected by inbreeding depression as a result of fishing pressure (Fernández-García, 2017). The findings will form a basis for establishing appropriate management units to enhance recovery and the species' survival and adaptations.

The demarcation of genetically structured populations that are treated as conservation units is considered important in spatially structured conservation efforts (von Der Heyden et al., 2014; Fernández-García, 2017). Furthermore, isolated evolutionary locations are deemed to be more defined when genetically structured patterns in populations are identified (von Der Heyden et al., 2014). Therefore, information on the genetic structure of populations will aid in the design of conservation units.

Lastly, recent aquaculture experimental projects in Kenya towards sea cucumbers have shown success with *Holothuria scabra* (Magondu et al., 2021). *H. fuscogilva* becomes a potential species for aquaculture, and a complete genetic understanding of wild populations is needed. Knowledge of the population genetic structure of *H. fuscogilva* is required to enable informed decisions on the sustainable management of its fisheries.

1.4 Objectives

The overall objective of the study was to characterize the genetic population structure of White teatfish, *Holothuria fuscogilva* at selected sites along the south-coast Kenya.

The specific objectives were to:

i. Determine genetic population structure of *H. fuscogilva* at the selected sites in south-coast Kenya;
ii. Determine the patterns of effective population sizes of *H. fuscogilva* based on mitochondrial data at the selected sites in south-coast Kenya and;
iii. Assess the influence of fishing pressure on genetic diversity of *H. fuscogilva* populations in south-coast Kenya.

1.5 Research questions

i. Is there population structure of *H. fuscogilva* from the different sites along the south-coast Kenya?
ii. Is there significant patterns of effective population sizes among individuals of *H. fuscogilva,* along the south-coast Kenya?
iii. Is there influence of fishing pressure on genetic diversity of *H. fuscogilva* populations in south-coast Kenya?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Species biology of *Holothuria fuscogilva*

The white teatfish, *Holothuria fuscogilva*, is, an oval-shaped, dorsally arched, and ventrally flattened sea cucumber (Figure 1). The mouth is surrounded by a collar of yellowish papillae and tentacles, while its anus is encircled by calcareous teeth (Palomares and Pauly, 2020). The species also varies in appearance, with the dorsal side being brown with distinct white spots, while the ventral side is whiter in color (Palomares and Pauly, 2020).

H. fuscogilva lacks cuvierian tubules and its spicules are in form of tables and buttons (Ramofafia et al., 2000; Purcell and Tekanene, 2006).

Figure 1.Dorsal view of *Holothuria fuscogilva* from Gazi, in south-coast Kenya. Source: Diana Karan, taken during field sampling in January 2021

The adult of *H. fuscogilva* has a mean length of 40 cm, with a maximum length reaching up to 60 cm (Palomares and Pauly, 2020). The species inhabits deep habitats and often appears on shallow sea grass beds (Ramofafia et al., 2000; Palomares and Pauly, 2020).Furthermore, reproduction occurs during the North East monsoon seasons (Muthiga and Kawaka, 2010; Palomares and Pauly, 2020).

2.2 Population genetics

Genetic approaches have evolved into important tools for studying marine organisms (Dudgeon et al., 2012). These methods have progressed to utilizing solely tissue samples to comprehend their genetic components (Dudgeon et al., 2012). Population genetics, as part of genetic approaches, intends to study how organisms change over time in response to their environment and adaptation (Upling, 2020). Moreover, population genetics has overcome the barrier of limited genetic data, making it easier to uncover previously unknown genetic population structures among populations (Zhang et al., 2020). Therefore, population genetics studies have become important in genetic studies in marine populations, for instance, with the sea cucumber populations, population genetic diversity, bottlenecks, and genetic structure have been studied (Brown et al., 2022). Studies on the family Holothuriidae have been conducted worldwide, with few studies including *H. fuscogilva*. These investigations have sought to comprehend genetic connectivity (Uthicke et al., 2001; Henriques, 2014; Soliman et al., 2016; Ochoa-Chávez et al., 2018), gene flow (Uthicke and Benzie, 2003; Borrero-Perez et al., 2011), genetic structure (Kim et al., 2008; Gardner and Fitch, 2012; Valente et al., 2015; Nowland et al., 2017), genetic diversity (Vergara-Chen et al., 2010; Ravago-Gotanco and Kim, 2019), and phylogenetic relationships (El-Naggar et al., 2008; Kamarudin et al., 2016).

Population genetics provides genetic diversity analyses that show the status of population densities, and consequently indicate key diversity hotspots (Upling, 2020). Low genetic diversity serves as a cautionary indication of overexploitation in fishery management and that the population requires immediate control of the species' exploitation (Upling, 2020; Ivanova et al., 2021; Pinsky et al., 2021). Fisheries management based on genetic diversity criterion may then propose the establishment of a no-take zone to ensure ecosystem replenishment (Upling, 2020). Conservation authorities also utilize genetic diversity information to make management decisions because it is a significant factor in how well

a species can adapt and evolve (Reynolds et al., 2012; von der Heyden et al., 2014). Therefore, population genetics assists in the development and conceptualization of conservation measures for ecologically and economically important species under anthropogenic fishing pressures (Upling, 2020; Pinsky et al., 2021).

Population genetic research on sea cucumbers in the Western Indian Ocean (WIO) region is extremely limited with none described for *H. fuscogilva* in Kenya (Uthicke et al, 2004; Ridgway and Sampayo, 2007; Oury et al., 2019). These studies have employed a diverse set of genetic markers, including the mitochondrial cytochrome oxidase subunit I (COI) gene, allozymes, and nuclear microsatellites (Uthicke et al., 2001; Uthicke et al., 2010; Oury et al., 2019).

2.2.1 Genetic Diversity

Genetic diversity is defined as the variation in genes and genotypes among organisms (Sjöqvist and Kremp, 2016). It is influenced by population size, with species with more alleles having high genetic diversity (Li et al., 2018; Lonsinger et al., 2018). Smaller populations are linked with inbreeding and genetic drift, which are fundamental to decreasing genetic diversity (Lonsinger et al., 2018). Furthermore, the amount of genetic diversity in a species depends on mating systems and the balance among evolutionary forces; such as mutation (Alam et al., 2015). High genetic diversity has been connected to an individual's fitness, which means they have a higher chance of survival, resistance to diseases, and more mating possibilities (Henriques, 2014; Saleky et al., 2016; Stewart et al., 2019). Furthermore, genetic diversity may influence genetic structure in response to different environmental conditions, resulting in intraspecific adaptation of different species to environmental changes (Henriques, 2014). Generally, genetic diversity is concerned with the variations of a population and is sensitive to the evolutionary rate

between genes and species whereas population genetic structure is concerned with the ordering of genetic variants in time and space (Mkare, 2013).

2.2.1.1 Fishing and genetic diversity

Fishing is a selective process that affects the size of the organism, alters the sex ratio by capturing one sex over another and increases the proportion of phenotypic change in wild populations (Kenchington, 2003; Pinsky et al., 2021). Extreme fishing pressure, for instance, has contributed to reducing the population of sea cucumbers to near extinction with extreme densities of 0.83 individuals per 100 m^2 as observed in Fiji (Jontila et al., 2017). Sea cucumbers may also display the Allee effect (decrease in reproduction success) at low densities, which could cause population collapse (Eriksson et al., 2012). Therefore, the population could be driven to extinction as a result of fishing pressure, which has drastically reduced the biomass of several populations of sea cucumbers (Eriksson et al., 2015; Rahman et al., 2015).

Genetic diversity is influenced by fishing when the population left behind has genetic alterations when compared with the previous one (Henriques et al., 2016). Species that experience strong events of fishing may have a reduction in their effective population size reducing the efficiency of mutation to balance out the loss of genetic diversity (Henriques, 2014; González-Wangüemert et al., 2015). Fishing at its extreme has therefore been linked to genetic diversity and the genetic structure of species (González-Wangüemert et al., 2015). Moreover, extreme fishing pressure where many individuals are removed can result in a bottleneck leading to an immense loss of genetic diversity and possible threat to extinction (Marty et al., 2014).

2.3 Population genetic structure

Genetic structure or genetic differentiation is defined as the aggregation of variation in allelic composition between entirely or partially isolated populations due to evolutionary influences (Abdul-Muneer, 2014; Upling, 2020). Therefore, population genetic structure is a field that looks at the accumulated differences among populations due to the interaction between ecological and genetic processes (Jin et al., 2020). It is a fundamental part of evolution, as species are divided into genetically distinct populations (Abdul-Muneer, 2014). Moreover, there are three patterns of genetic differentiation recognized: no differentiation, continuous genetic change, and complete differentiation (Mkare, 2013). Genetic differentiation in marine populations is usually expected to be low (Ward, 2000). The low levels of genetic differentiation over a large geographical scale are linked to the high dispersal among their life stages and the lack of physical barriers in the open sea (Yang and Xin-Zheng, 2018). Genetic structure is also influenced by local geographical and oceanographic characteristics (Nowland et al., 2017). These geographical and oceanographic characteristics may include landmasses, temperature gradients, ocean currents, and ocean depths that restrict the dispersal of pelagic larvae, giving rise to structured populations leading to speciation (Holsinger and Weir, 2009; Vergara-Chen et al., 2010; Michonneau et al., 2015). Vicariance events, whereby a barrier results in fragmented populations, in the marine environment can also limit dispersal, resulting in the formation of genetic structure among marine taxa (Mkare et al., 2017). In addition, spatial and temporal separation caused by differences in spawning seasons has also been found to influence the sea cucumber population, resulting in reproductive separation leading to gene differentiation (Kang et al., 2011).

Marine ecosystems are deemed to be connected, with fewer barriers to gene flow, so populations are assumed to be homogenous (Yang and Xin-Zheng, 2018). However, changes in environmental conditions and fishing demands on marine resources may cause

varied responses among their populations, making many of the populations to be structured. Population genetic structure is generally, therefore, influenced by two factors: gene flow and genetic drift.

2.3.1 Gene flow

The movement of migrants between populations and their effective contribution to a gene pool is referred to as "gene flow" (Henriques, 2014). Gene flow among marine species can be constrained by geographical barriers such as waterways and influenced by ocean currents (Zulliger et al., 2009), leading to genetic structure. In addition, spatial homogeneity in marine populations is thought to be possible, especially for species with pelagic larval stages (Choi et al., 2021) through larval dispersal. A planktonic larval stage, according to population genetic theory, leads to increased gene flow and lower levels of population structure (Ye et al., 2015).

Marine invertebrates with high dispersal capabilities are expected to show high levels of gene flow and a low population genetic structure over small spatial scales (Vergara-Chen et al., 2010). Therefore, dispersal patterns of the population and the dynamic migratory behavior of adults and juveniles are major factors that potentially influence the spatial scale of population structure (Ciannelli et al., 2013). Gene flow allows individuals that migrate into a new population to contribute unique alleles that are not found in that population (Dudu et al., 2012), inhibiting genetic structure among populations and making them genetically similar (Vergara-Chen et al., 2010; Dudu et al., 2012).

2.3.1.1 Isolation by distance relationship with genetic structure

Geographical distance and habitat complexity can dictate gene flow for a particular species (Saleky et al., 2016). Genetic structure is expected to increase with increasing geographic distance when there is limited larval exchange among populations

(Michonneau et al., 2015). Therefore, assuming that dispersal reduces with distance from the source, the stepping stone model of population genetics predicts that as the distance grows, migrant population mixing will be reduced, inducing genetic structure among them (White et al., 2010). Consequently, marine organisms of low population size across wide geographical scales that are isolated face high extinction rates over time (Saleky et al., 2016). Geographic distance is, therefore, a significant contributor to genetic structuring; however, for some populations, this may not be the case, as shown by sea cucumber, *Holothuria atra* (Skillings et al., 2010). *H. atra* had significant population structuring within proximity to the Hawaii archipelagos (Skillings et al., 2010).

2.3.2 Genetic Drift

Genetic drift is defined as the shift in gene frequencies induced by random variations in the parental individuals determining reproduction (Jones and Wang, 2012). Genetic drift can be classified into two types: bottleneck and founder effect. Genetic bottlenecks are evolutionary events where populations are drastically reduced in size, followed by a decline in diversity and the number of genes (Ali and Roossinck, 2008; Horing, 2014). Bottleneck events may result in the formation of a new population from a small number of individuals, resulting in population structure (Horing, 2014). The founder effect, on the other hand, is a phenomenon where individuals colonize a new region and evolve independently (Arizmendi-Mejia et al., 2015). The founder effect influences genetic structuring among populations since the new populations become genetically distinct from the original population (Hatmaker et al., 2015). Genetic drift, as an evolutionary force, contributes to genetic structuring among marine populations. Genetic drift causes gene frequencies to shift unpredictably over a few generations, which is its direct influence (Dudu et al., 2012; Arizmendi-mejía et al., 2015). Furthermore, genetic drift over time allows for the loss of genetic diversity, as some of the alleles present in the parent

generation may not be passed on to their offspring (Dudu et al., 2012). As a result of genetic drift, various genes become more widespread or fixed in different populations, resulting in populations that are genetically different from one another (Dudu et al., 2012; Arizmendi-mejía et al., 2015).

Genetic structure studies then become crucial in assessing these marine populations to aid fisheries management and track population stocks (Zhang et al., 2020). These populations are likely to be impacted by fishing pressure as an external force (Zhang et al., 2020). Population genetic structure studies on sea cucumbers have been done using mitochondrial markers with low genetic structure among them linked to unrestricted gene flow (Vergara-Chen et al., 2010; Soliman et al., 2016). The absence of genetic structure is typically understood as a well-mixed population or a population with recent demographic expansion following a genetic bottleneck event that left little or no imprint in the genome (Yan et al., 2020).

Marine habitats may also influence organism genetic structure; for example, coastal lagoons are expected to fluctuate in both space and time due to their heterogeneous environmental conditions (Vergara-Chen et al., 2010). Genetic structure was investigated for *Holothuria polii* at the Mar Menor coastal lagoon in Spain and for *Holothuria arguensis* at the Ria Formosa coastal lagoon in Portugal (Vergara-Chen et al., 2010; Rodrigues et al., 2015). The mitochondrial gene fragment cytochrome oxidase I (COI) used showed non-significant differences in genetic structure, revealing high connectivity among the species (Vergara-Chen et al., 2010; Rodrigues et al., 2015), even in fluctuating environments such as lagoons.

On Okinawa Island in Japan, for instance, a population genetic structure study was carried out to investigate populations of *Holothuria edulis* given that they were highly exploited in the area (Soliman et al., 2016). The mitochondrial cytochrome oxidase subunit I (COI) marker, among other markers, was used at six locations across Okinawa Island. The

overall population structure from the markers was significant and was related to the coastal developments influencing ecosystem degradation and the observed population structure (Soliman et al., 2016). The study also highlighted that the unique features of Okinawa's Main Island would have promoted such patterns of structure and that barriers may have led to genetic structuring.

Population structure among sea cucumbers as observed in *Holothuria atra* is being driven primarily by barriers (Skillings et al., 2014). The barriers among the archipelagos where the *H. atra* were sampled showed patterns of restricted gene flow between the Hawaii sites. Barriers in marine environments are sources of population differentiation, as shown in the Hawaiian Islands (Skillings et al., 2014). In Seychelles, appropriate population-specific fishery management regulations were implemented, and a study of the genetic diversity and connectivity of the natural and exploited populations of three taxa was done. The results showed that species, including *H. fuscogilva*, had high levels of inbreeding among them (Oury et al., 2019). Apart from these microsatellite-marker-based studies, population genetic studies based on *H. fuscogilva* are lacking in Kenya and in the WIO region.

2.3.3 Estimating population genetic structure

Genetic population structure is expected to reflect reproductive isolation (Ward, 2000). Moreover, population genetic structure is influenced by genetic drift and gene flow and can be detected by variations in gene frequencies (Henriques, 2014).The Fishers Wright statistics (F_{ST}) is normally used to quantify the genetic difference among populations using gene frequencies (Bird et al., 2011; Ma et al., 2015). The F_{ST} estimators are known to examine the ratio of population variance to the total variance in gene frequencies (Kitada et al., 2021). Population-specific F_{ST} estimators are used to estimating population structure and are classified as Global F_{ST} and Pairwise F_{ST} (Kitada et al., 2021). Pairwise F_{ST} values frequently represent the current population structure of that specific population

(Kitada et al., 2021). Global F_{ST} values measure the overall population structure, whereas Pairwise F_{ST} values measure values between pairs of population samples. Therefore, with F_{ST} values being indicators of population structure, genotype differences will show subdivision patterns among populations (Henriques, 2014). Studies of population genetic structure in marine populations show a complicated nature across geographical scales (Zhang et al., 2020). Determining the level of genetic structure using F_{ST} to show variations within and between populations becomes important since it highlights the effects of mutation, natural selection, and genetic drift (Kang et al., 2011; Soliman et al., 2016; Nowland et al., 2017).

2.4 Effective population size

The effective population size (N_e) is a theoretical measure of an idealized population size that would be expected to experience the same rate of genetic diversity loss as the actual population (Lonsinger et al., 2018). It determines the level of inbreeding and the level of genetic loss in a population (England et al., 2006; Henriques, 2014; Wang et al., 2016). Effective population size estimates are useful for fisheries management because they integrate genetic influences with species' life histories and provide forecasts of a population's present and future survival (Hare et al., 2011).

Individuals with a smaller effective population size are a result of founder and bottleneck effects (Henriques, 2014). Small effective population size (N_e) allows the genetic diversity of a population to be reduced through evolutionary mechanisms (Hare et al., 2011). Therefore, a decline in N_e increases genetic drift gradually overpowering natural selection and leading to inbreeding depression for populations in isolation (Hare et al., 2011; Wang et al., 2016). The outcome of the imbalance between genetic drift and natural selection is fixed detrimental mutations (Hare et al., 2011), and natural selection is weakened and hence unable to balance the mutations out (Hare et al., 2011; Wang et al., 2016). Selective

stress, such as fishing pressure, tends to increase the stochastic loss of genetic diversity, with the rate of mutations becoming unbalanced in comparison to genetic drift (Henriques, 2014; Lonsinger et al., 2018), resulting in the reduction of population fitness to environmental changes.

2.4.1 Estimating effective population size (N_e)

Effective population size (N_e) determines the rate at which diversity is declining and the extent of inbreeding (Luikart et al., 2010; Hare et al., 2011). Conversely, it is challenging to estimate N_e directly from demographic data and indirect genetic estimators have then been used (Luikart et al., 2010). Given the rapid advances in genomic and computational technologies (Luikart et al., 2010), for instance the concepts of Coalescent Bayesian methods, estimation of N_e is now possible. Contemporary and historical effective population size estimates have emerged from the diverging methodological approaches (Nadachowska-Brzyska et al., 2021). Additionally, contemporary estimation of N_e uses both direct and indirect genetic methods. Direct methods require knowledge on population's parameters of census size, sex ratio, variance in reproductive success and mating system (Nadachowska-Brzyska et al., 2021). The use of direct methods is however limited with sampling generations needed to acquire their temporal data such as variance in reproductive success (Hare et al., 2011; Nadachowska-Brzyska et al., 2021). The indirect methods on the other hand, require information on genetic variation that include; allele frequency changes over time, estimation of heterozygosity excess among other parameters which can be obtained from markers such as microsatellites and Single Nucleotide Polymorphisms (SNPs).

Historical N_e estimations on the other hand requires only genetic data to make conclusion about the population (Hare et al., 2011). Historical estimates draws conclusion from demographic events such as population expansions, and population structure, since they

leave distinct traces in their genome's (Hare et al., 2011; Nadachowska-Brzyska et al., 2021). Additionally, historical N_e estimations can be traced accurately with mitochondrial markers such as Cytochrome oxidase I (COI) and Cytochrome b (Cyt b).

Coalescent theory, a concept based on tracing backwards in time to identify events that occurred since the most recent common ancestor, allows for the estimation of historical effective population size using genetic data (Atkinson et al1q1., 2008; Nordborg, 2019). Mitochondrial DNA (mtDNA) markers such as cytochrome oxidase I (COI) are suitable option for coalescent inference of population size because of its high number of genes, lack of recombination, and rapid substitution rate with a clear genealogical tree (Atkinson et al., 2008). Additionally, mitochondrial markers are ideal in recording signatures of population structure and expansions with departures from neutrality often linked to changes in effective population size. Estimation of N_e comes with a set of conditions to allow accurate measurements of the parameter. For instance, when estimating N_e, it is crucial to take-into account the Wright-Fisher population assumptions because overlapping generations and migration might lead to incorrect N_e estimations (Dudgeon et al., 2012).

Generally, in conservation particularly in regards to conservation genetics, the concept of effective population size is important as it highlights the history of the population in regards to genetic drift and inbreeding (Wang et al., 2016). The N_e assessment also is an essential population evaluation tool for conservation and fishery management as it offers information on both breeding population size and population genetic fitness (Dudgeon et al., 2012). It is proposed that to maintain immediate effects of inbreeding, the contemporary effective population size should be greater than 50 individuals and for future adaptation to environmental changes then N_e should be between 500-5000 individuals (Watts et al., 2007). Therefore, most of the studies have examined the effective

population sizes on *Holothuria spp* using contemporary microsatellite markers (Henriques, 2014; Ravago-Gotanco and Kim, 2019; Brown et al., 2022).

2.5 Genetic markers

Genetic markers or molecular markers are great tools used to detect the genetic uniqueness of individuals, species or populations (Chauhan and Rajiv, 2010). Markers have been used in marine fisheries to investigate commercially exploited invertebrates and have had a significant contribution to their conservation as well (Abdul-Muneer, 2014; von Der Heyden et al., 2014; Grünwald et al., 2017). Markers also allow assessment of distribution of genetic variation among animal populations (Chauhan and Rajiv, 2010). Genetic markers are classified based on their functionality and position (Dudu et al, 2012; Beer, 2014). Markers on functionality are categorized as Type I Markers and Type II markers (Chauhan and Rajiv, 2010). Type I include allozymes markers while Type II include Random Amplified Polymorphic DNA.(RAPD), microsatellites, Single Nucleotide Polymorphisms(SNPs) and Amplified Fragment Length Polymorphisms (AFLPs) (Chauhan and Rajiv, 2010; Abdul-Muneer, 2014). Additionally, the markers location in the cell, is another way to categorize them (Dudu et al, 2012). Therefore, distinguishing between nuclear and mitochondrial DNA (mtDNA) markers is based on where they are found on the cell (Chauhan and Rajiv, 2010).

Mitochondrial DNA (mtDNA) markers for example include Cytochrome oxidase sub unit I, Cytochrome b, and 16s gene. They exhibit a non-Mendelian pattern of inheritance (maternally inherited) and can record mutations over time. These mtDNA markers are preferred for population genetics work due to their multiple copies that can be amplified easily (Beer, 2014; Horing, 2014). Moreover, mitochondrial markers provide inference about the evolutionary history given their mutation rate that exhibits genetic variation among populations (Beer, 2014; Grünwald et al., 2017). Therefore, mitochondrial

markers, are important for understanding population genetics, because they can acquire mutations through time, showing divergence times in a population (Semina et al., 2007; Mkare, 2013).

Cytochrome Oxidase subunit I (COI), a common mitochondrial marker evolved differently from nuclear DNA (Ismail, 2013). In addition, when compared to other genetic markers, it has a fast evolutionary rate and a wide intraspecific polymorphism (Li et al., 2015). The marker has frequently been employed to examine questions of genetic diversity and speciation processes among species (Xu et al., 2014; Soliman et al., 2016; Coykendall et al., 2017; Hamamoto et al., 2021). COI is less conserved with higher mutation rates, therefore, provides better genetic structure at a contemporary level of a species (Rodrigues et al., 2015). Investigating genetic structure using COI among the family of Holothuriidae has been documented for different Holothuria *spp.* (Vergara-Chen et al., 2011; Soliman et al., 2016; Hamamoto et al., 2021).

The rapid improvement of computational power and statistical models have enabled conclusions to be drawn from the genetic data derived from various markers (Chauhan and Rajiv, 2010). In addition, genetic markers have the advantage of being directly related to reproduction as well as other biological factors, allowing access to genetic information on population structure that traditional approaches do not provide (Knutsen et al., 2015). Therefore, genetic markers such as COI gene would adequately provide population structure information on *H. fuscogilva* as aided by the advanced computational and statistical approaches.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was conducted at three selected sites.The sites selected represented the distribution range of the species in the country and also have the highest catch landings of the species. These selected sites were Gazi, Shimoni, and Vanga (Figure 2). Gazi and Shimoni are some of the areas where the species is heavily exploited in south-coast Kenya (Ochiewo et al., 2010; Muthiga and Conand, 2014), with Shimoni lying adjacent to the Kisite-Mpunguti Marine Park and Reserve. Vanga is located on the Kenyan coast's southernmost tip, bordering Tanzania. Vanga location is characterized by migrant fishers due to its fairly rich fishing grounds. Given the conservation status of the species, a sampling permit was obtained from the Kenya Wildlife Service

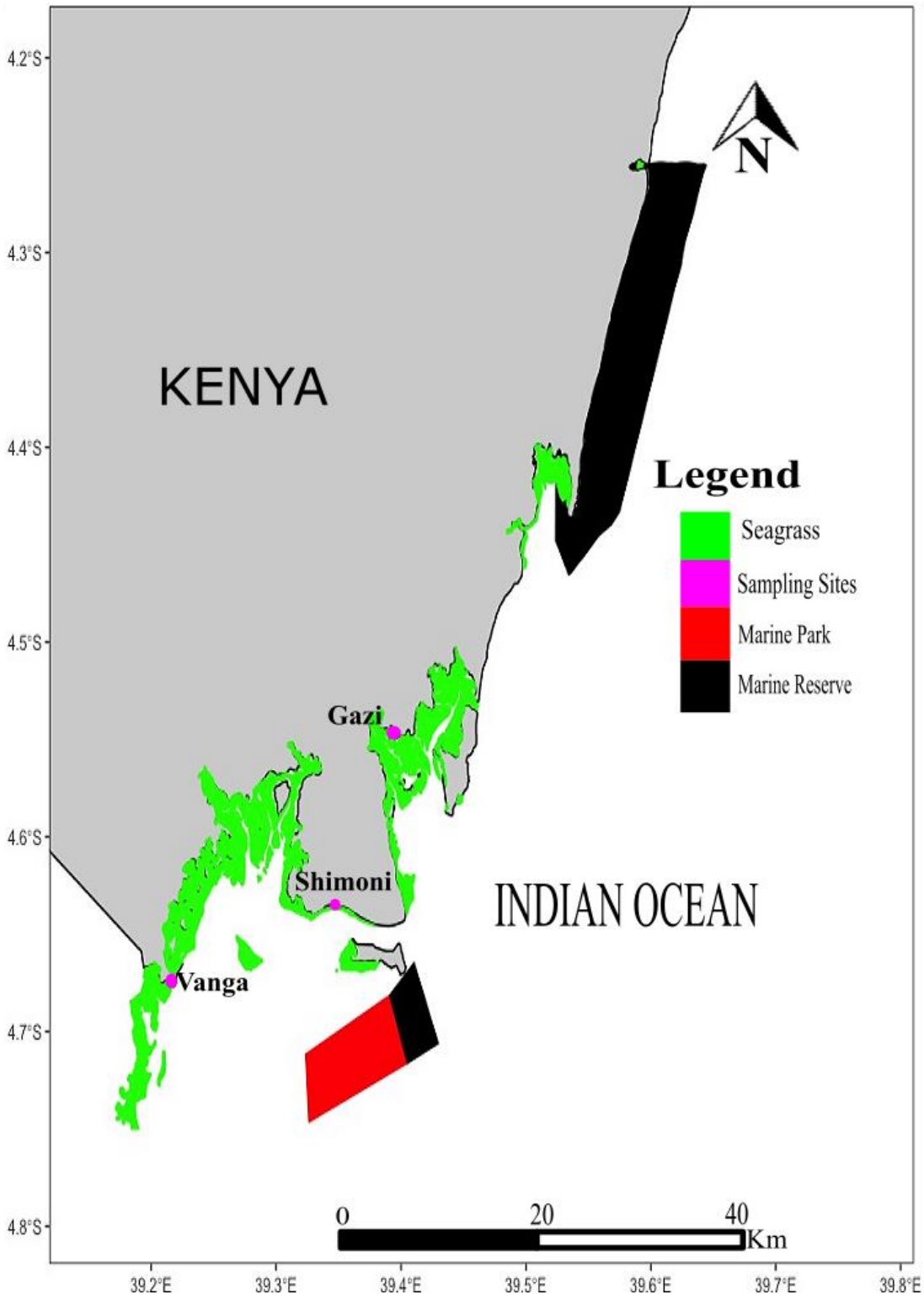

Figure 2. Map of Kenyan south-coast showing sampling sites for the *Holothuria fuscogilva* study

3.2 Sampling

Sample collection of *Holothuria fuscogilva* were acquired with assistance from fishers at the fishing grounds. Convenience sampling was used to conduct the sampling surveys over the course of two trips, one in November 2020–April 2021 and the other in February 2022, due to the difficulty in getting *Holothuria fuscogilva* species and the difficulties involved with tissue DNA extraction. A total of 60 individuals of *H. fuscogilva* were obtained: 25 individuals from Gazi, 27 individuals from Shimoni and 8 from Vanga (Table 1) with identities confirmed by the presence of white teats. A few individuals as representative samples of each site were photographed before obtaining the tissue samples for further reference.

Table 1. Sampling locations and number of individuals for *Holothuria fuscogilva* collected for the population genetic structure study.

Site	Latitude	Longitude	Number of Individuals
Gazi	4˚2542 S	39˚3058E	25
Shimoni	4˚3910 S	39˚2229E	27
Vanga	4˚3931 S	39˚1403E	8
Total			60

3.3 DNA extraction

Tissue samples of *H. fuscogilva* were obtained from a small section of body wall muscle using a sterilized scalpel and preserved in 100% absolute ethanol while in the field (Soliman et al., 2016).All DNA extractions were conducted using DNeasy Blood and Tissue extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and then stored at -20 ºC prior to analyses. DNA concentration was quantified using

NanoDrop1000 (Thermo Scientific) spectrophotometer. Purity of DNA from protein and polysaccharide contamination was also assessed by estimating the absorbance ratio at A260/A280 and A260/A230 respectively. The extracted DNA after quantification was checked for quality of bands by electrophoresis separation on 1% Agarose gel (Appendix 1).

3.4. Polymerase Chain Reaction (PCR)

3.4.1 Primer design

Quality DNA samples were initially amplified by using published echinoderm universal primers CO1-eF, 5′-ATAATGATAGGAGGRTTTGG-3′ and CO1-eR, 5′-GCTCGTGTRTCTACRTCCAT-3′ (Uthicke et al., 2010), however under a series of runs they could not amplify consistently hence new primers were designed and ordered for use (Appendix A).

3.4.2 PCR amplification

A pair of the designed primers; HFC-F, 5′-ATGAAAACATGAGATTTTG-3′ and HFC-R, 5′-ATGGTTCTTGCTTTCCTCT-3’ were used to amplify a fragment of 515 base pairs of the mitochondrial cytochrome oxidase subunit I (COI) gene. Polymerase chain reaction (PCR) amplification was performed on an Applied Biosystems thermo cycler (Thermo Fisher Scientific Inc., MA, USA) using a total volume of 25 ul with 12.5 ul of ONE TAQ Master Mix (New England Biolabs, MA, USA), 2.5 um of each primer, 1 x Bovine Serum Albumin (BSA) (Invitrogen, Thermo Fisher Scientific Inc., MA, USA) and 20–80 ng of DNA template. The thermal profile consisted of an initial denaturation step at 95 °C for 60 secs, 40 cycles of denaturation at 94 °C for 30 secs, annealing at 50 °C for 30 secs, and extension at 72 °C for 80 secs, and a final extension step at 72 °C for 10 min. The quality of the PCR products as correctly sized products was checked via electrophoresis (110–

120 V, 45 min) on a 1% agarose gel in TRIS-Borat-EDTA buffer (1x TBE buffer)(Sigma-Aldrich, MI, USA). In addition, for every sample, 4 ul of the PCR product was used with 1 ul of 6X loading dye (New England Biolabs, MA, USA) applied to the gel. The amplified products resulted in 52 successful products that, after being checked for quality and quantity, were sent to the Macrogen Europe DNA sequencing facility in the Netherlands for bidirectional Sanger sequencing.

3.5 Bioinformatics

3.5.1 Quality control

Chromatograms were visualized and analyzed using BioEdit software version 7.0.4.1 (Hall, 1999) based on the nature of their peaks. In each sea cucumber individual sample, nucleotide sequences derived from both forward and reverse sequences were assembled to get consensus sequences. Thereafter, the consensus sequences were aligned using Clustal W in Molecular Evolutionary Genetic Analysis (MEGA) X (Kumar et al., 2018) and visually checked with over hangings cropped out. The validity and integrity of the specimens acquired as belonging to the *H. fuscogilva* species were established by comparing each sequence with mitochondrial COI sequences of other sea cucumbers in GenBank using NCBI Blast-N (http://blast. ncbi.nlm.nih.gov).

3.5.2 Genetic diversity

Genetic diversity indices of the sea cucumber population such as; numbers of haplotypes (nh), haplotype diversity *(h*), number of polymorphic sites (s), and nucleotide diversity (π) were estimated with DNA Sequence Polymorphism (DnaSP) v.5.10.01) (Librado and Rozas, 2009) and Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). Since, the populations had different sample sizes, haplotype richness was calculated using the Allelic Diversity Analyzer (ADZE) based on the rarefaction method (Szpiech et al., 2008).

Fu Fs and Tajima's D neutrality tests were used to detect deviations from the neutral theory's expectations (Fu and Li, 1993; Beer, 2014). Moreover, population expansion was examined using neutrality tests, Harpending's raggedness index, and mismatch distribution (Villamor et al., 2014) in DnaSP and Arlequin version 3.5.2.2 (Librado and Rozas, 2009; Excoffier and Lischer, 2010). Negative Fu Fs test, values are deemed to pinpoint recent population expansion, whereas positive values show a recent bottleneck effect. Tajima's D scores when positive show a state of neutrality (evolving randomly) with natural selection acting upon them, and when negative show a population that has experienced a recent bottleneck or a demographic event (Tajima, 1989; Hamamoto et al., 2021).

3.5.3. Genetic structure/Genetic differentiation

To undertake genetic population structure analysis, sampling sites as indicated on the study area map were considered as distinct groupings. The groupings were then tested for genetic differentiation using Arlequin version 3.5.2.2 software (Excoffier and Lischer, 2010) under null assumptions of no differentiation as adopted from Mkare (2013). Analysis of Molecular Variance (AMOVA) (Beer, 2014) and the computation of F_{ST} values were conducted in Arlequin version 3.5.2.2 software (Soliman et al., 2016). Wright Fisher Fixation index (F_{ST}) is usually an appropriate measure of genetic differentiation among locations where migration is potentially, in theory, occurring at a faster rate than mutation (Bird et al., 2011).If there was significant differentiation, isolation by distance analyses we performed using the Mantel test in Arlequin software.

3.5.4. Phylogenetic analyses

The evolutionary relationship between haplotypes derived from the sampled *H. fuscogilva* populations was demonstrated using a haplotype network and phylogenetic

trees. The Neighbor-joining (NJ) and Maximum Likelihood (ML) were used to reconstruct phylogenetic trees, with each relying on different mathematical models, thus providing complementary results. The haplotype network was constructed using a median-joining haplotype network as implemented in the Population Analysis with Reticulate Trees (POPART) software (Leigh and Bryant, 2015). The phylogeographic patterns were reconstructed based on the genetic linkages between samples to investigate the relationship between the geographical region and genetic connection (Beer, 2014). Therefore, phylogenetic analyses were used to investigate the relationship between the sampled individuals and *H. fuscogilva* specimens from different geographic locations around the world. The out-group was *Holothuria whitmaei*. Moreover, the sequences from this study were then defined by haplotypes, with global sequences and out-groups obtained from GenBank, see (Appendix 4).

Sequence alignment was carried out in MEGA.X (Kumar et al., 2018), whereby haplotype sequences were pooled into a single file with those obtained from GenBank and realigned to generate a phylogenetic tree. The phylogenetic trees were constructed with NJ and ML using MEGA.X (Kumar et al., 2018). ML analysis was performed using 1000 bootstraps support. The jModel test (Darriba et al., 2012) was used to estimate the appropriate ML model of DNA substitution.

3.5.5. Estimation of historical effective population size

The historical effective population size of the female genealogy was estimated using Bayesian skyline plots (BSPs). BSPs assume that a population is panmictic and use inferred patterns of coalescence to fit a demographic model to the set of sequence data provided (Drummond et al., 2005). Therefore, effective size analyses were performed using sequences generated from this study by generating Bayesian skyline plots (BSPs) using the Bayesian evolutionary analysis by sampling trees (BEAST) program

(Drummond et al., 2012). Estimates of expansion time and historical changes in effective population sizes (N_e) were also deduced through Markov Chain Monte Carlo (MCMC) simulations (Porretta et al., 2012; Villamor et al., 2014; Coscia et al., 2016; Li et al., 2018). Using the jModel test (Darriba et al., 2012), the best-fit nucleotide substitution models were Hasegawa-Kishino-Yano (HKY) for the species' data set chosen based on the lowest Akaike Information criterion (AIC) score. Under the clock option, a strict molecular clock with a fixed rate of 0.84% substitution per million years (Borrero-Pérez et al., 2010) was chosen, and data was partitioned into the first, second and third codon respectively using BEAUTi v.1.10 (Drummond et al., 2012).

Three independent analyses with 30 000 000 generations were carried out, with log and tree parameters logged every 1000 generations. TRACER (Rambaut et al., 2018) was then used to construct the confidence interval for N_e over time, based on the posterior probability distribution (Porretta et al., 2012; Villamor et al., 2014). To eliminate issues of convergence, species were treated to multiple distinct runs with 10% burn-in until the effective sample size reached 200, as specified in the user's manual (Coscia et al., 2016). TRACER was also used to reconstruct the population dynamic over time through the Bayesian Skyline construction option (Porretta et al., 2012). Graphical representations that included phylogenetic trees were visualized in Fig Tree v1.4.0 (Rambaut, 2009).

CHAPTER FOUR

4.0 RESULTS

4.1 Quality control

Out of the 60 samples that were collected, 8 failed to amplify using the PCR and were eliminated, leaving behind 52 samples that were amplified and analysed. Electropherograms obtained from the primers used did not show any signs of double reads as evidence of pseudo genes. The validity and integrity of the sequences were compared to published sequences in the GenBank database using the BLAST-N option in NCBI to ascertain the accuracy of COI sequences analyzed belonging to *H. fuscogilva.* The BLAST-N results lead to one sequence being excluded resulting to 50 individuals exclusively belonging to *H. fuscogilva*. From the 51 individuals a total of 47 individuals had high quality sequences and were analyzed after a final alignment of 518 nucleotides were drawn from consensus of HFC-F and HFC-R.

4.2 Genetic diversity

The 47 sequences yielded 22 haplotypes that were defined by 38 polymorphic sites among the populations. The overall nucleotide and haplotype diversity estimates were 0.008±0.001 and 0.922±0.021, respectively. Fifteen haplotypes were identified for Shimoni ten haplotypes for Gazi and four haplotype among the Vanga samples. Individually, among the populations the nucleotide diversity and haplotype diversity ranged from 0.001 (Vanga) to 0.009 (Gazi) and 0.900 (Vanga) and 0.933 (Gazi) respectively (Table 2).

Table 2. Genetic Diversity indices for the 47 samples population of *Holothuria fuscogilva* from south-coast Kenya

Locality	***n***	***s***	***nh***	***π***	***h***	***Ar***
Gazi	15	20	10	0.009±0.002	0.933±0.024	6.675
Shimoni	27	26	15	0.008±0.001	0.929±0.028	6.155
Vanga	5	4	4	0.001±0.010	0.900±0.048	1.790
All pop	47	38	22	0.008±0.001	0.922±0.021	14.62

n represents Sample size, s represents Polymorphic Sites, nh represents Haplotypes**,** π represents Nucleotide Diversity, h represents Haplotype diversity, A_r represents Haplotype Richness.

4.2.1 Substitution patterns in *Holothuria fuscogilva* populations

A total of 42 changes were required to account for all 22 haplotypes; these changes included 35 transitions and 18 transversions with the values giving a transition/transversion (ti/tv) ratio of 1.945 (Table 3), with no insertion or deletions events observed among the individuals. Transversion usually occurs when the nucleotide interchange between purine (Adenine (A) or Guanine (G)) with pyrimidines (Cytosine (C) or Thymine (T)) while transition is a substitution mutation where a purine base (A) is interchanged for another purine (G) or pyrimidine (C) is interchanged for another pyrimidine (T) base (Saleky et al., 2016). The ti/tv ratios ranged from (0.33-3) among the *H. fuscogilva* sequences and the mean frequencies of the C residues (33.28) were higher with G residues (5.31) being the least as observed among the populations (Table 3). Across all *H. fuscogilva* populations GC content averaged at 38.57% while AT content was at 61.40%.

Table 3. Nucleotide bases composition in amplified mitochondrial COI gene for *Holothuria fuscogilva* populations

Populations	%Bases				GC cont.%	AT cont.%	ts	tv	ts/tv
	A	**C**	**G**	**T**					
Gazi	25.79	34.56	5.44	34.21	40	60	15	5	3
Shimoni	25.44	33.72	5.75	35.09	39.47	60.53	19	10	1.9
Vanga	25.26	31.58	4.74	38.42	36.32	63.68	1	3	0.33
Total(mean)	**25.50**	**33.28**	**5.31**	**35.91**	**38.57**	**61.40**	**11.67**	**6**	**1.945**

4.2.2 Haplotype richness in *Holothuria fuscogilva* populations

Sample size has an influence on haplotype richness and may be adjusted using rare-faction techniques. The rare-faction approach, can only be employed when sample sizes are unequal since it predicts haplotype richness for a uniform number of observed genes (g). Haplotype richness from the three *H. fuscogilva* population showed that it increased steadily (Figure 3), with varying mean haplotype richness (Table 2). The highest levels of mean haplotype richness were shown in the Gazi populations following rarefaction adjustment (6.675). Shimoni populations also exhibited high levels of mean haplotype richness (6.155), while Vanga populations had the least genetic diversity (1.790).

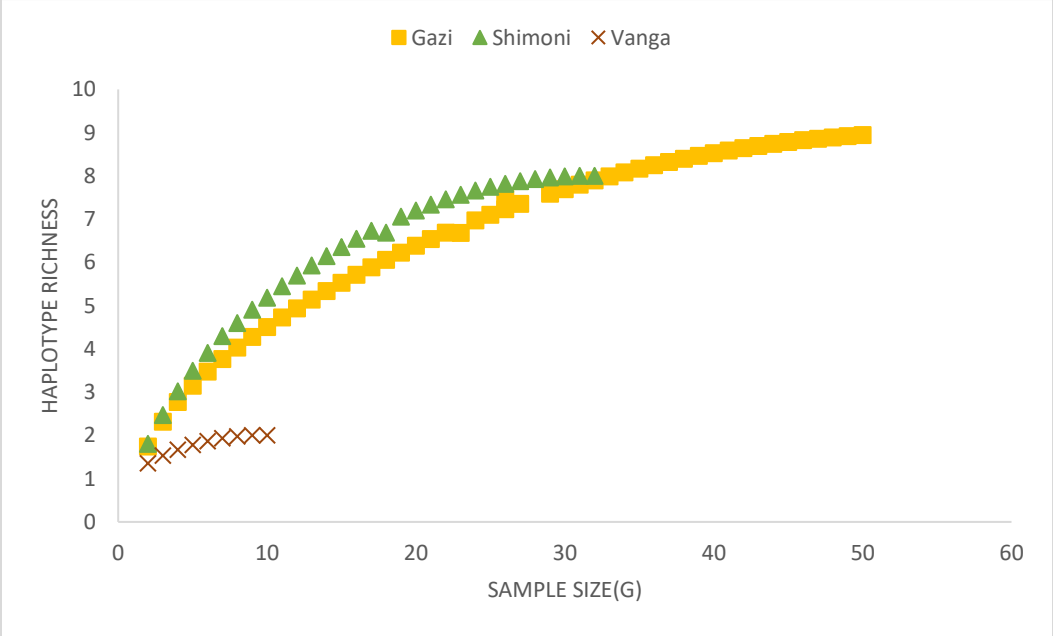

Figure 3. Haplotype Richness analyses among *Holothuria fuscogilva* populations based on ADZE program

4.2.3 Population dynamics among *Holothuria fuscogilva* populations

Negative Fu and Tajima D tests provided statistical support for recent demographic changes. Gazi and Shimoni showed significant Fu values of (-9.558 (0.003) and -5.751 (0.002)) for *H. fuscogilva* (Table 4) with Vanga having insignificant negative values. These revelations highlight the occurrence of a number of mutations that hint out a population that has undergone a population expansion. Moreover, Shimoni Tajima D score (-1.889, p=0.05) were statistically significant compared to Gazi and Vanga populations. Overall, the Tajima D scores (-2.095, p=0.05) and Fu test (-10.862, p=0.003) were negative for all the populations, an indication that population size has experienced demographic events.

The raggedness index used to evaluate the smoothness of mismatch distribution ranged from 0.015 to 0.120 for the three populations, with Vanga populations' raggedness index (0.120, p =0.029) being statistically significant (Table 4). The whole population had low and not significant ragged index of (0.025, p=0.08**).** The significance of Tajima D and Fu

tests based on mitochondrial Cytochrome Oxidase I (COI) for *H. fuscogilva* as observed on (Table 4), indicate that population expansion events may have been experienced especially on Shimoni population. In addition, Gazi population on the other hand, recorded the highest negative Fu Fs test values an indicator of excess new haplotypes hence population growth.

Table 4. Population dynamics based on neutrality tests for *Holothuria fuscogilva*

Populations	***D*** **(p-value)**	***Fu*** **(p-value)**	R **(p-value)**
Gazi	-1.195(0.10)	**-9.558(0.003)**	0.015(0.200)
Shimoni	**-1.889(0.05)**	**-5.751(0.002)**	0.036(0.167)
Vanga	**-1.323 (0.001)**	-1.195(0.232)	**0.120(0.029)**
All populations	**-2.095 (0.05)**	**-10.86(0.003)**	0.025(0.08)

Statistically significance values shown in bold (*p* < 0.05)

D represents Tajima test, FU represents FU'S FS statistics and R represents Harpending's raggedness index

Mismatch Distributions was pooled and calculated for all populations. Unimodal distributions was observed by the nature of having one peak in the distribution (Figure 4) with this distribution generally associated with sudden expansion among the populations.

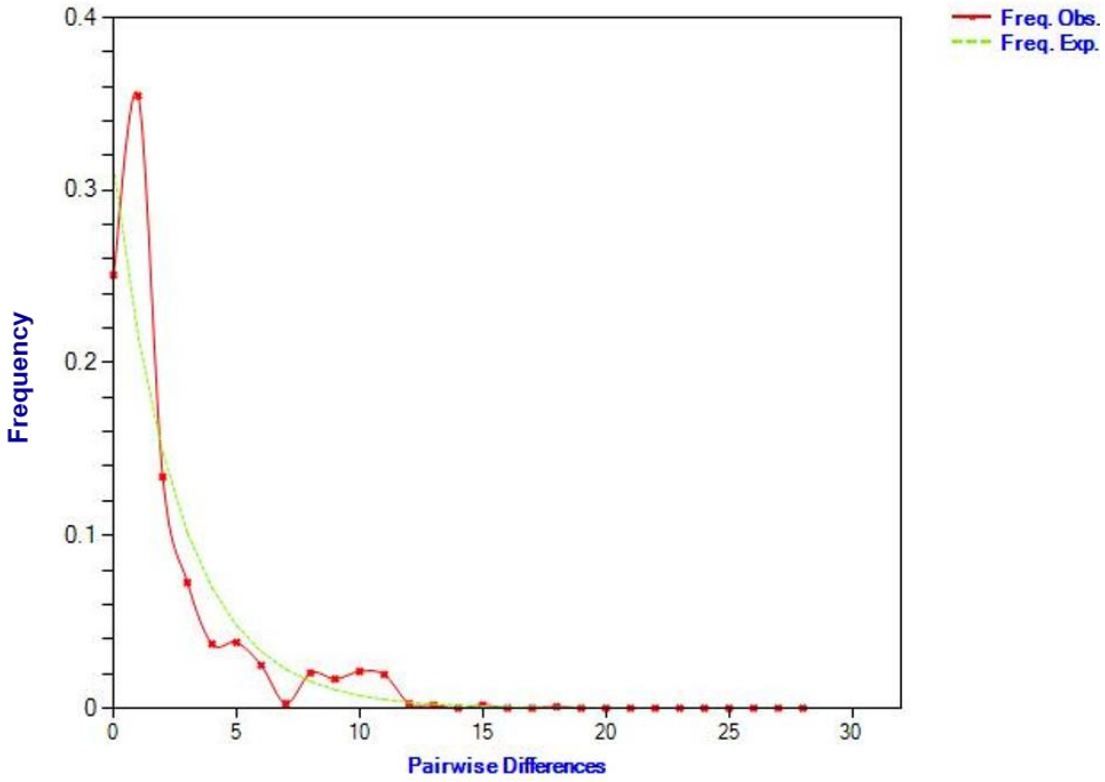

Figure 4. Mismatch graph generated using observed and expected pairwise frequencies. The solid green line depicts the projected distribution in the event of population growth.

4.3 Phylogenetic analyses

4.3.1 Haplotype networks

Median joining network based on haplotype networks showed a star-burst formation with Hap_2 being centrally placed for all networks hence shared among all the populations (Figure 5). These haplotypes were not distributed based on a geographic pattern as they were shared amongst the three locations (Gazi, Shimoni and Vanga) that herein are treated as populations (Figure 5). Therefore, Hap_1, Hap_2, Hap_3, Hap_4, Hap_5, and Hap_11 were shared by all three populations. The rest of the haplotypes were exclusive to a specific population. All haplotypes were separated by ten or fewer mutations from each other as displayed by crossbars (Figure 5). Hap_21 for instance exhibited significant increase of evolution as shown by the increase in the mutational steps compared to other

haplotypes. Additionally, the geographical distribution of haplotypes differed with the highest haplotype diversity displayed in Gazi (0.933) with the lowest haplotype diversity in Vanga population (0.900).

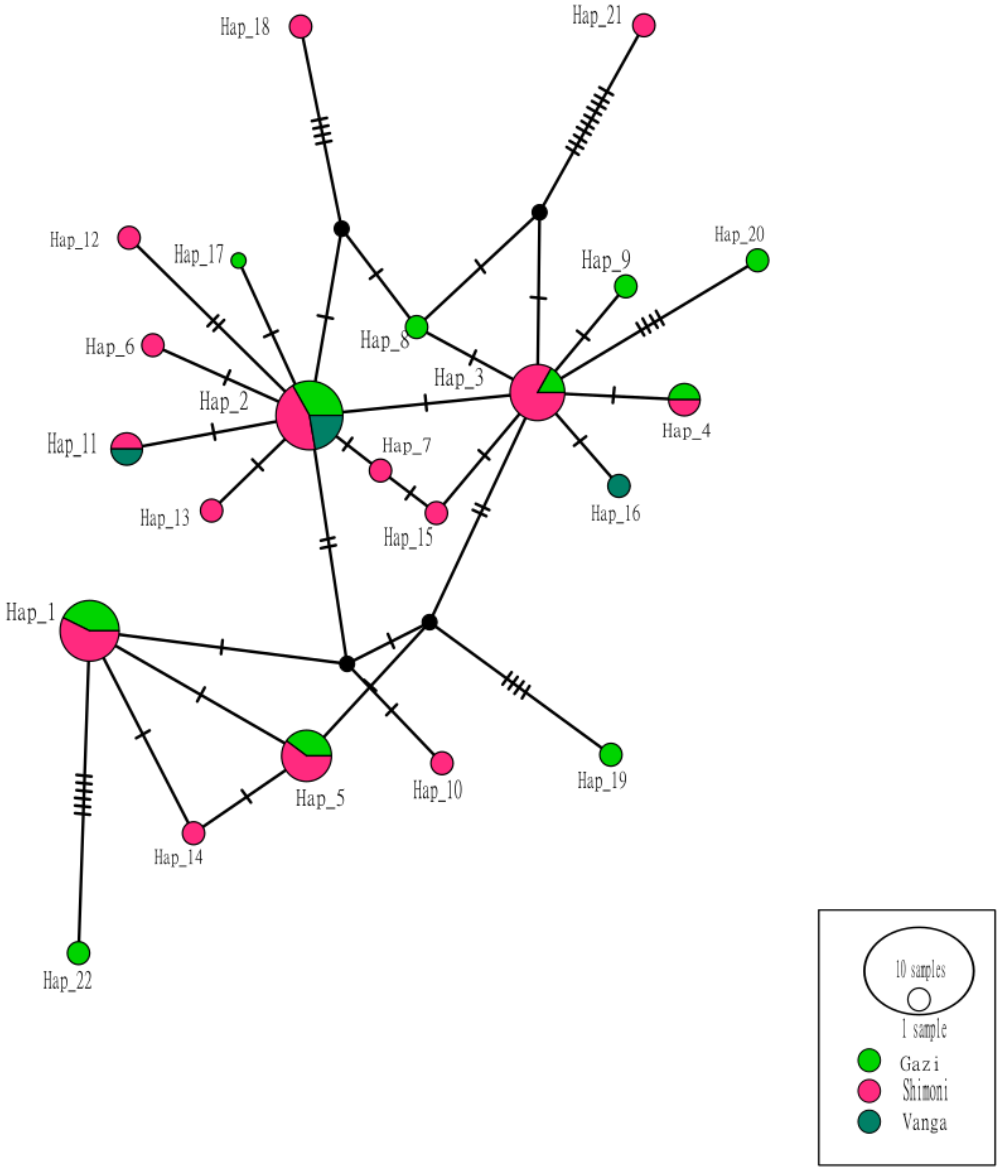

Figure 5. Median joining network show phylogenetic relationships among the haplotypes found. Haplotypes are shown as circles, the size of the circle indicates the number of individuals sharing the haplotype with dark circles being those that were not sampled.

4.3.2 Phylogenetic Trees

Neighbor-joining (NJ) tree established evolutionary relationship among the *H. fuscogilva* sequences based on their genetic distances, and determined that sequences evolutionary

inference formed two clusters (Figure 6). Specimens of *H. fuscogilva* from global sequences showed high sequence divergence with sequences from south coast Kenya suggesting that they formed two different type of species (Figure 6). The Maximum likelihood (ML) inferences also clustered *Holothuria* species into two main groups (Appendix 5). The Bayesian consensus ML analyses also had a similar topology to trees generated by NJ. They potrayed that *Holothuria* spp from this study and global sequences formed two clades. In addition, all *H. fuscogilva* populations were monophyletic with respect to the outgroup.

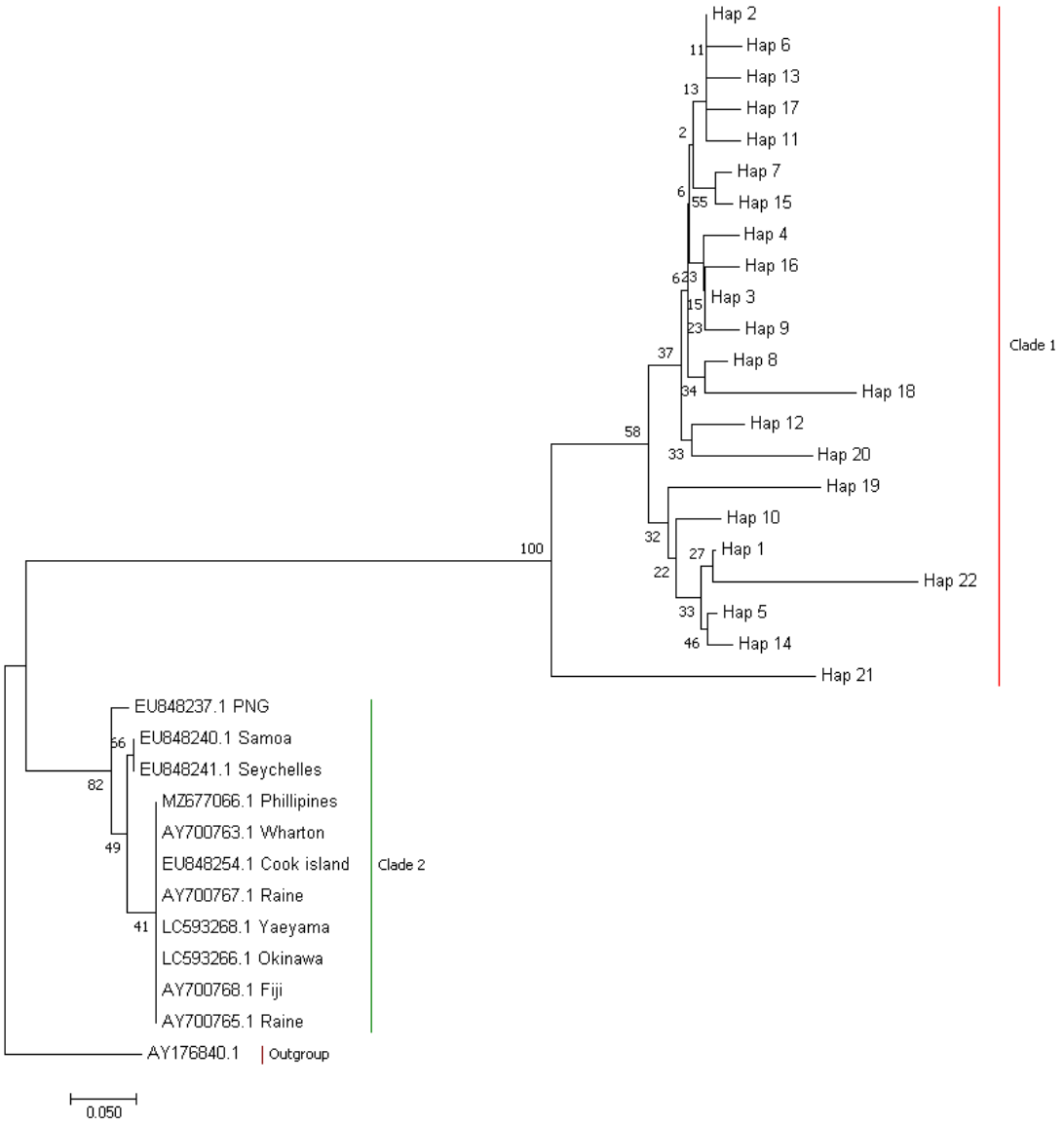

Figure 6. A Neighbor-Joining tree showing the evolutionary history of *Holothuria fuscogilva* populations. *Holothuria whitmaei* (AY176840.1) was used as the out-group. Clade 1 represents the sampled haplotypes in this study, while Clade 2 represents the already published and downloaded global sequences. The proportion of duplicate trees in which the connected clustered together in the bootstrap test (1000 replications) is shown next to the branches. The scale bar at the bottom shows substitutions per site.

4.4 Genetic Structure

Based on Analyses of Molecular Variance (AMOVA) results the level of variation among populations was not significant (1.19%) while within populations the variation was at

(98.81%) (Table 5). AMOVA indicated that variations occurred within individuals compared to their geographical regions hence the populations were not structured. The F_{ST} value (0.01186, p.value = 0.29069) show that there is no difference between the three populations and that they lack population structure with values of 1 implying genetic variation. The results therefore show the highest variance was within populations than among them.

Table 5. AMOVA analyses of sequences of *Holothuria fuscogilva* populations sampled in south coast Kenya.

Source of variations	**d.f**	**Sums of squares**	**Variance of components**	**Percentage of variations**	**F_{ST}**
Among Populations	2	4.281	0.02220 Va	1.19	0.01186 (p.value = 0.29069)
Within populations	44	81.400	1.85000 Vb	98.81	
Total	46	85.681	1.87220		

4.5 Historical Effective Population Size

Bayesian skyline plot (BSP) usually allows estimation of effective population size (N_e) in a piecewise fashion at coalescent events. Given that mitochondrial marker, Cytochrome Oxidase subunit I (COI), was used in BSP estimates they would reflect the maternal effective population size. Therefore, estimation of historical female N_e began with the calculation of the start of the population expansion using *Holothuria* spp locus mutation rate per year μ= 0.00084 substitutions per site per million years as prior information. The BSP estimates showed *H. fuscogilva* populations had a stable historical N_e until 4.0 x 10^6 Mya then it was followed by sudden demographic event (Figure 7). Therefore, BSP estimated that population expansion started roughly in 6.5 x 10^6 Mya (demographic

expansion might have started from $6.0x10^6$ to $7.0x10^6$ Mya as inferred from the 95% HPD regions drawn in Figure 7) and that led to the populations rapidly increasing to the present size. The BSP also yielded particularly higher estimates for the maternal N_e (greater than 10 on a logarithmic scale; Figure 7) reflecting overall higher genetic diversity which can be interpreted as an approximation of changes in relative effective population size.

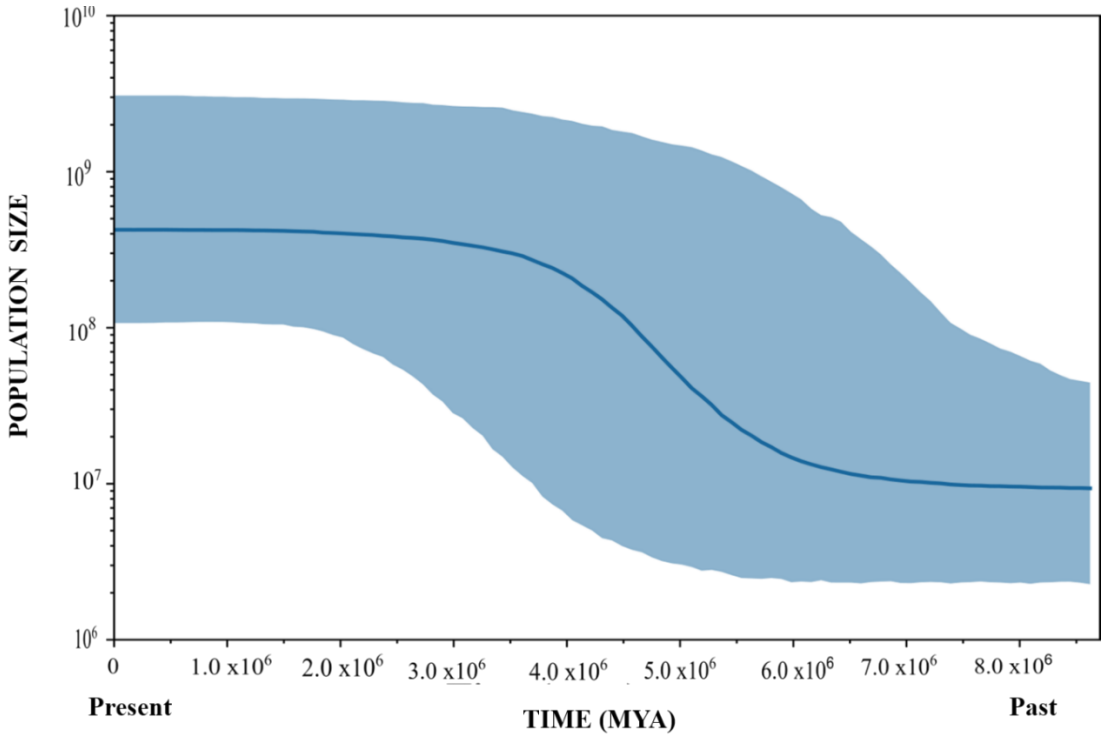

Figure 7. Population size (y-axis) is measured as effective population size per generation with (x- axis) expressed in million years ago. The solid line is the median estimate, and the blue areas show the 95% Highest Posterior Density (HPD) limits.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Genetic diversity

5.1.1 Nucleotide composition

The findings of the study show that the nucleotide composition content of A+T (50.89%) was higher than that of C+G (49.11%) for the COI gene in *H. fuscogilva*. These results were consistent with those of other sea cucumbers species with higher AT content on their mitochondrial genomes, including *Holothuria scabra* (A+T, 59.74%), *Holothuria fuscocinerea* (A+T, 60.30%), *Holothuria leucospilota* (A+T, 57.6%), and *Holothuria spinifera* (A+T, 60%) (Xia et al., 2015; Zhong et al., 2019; Ding et al., 2020; Sun et al., 2020). The higher Adenine (A) and Thymine (T) composition over Guanine (G) and Cytosine (C) in *H. fuscogilva* corroborates the findings of Alcudia-catalma et al., (2020) that also had the same trend. Echinoderms that include *H. fuscogilva* tend to utilize only the ATG codon for methionine function, hence the bias for AT being higher, as reflected in the codon usage in the COI sequence of all sea cucumbers (Alcudia-catalma et al., 2020).

5.1.2 Optimum sample sizes with rarefaction

Rarefaction curves qualitatively evaluate the amount of haplotype variety sampled in order to assess whether sample sizes accurately represent population variation (Geraghty et al., 2013). A trend toward an asymptotic relationship implies haplotype saturation, as was indicated by the Shimoni population. Therefore, Shimoni had the majority of the genetic diversity present sampled adequately at that site. Further, the steep slope (Figure 4) shown by Gazi and Vanga usually denotes that a significant portion of the potential haplotype diversity remains largely unexplored (Geraghty et al., 2013). Rarefaction curves revealed that the optimum sample size required to accurately represent levels of genetic

variation was site-dependent, with sample sizes in excess of 50 required for robust comparisons, whereas a sample size of around 30 appeared sufficient for Shimoni.

5.1.3 Genetic variation among *Holothuria fuscogilva* populations

Genetic diversity within and among populations provides the basis for population fitness and adaptability to ecological dynamic forces (Saleky et al., 2016; Stewart et al., 2019). Haplotype and nucleotide diversity are important genetic variety indicators; the higher the proportion, the greater the genetic diversity within the population (Zhang et al., 2020). Moreover, values that determine a high level of haplotype diversity and nucleotide diversity are above 0.5 and 0.005, respectively (Fang et al., 2022). Therefore, using mitochondrial COI, this study shows that *H. fuscogilva* population in south coast Kenya has high haplotype diversity coupled with low nucleotide diversity ($h = 0.922$, $\pi = 0.008$). This trend is also notable in other *Holothuriidae* species such as *Holothuria edulis (h* = 0.54-0.77, π = 0.0015–0.0039), *Holothuria arguinensis (h* = 0.8526-0.9474, π = 0.0101–0.0128), and *Holothuria polii (h* = 0.8889–0.9708, π = 0.0038–0.0062) (Rodrigues et al., 2015; Valente et al., 2015; Soliman et al., 2016). The high haplotype-low nucleotide diversity trend is linked with populations that have had recent population expansion (Maggi and González-Wangüemert, 2015).

Gazi had higher haplotype diversity (*h = 0.933*) compared to the other two populations based on this study. Based on high haplotype-low nucleotide diversity, it can be assumed that Gazi has experienced population expansion events, as shown by the emergence of haplotypes leading to the high haplotype diversity. Gene flow as a result of ocean currents may be the driver of the haplotype distributions observed. The high genetic diversity of *H. fuscogilva* in Gazi may reflect large effective population sizes. Moreover, the neutral theory of molecular evolution suggests that species with large effective population sizes

tend to reach mutation–random drift equilibrium and retain high levels of genetic diversity (Kenchington, 2010); this might be the case for Gazi.

Higher haplotype diversity and ultimately high genetic diversity for other sea cucumbers have been linked to environmental variables like salinity and temperature being different for a particular location as compared to its adjacent locations (Rodrigues et al., 2015). Marine invertebrates have also been linked with anthropogenic disturbances leading to population size increases that influence haplotype rise through mutation (Yang and Xin-Zheng, 2018). On the other hand, the nucleotide diversity tends to be low since they need time to accumulate sequence differences as compared to haplotypes (Yang and Xin-Zheng, 2018). These observations may explain the varied haplotype diversity among the study sites, given their proximity to each other. In addition, the geographical distribution of haplotypes in a given population is known to be influenced by gene flow and its population history (Uthicke and Benzie, 2003). In close locations at a microgeographic scale, as is the case with the study sites, genetic diversity can also often be influenced by environmental heterogeneity and population expansion that may in turn affect the natural selection and gene flow of that population (Rodrigues et al., 2015). Lower genetic diversity, on the other hand, can be driven by the population being subjected to fishing pressure coupled with ecological variations that may promote local extinctions (Kenchington, 2010).

5.1.4 Phylogenetic diversity

Phylogenetic reconstruction indicated that genetic homogeneity among the population of *H. fuscogilva* sampled along the Kenyan south coast, therefore forming one clade. The distinct groupings of *H. fuscogilva* sampled with those accessed from GenBank that represented the global distribution could be explained on the basis of the unique differences in eco-geographical characteristics of *H. fuscogilva* at different spatial scales.

According to Uthicke et al. (2004), *H. fuscogilva* in the Indian Ocean appears to differ geographically from those in the Pacific and Atlantic Oceans. The phylogeny of species, when compared with pairwise distances, showed different clades, with clade 1 being exclusively *H. fuscogilva* samples from this study (Figure 6). Furthermore, despite being from the Western Indian Ocean (WIO), there was a distinction with sequences from Seychelles, implying that species from the Indian Ocean are distinct among themselves and also from those from the Pacific and Atlantic Oceans (Figure 6). These trends suggest that *H. fuscogilva* from the Kenyan coast are unique from those clades of Indo-Pacific and Atlantic Ocean areas. Therefore, phylogenetic tree diagrams highlighted the distinctiveness of *H. fuscogilva* populations based on their genetic differences.

Populations with a high degree of isolation reduce the exchange of their genetic pool with their adjacent populations, leading to inbreeding (Rodrigues et al., 2015). Populations with high genetic connectivity exhibit shared haplotypes among themselves (Rodrigues et al., 2015), as shown by the haplotype network from this study. The haplotype network in this study revealed that the majority of the haplotypes had a common ancestor that gave rise to other shared and single haplotypes. Haplotypes arising from common haplotypes with mutations distinguish them as unique haplotypes (Vergara-Chen et al., 2010), similar to haplotype networks in this study. The haplotype network pattern of this study was also the case with *H. scabra* populations (Gardner and Fitch, 2012). *H. scabra* populations as noted by Gardner and Fitch (2012) had haplotypes that were abundant and giving rise to rare haplotypes. A star-like shape network indicates population expansion from one ancestor (Vergara-Chen et al., 2010) and was also the same case with *H. polii* in the Mediterranean sea (Valente et al., 2015). The star-shaped networks are also associated with a population that has undergone a founder effect (Gardner and Fitch, 2012; Maggi and González-Wangüemert, 2015). Gazi specifically presented the highest genetic diversity with plenty of unique haplotypes on the haplotype network, compared to the

other populations. Mutations often show as haplotypes that are distantly related and appear on recent haplotypes and are linked to changes in effective population sizes (Rodrigues et al., 2015). The mutations shown in the haplotype network of this study had abundant single unique haplotypes with mutations from the Gazi and Shimoni populations as part of effective population size changes. Therefore, *H. fuscogilva* populations from this study based on the haplotype network linkages indicated a high rate of gene flow, and the exclusive haplotypes with mutations indicated population changes.

5.1.5 Population demography history in *Holothuria fuscogilva* populations

The mitochondrial marker Cytochrome Oxidase I (COI), which has a slower evolutionary rate, can accurately reveal the population dynamics of a given population based on the Tajima and Fu Fs tests (Fang et al., 2022). A significant negative neutrality test frequently implies that the nucleotide sequences have more nucleotide modifications than the expected neutral evolution model, implying a historical population expansion event (Zhang et al., 2021). *Holothuria fuscogilva* from this study revealed that the population had recent population expansion through a previous bottleneck event based on significant negative values of Tajima (**-2.44,** p = 0.01**)**. Sea cucumber species in the family Holothuriidae also showed Tajima test as negative values for *H. atra* **(–2.331**, p = 0.001), *H. mammata* (**-2.070**, p = 0.001), *H. arguinensis* (−**1.5364**, p = 0.0240), *H. polii* **(–2.116,** p = 0.001) *H. scabra* (**-2.101**, p = 0.05) and *Parastichopus regalis* (**-1.1833**, p = 0.106) (Vergara-Chen et al., 2010; Borrero-pérez et al., 2011; Gardner and Fitch, 2012; Maggi and González-Wangüemert, 2015; Rodrigues et al., 2015; Hamamoto et al., 2021). The Fu Fs test was also negative and significant (**-5.389,** p = 0.003**)** for the *H. fuscogilva* sampled. Species for other holothurians have also shown significant and negative results, Fu's Fs test for *H. arguinensis* (−19.4182, p = 0.0000), *H. polii* (–26.732, p = 0.000) and *H. scabra* (-48.932 p = 0.01) (Vergara-Chen et al., 2010; Gardner and

Fitch, 2012; Rodrigues et al., 2015). The Fu's Fs tests returning negative results for all samples could also be indicating an abundance of rare haplotypes (Kennington et al., 2009). The evidence from neutrality tests revealed by *H. fuscogilva* populations show they have experienced population expansion, hence could be the primary reason for the absence of a population structure as also shown in other marine populations (Fang et al., 2022). The findings of neutrality tests are consistent with observed patterns of high haplotype diversity and low nucleotide diversity, as well as the "star-like" shape of the haplotype network attached to populations that have experienced demographic events as suggested to be the case in most marine organisms (Fang et al., 2022).

The Mismatch graph distribution compares observed with the simulated pairwise frequencies under the model of demographic expansion. The model of demographic expansion assumes that populations suddenly expand in population size and increase the total number of individuals (Harpending, 1994; Schneider and Excoffier, 1999; Vergara-Chen et al., 2010). The presence of rapidly evolving mutation sites will lead to unimodal mismatch distributions, with the shape of the mismatch often influenced by the population structure of the population (Schneider and Excoffier, 1999; Vergara-Chen et al., 2010). The *H. fuscogilva* populations showed peaks that showed a unimodal distribution (Figure 4), possibly defined by the population structure, providing strong evidence for sudden population expansion (Harpending, 1994). Moreover, the exhibited negative and significant neutrality test values corroborated the existence of population expansion. *H. polii* (Vergara-Chen et al., 2010; Valente et al., 2015), *H. arguensis* (Rodrigues et al., 2015), and *H.mammata* populations (Borrero-Perez et al., 2011) also showed a unimodal distribution similar to *H. fuscogilva* populations from this study.

5.2 Genetic structure among *Holothuria fuscogilva* populations

Marine organisms' genetic structure is associated with the presence of barriers and events inhibiting gene flow (Rodrigues et al., 2015; Yang and Xin-Zheng, 2018). The presumed location of marine populations is often not well defined, increasing genetic diversity within their genetic borders (Valente et al., 2015). The F_{ST} values, as estimates of genetic structure, show levels of inbreeding and the level of gene flow among populations (Ye et al., 2015). This study revealed an insignificant low genetic structure based on F_{ST} (F_{ST} = 0.01186, p.value = 0.29069) between the *H. fuscogilva* populations using the COI marker. F_{ST} values with ranges below 0.05 often indicate insignificant and low genetic structure, whereas values greater than 0.25 indicate high genetic structure within the analyzed population (Ye et al., 2015; Zhang et al., 2020). F_{ST} values among the sea cucumber population based on COI markers have been varying, with indications of low genetic structure among *H. polii* (F_{ST} = 0.0036, p. value = 0.008), *H. edulis (*F_{ST} = 0.0077, p. value = 0.400), and *H. arguinensis* populations (F_{ST} = 0.00946, p. value = 0.213) (Rodrigues et al., 2015; Valente et al., 2015; Soliman et al., 2016). These populations, along with the *H. fuscogilva* populations from this study all lacked significant genetic structure among them. The *H. arguinensis* populations for instance with restrictive geographical distribution linked to their genetic connectivity still showed no differentiation among their populations (Valente et al., 2015). The findings of a lack of population structure within shorter spatial scales were also observed for H. polii and were associated with unrestricted gene flow (Vergara-Chen et al., 2010). The FST values from H. fuscogilva populations show evidence of high gene flow with the postulation that there could be a lack of geographic barriers among the Gazi, Shimoni, and Vanga populations. Therefore, the Kenyan south coast does not establish a significant barrier that would shape the population's genetic structure. This observation of a lack of significant barriers as the findings show agree with those of Mkare (2013) who pinpointed that the East African

coast, lacked barriers that would have contributed to structuring among marine populations. Therefore, the low values for F_{ST} as observed in H. fuscogilva could be as a result of haplotype sharing of both ancestral and newly derived haplotypes among the populations sampled (Geraghty et al., 2013).

The AMOVA findings largely supported the presence of genetic variations within populations of *H. fuscogilva*. The observation of variations within populations was also the case with species of *H. scabra* (Gardner and Fitch, 2012; Ravago-Gotanco and Kim, 2019), *H.mammata* (Valente et al., 2015), and *H. halodeima* (Hamamoto et al., 2021. The variation in intra-population levels could be explained by the possibility of high gene flow among the locations that make up the populations as also suggested for *H. polii* populations that were sampled within smaller geographic scales (Vergara-Chen et al., 2010). The maintenance of the intra-population genetic diversity could be explained by the population being robust and able to regenerate the lost individuals that end up being harvested over time (Gardner and Fitch, 2012). Thus, sea cucumber populations that are heavily exploited like *H. fuscogilva* are expected to have low within-population genetic diversity as the result of the effects of bottlenecks and extreme genetic drift events (Nowland et al., 2017). However, AMOVA analyses showing high intra-variation suggest the robustness of *H. fuscogilva* populations to recolonize areas that are affected by fishing pressure, with these populations showing great genetic maintenance within the populations from extreme bottleneck events (Uthicke and Benzie, 2003; Nowland et al., 2017).

Generally, genetic structure among holothurians has often been associated with physical barriers, oceanographic currents, and the influence of natural events. In the populations of Gazi and Shimoni, they had the majority of shared haplotypes, which may have led to an influence of high genetic connectivity potentially driven by ocean currents between the spatial scales. In addition, other sea cucumbers in other regions have also attributed the

lack of genetic structure (Rodrigues et al., 2015; Panithanarak, 2022) and connectivity patterns (Henriques, 2014) to the influence of ocean currents. In addition, the East African Coastal Current (EACC) may also have an impact on determining the structure of organisms in the WIO region (Otwoma, 2018) which covers the sampling sites for this study. *Acanthurus leucosternon*, an echinoderm species, demonstrated EACC influence on its gene flow to establish genetic homogeneity, resulting in populations lacking population structure (Otwoma, 2018).

Marine populations with long-distance dispersal of pelagic larvae tend to facilitate the lack of genetic structure seen among them (Mkare, 2013). The genetic breaks between locations may typically occur on benthic species that have an averagely short (< 2 weeks) Planktonic Larval Duration (PLD) (Pascual et al., 2017). Holothurian's planktonic larval phase is recorded at 16–21 days and may promote dispersal leading to a well-mixed population and low genetic structure among the populations (Valente et al., 2015). Sea cucumbers like *H. fuscogilva*, with their life histories that include pelagic phases, are expected to show high levels of gene flow and low population genetic structure over small spatial scales (Vergara-Chen et al., 2010). *H. fuscogilva* populations as observed within the short spatial scale were therefore not genetically structured as observed from F_{ST} and AMOVA.

5.3 Historical effective population size in *Holothuria fuscogilva* populations

Effective population-size estimates incorporating genetic influences on species' life histories over time are useful in determining the survival and fitness of a population to environmental changes (Hare et al., 2011). The Bayesian skyline plots (BSP) incorporates coalescent techniques such as Markov chain Monte Carlo (MCMC) techniques to estimate the distribution of effective population sizes (N_e).In addition, the estimation of N_e distribution is calculated through time directly from gene sequences

showing how Ne has evolved based on coalescent events (Drummond et al., 2005; Miller et al., 2021).

BSP analyses based on this study indicated that the *H. fuscogilva* population has been growing steadily until 4.0 x 10^6 Mya, where their population experienced an increase in effective population size until recently (Figure 7). Similarly, *Acanthurus leucosternon* and *Acanthurus triostegus* belonging to the phylum Echinodermata same as sea cucumbers had similar BSP illustrations (Otwoma, 2018). A flat BSP often with genetic sequences shows the population has been constant over a period of time (Ho and Shapiro, 2011). BSP analyses in this study indicated that until recently the population sizes had experienced growth of Ne hence the BSP illustration was not flat. The star-burst haplotypes may not have their BSP illustration portrayed in this manner (Grant et al., 2012). *Holothuria fuscogilva* populations based on haplotype networks showed a star-burst pattern with unique haplotypes therefore a population expansion event may have occurred resulting in an increase in N_e. Additionally, to support the nature of the *Holothuria fuscogilva*, BSP illustration, a marine invertebrate, *Halocynthia papillosa* indicated stable Ne with significant population genetic structure, positive neutrality tests, and a flat-shaped BSP (Villamor et al., 2014). The use of historical N_e estimates, from the Cytochrome Oxidase I (COI) marker for this study is informative towards fisheries management since they highlight the pre-exploitation estimates of abundance of the *Holothuria fuscogilva* stock and contribute to its demographic data. Historical N_e estimates are essential in providing the nature of populations' adjustment to fishing pressure through time and the state of the resources as they are still being exploited as has been done on other marine stocks (Henriques, 2014). Therefore, the impacts of increasing fishing and any anthropogenic activity that can impact their habitats and the population were left as signatures on the COI markers with *H. fuscogilva* populations and its genetic processes showing an increase of effective population size through time.

5.4 Impact of fishing on genetic diversity of *Holothuria fuscogilva* populations

High genetic diversity within a given population improves the population's capacity to adapt to changes in the environment (Saleky et al., 2016). The *H. fuscogilva* populations showed high haplotype diversity and low nucleotide diversity. This phenomenon of high haplotype-low nucleotide diversity is linked to an increase in population growth. Moreover, an increase in accumulations of mutations leads to haplotype increase incomparably to nucleotide polymorphism (Zhang et al., 2020). The higher haplotype diversity and ultimately high genetic diversity for other sea cucumbers have been linked to increase in their biomass and belonging to areas associated with less fishing activity (González-Wangüemert et al., 2015). The shared haplotypes shown in haplotype networks among the *H. fuscogilva* populations could be that there is high larval dispersal (Saleky et al., 2016) and therefore extensive gene flow. In marine environments, organisms with limited gene flow are likely to experience the impacts of overfishing when they experience stronger exploitation levels over time (Henriques et al., 2016). *H. fuscogilva* as shown in this study has the capacity to counteract the adverse effects of fishing due to extensive gene flow shown by their interlinked connectivity from their haplotypes, and the high genetic diversity helps the population fit against extreme environmental disturbances.

The status of Kenya's fisheries on sea cucumber from FAO reports as of 2020 shows that 13,239 kg of sea cucumber were exported into Hongkok (Conand et al., 2022). *Holothuria fuscogilva* as a commercially exploited sea cucumber, make up Kenya's sea cucumber exports and that may influence their diversity over time. At present, based on neutrality tests, fishing activities among the three *H. fuscogilva* populations have little influence on their haplotype diversity. Shimoni for instance, had significantly higher negative Fu tests compared to the other populations while Gazi had significantly negative Tajima D values. Shimoni populations in that case are interpreted to be growing with unique haplotypes. Moreover, the negative Tajima D values in Gazi could be interpreted as a population that

has expanded with the new haplotypes. Based on genetic structure estimates, intra-population genetic diversity was evident and could be explained by the population being robust and able to regenerate the lost individuals after a fishing pressure event (Gardner and Fitch, 2012). Therefore although fishing contributes to the reduction of its population there is the robustness of populations to recolonize areas affected. In addition, the genetic connectivity among them allows for interbreeding among them maintaining these populations from extreme bottleneck events (Uthicke and Benzie, 2003; Nowland et al., 2017).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

Holothuria fuscogilva showed high genetic diversity within populations and no significant genetic structure, according to this study. The mitochondrial COI marker used revealed low differentiation of *H. fuscogilva* populations. As a result, the observed weak population structure may indicate panmictic (well-mixed) population due to current gene flow and previous connections of the populations. In addition, the patterns of effective population sizes from the past showed an increase indicating occurrence of population expansion. Population expansion events are corroborated by the negative results of neutrality tests, and unimodal mismatch distribution which shows rapid growth in population size. The observation of intra-population genetic diversity could be explained by the population being robust and able to regenerate the lost individuals after fishing pressure. Although, *H. fuscogilva* is vulnerable to fishing the genetic connectivity showed by shared haplotypes shows that the effects of fishing are able to be balanced out reducing extinction and inbreeding among them. The study also revealed the uniqueness of the Kenyan population compared to other populations from other different geographical regions.

Our findings provide information on the genetic composition of *H. fuscogilva* populations, as well as population genetic estimates that enable the detection of genetic differences between them. We anticipate that these findings will aid in population management and the preservation of *H. fuscogilva* genetic diversity. The negative impact of fishing pressure was not evident based on genetic data but fishing should still be monitored and *H. fuscogilva* conservation prioritized apart from using the CITES listing as a tool to monitor its trade. Furthermore, *H. fuscogilva* distinction from other species around the world emphasizes the importance of conservation at the local

level. Management units are important in managing wild populations with the genetic structure being a key prerequisite for the delineation of those populations.

The study has provided information to elucidate genetic structure within a region of the Kenyan coast and studies on the rest of the coast to fully delineate the species are recommended to provide comprehensive countrywide data. We recommend that to fully display the nature of genetic diversity among *Holothuria fuscogilva*, populations sample sizes of 30 to 50 would be ideal.

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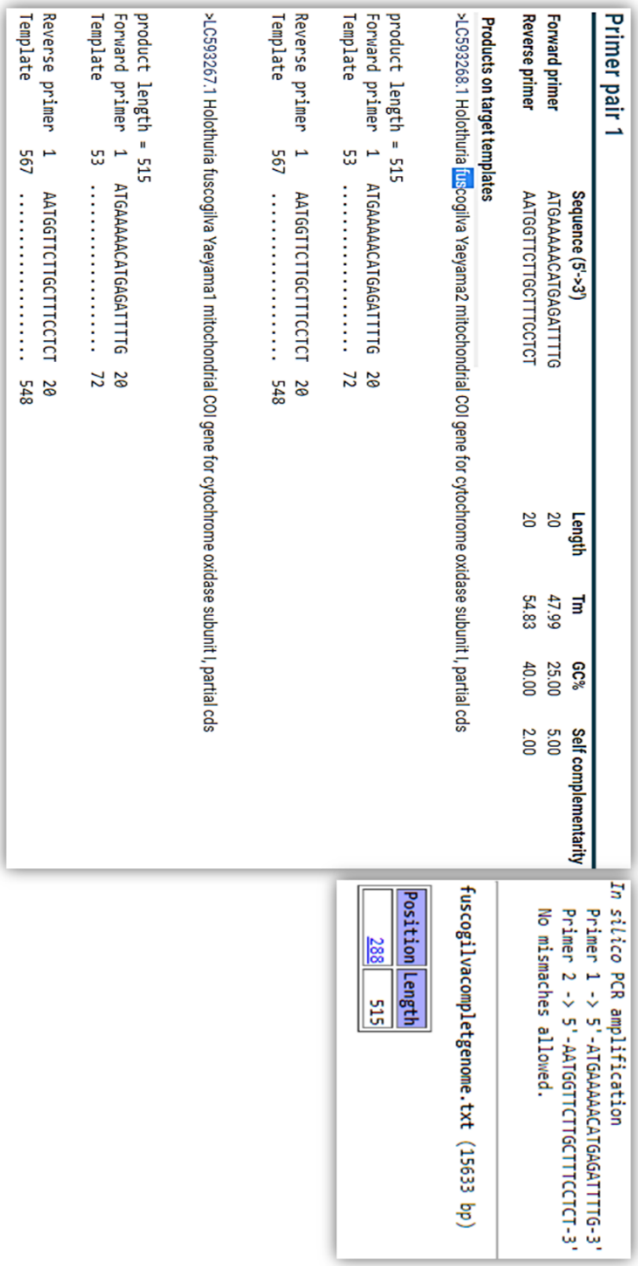
APPENDICES

Appendix 1

Primer design process as done on NCBI platform, which began with acquiring different COI genes for *H. fuscogilva* species from NCBI, then all sequences were aligned using Clustal W. Based on the aligned sequences a region was picked for the forward primer and another was picked for the reverse. With the flanking region widened to avoid overlapping and was ensured that the region picked were approximately 500 base pairs apart. The reverse region were then reverse complemented using NCBI Reverse complement. The designed pair primers were then checked for the specificity to amplify the intended species by uploading them in Primer BLAST in NCBI. For the broadest coverage, the nr database was chosen. Then followed by choosing the "Get Primers" button then submit the search and retrieve specific primer pairs .A virtual e-PCR amplification was carried out using In silico PCR. The mitochondrial genome of *H. fuscogilva* and *H. scabra* were loaded prior to accessing the PCR slot. The parameters were set after the forward and reverse primers were loaded with no mismatch in primer sequence allowed. The results were investigated for the ability to amplify when no mismatch was allowed. Once the primers amplified exclusively to H. fuscogilva without allowing mismatches among them they were picked as the preferred rimer.

The primer pair of 515 bp were selected and ordered for their manufacturing by Macrogen Europe DNA sequencing facility, Netherlands.



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity
Forward primer	ATGAAAAACATGAGATTTTG	20	47.99	25.00	5.00
Reverse primer	AATGGTTCTTGCTTTCCTCT	20	54.83	40.00	2.00

Products on target templates

>LC593268.1 Holothuria fuscogilva Yaeyama2 mitochondrial COI gene for cytochrome oxidase subunit I, partial cds

```
product length = 515
Forward primer  1    ATGAAAAACATGAGATTTTG  20
Template        53   ....................  72

Reverse primer  1    AATGGTTCTTGCTTTCCTCT  20
Template        567  ....................  548
```

>LC593267.1 Holothuria fuscogilva Yaeyama1 mitochondrial COI gene for cytochrome oxidase subunit I, partial cds

```
product length = 515
Forward primer  1    ATGAAAAACATGAGATTTTG  20
Template        53   ....................  72

Reverse primer  1    AATGGTTCTTGCTTTCCTCT  20
Template        567  ....................  548
```

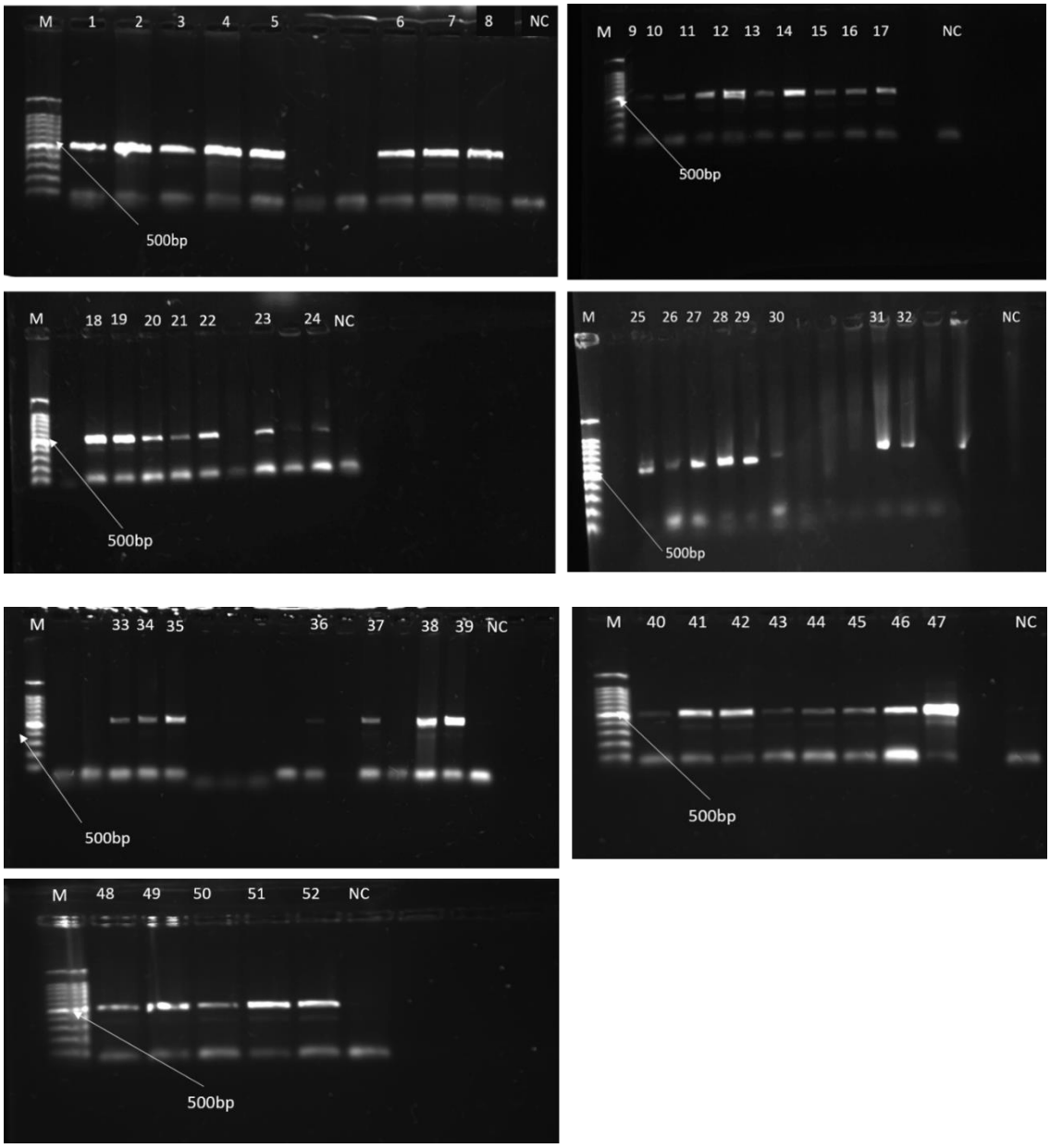
In silico PCR amplification

Primer 1 -> 5'-ATGAAAAACATGAGATTTTG-3'
Primer 2 -> 5'-AATGGTTCTTGCTTTCCTCT-3'
No mismatches allowed.

fuscogilvacompletgenome.txt (15633 bp)

Position	Length
288	515

Snapshot of NCBI primer blast with the primers inputted and Snapshot of In silco PCR analyses with primers against the mitochondrial genome. The intended *Holothuria fuscogilva* species was targeted with no mismatches.



Different gel images as results of PCR, M is the 100bp ladder.1 -52 are *H. fuscogilva* samples from this study and NC is the negative control on the gel with amplicons visible at the desired 500bp mark.

Appendix 2

Results of raw sequences as blasted on Genbank

The correctness of specimens sampled as belonging to *H. fuscogilva* was confirmed by blasting each of the COI sequences using GenBank (http://blast.ncbi.nlm.nih.gov). One sample showed it belonged to *H. nobilis* and was excluded from the study

Raw data		**GenBank blast search results**							
Specimen Code	**Species name**	**Species name**	**Max score**	**Total score**	**Query coverage**	**E-value**	**% identity**	**Accession length**	**Accession number**
Holo_G01	*Holothuria fuscogilva*	*Holothuria fuscogilva*	915	915	96	0	98.65	601	EU848241.1
Holo_G02	*Holothuria fuscogilva*	*Holothuria fuscogilva*	883	883	96	0	98.22	601	EU848241.1
Holo_G03	*Holothuria fuscogilva*	*Holothuria fuscogilva*	935	935	95	0	99.42	601	EU848241.1
Holo_G04	*Holothuria fuscogilva*	*Holothuria fuscogilva*	913	913	97	0	98.64	601	EU848241.1
Holo_G05	*Holothuria fuscogilva*	*Holothuria fuscogilva*	911	911	96	0	98.83	601	EU848241.1

Holo_G06	*Holothuria fuscogilva*	*Holothuria fuscogilva*	920	920	97	0	98.84	601	EU848241.1
Holo_H01	*Holothuria fuscogilva*	*Holothuria fuscogilva*	869	869	97	0	97.09	601	EU848241.1
Holo_H02	*Holothuria fuscogilva*	*Holothuria fuscogilva*	911	911	98	0	98.46	601	EU848241.1
Holo_H03	*Holothuria fuscogilva*	*Holothuria fuscogilva*	904	904	97	0	98.26	601	EU848241.1
Holo_H04	*Holothuria fuscogilva*	*Holothuria fuscogilva*	920	920	95	0	99.22	601	EU848241.1
Holo_H05	*Holothuria fuscogilva*	*Holothuria fuscogilva*	876	876	97	0	97.48	601	EU848241.1
Holo_H06	*Holothuria fuscogilva*	*Holothuria fuscogilva*	904	904	97	0	98.6	601	EU848241.1
Holo_E01	*Holothuria fuscogilva*	*Holothuria fuscogilva*	920	920	97	0	99.03	601	EU848241.1

	Holothuria	*Holothuria*							
Holo_E02	*fuscogilva*	*fuscogilva*	907	907	96	0	98.64	601	EU848241.1
Holo_E03	*Holothuria*	*Holothuria*	929	926	96	0	99.22	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_E04	*Holothuria*	*Holothuria*	889	889	95	0	98.23	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_E05	*Holothuria*	*Holothuria*	918	918	97	0	98.84	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_E06	*Holothuria*	*Holothuria*	904	904	94	0	98.82	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_E07	*Holothuria*	*Holothuria*	898	898	95	0	98.07	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_F01	*Holothuria*	*Holothuria*	845	845	99	0	98.94	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_F02	*Holothuria*	*Holothuria*	904	904	96	0	98.26	601	EU848241.1
	fuscogilva	*fuscogilva*							

	Holothuria	*Holothuria*							
Holo_F03	*fuscogilva*	*fuscogilva*	274	274	97	4e-69	89.72	601	EU848241.1
Holo_F04	*Holothuria*	*Holothuria*	870	870	97	0	97.42	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_F05	*Holothuria*	*Holothuria*	900	900	99	0	98.44	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_C01	*Holothuria*	*Holothuria*	911	911	97	0	98.64	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_C02	*Holothuria*	*Holothuria*	900	900	98	0	98.44	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_C04	*Holothuria*	*Holothuria*	887	887	95	0	98.42	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_C05	*Holothuria*	*Holothuria*	929	929	96	0	99.22	601	EU848241.1
	fuscogilva	*fuscogilva*							
Holo_C06	*Holothuria*	*Holothuria*	898	898	98	0	98.62	601	EU848241.1
	fuscogilva	*fuscogilva*							

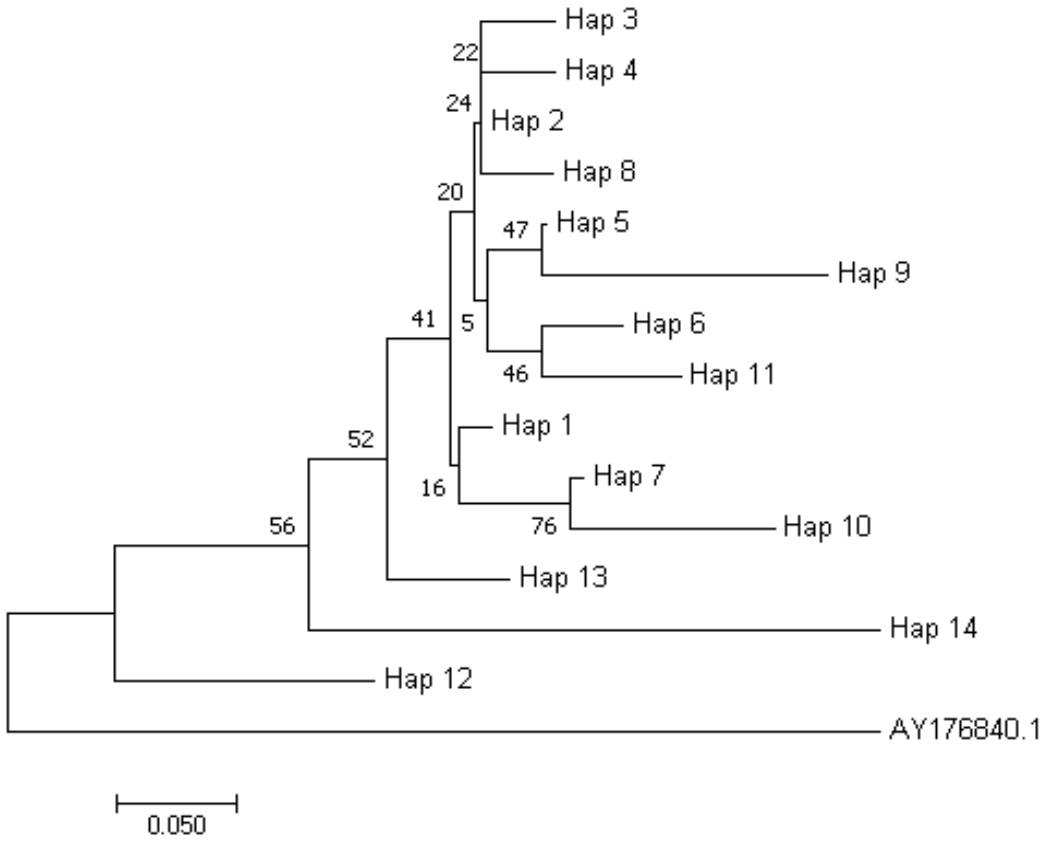
Holo_C07	*Holothuria fuscogilva*	*Holothuria fuscogilva*	920	920	96	0	98.84	601	EU848241.1
Holo_D01	*Holothuria fuscogilva*	*Holothuria fuscogilva*	918	918	96	0	99.03	601	EU848241.1
Holo_D02	*Holothuria fuscogilva*	*Holothuria fuscogilva*	911	911	95	0	99.40	601	EU848241.1
Holo_D03	*Holothuria fuscogilva*	*Holothuria fuscogilva*	846	846	95	0	99.33	601	EU848241.1
Holo_D04	*Holothuria fuscogilva*	*Holothuria fuscogilva*	913	913	95	0	98.83	601	EU848241.1
Holo_D05	*Holothuria fuscogilva*	*Holothuria fuscogilva*	924	924	96	0	99.03	601	EU848241.1
Holo_D06	*Holothuria fuscogilva*	*Holothuria fuscogilva*	887	887	95	0	98.80	601	EU848241.1
Holo_D07	*Holothuria fuscogilva*	*Holothuria fuscogilva*	920	920	97	0	98.84	601	EU848241.1

Holo_C03R	*Holothuria fuscogilva*	*Holothuria fuscogilva*	196	196	97	5e-46	93.85	601	EU848241.1
Holo_A01	*Holothuria fuscogilva*	*Holothuria fuscogilva*	929	929	96	0	99.22	601	EU848241.1
Holo_A02	*Holothuria fuscogilva*	*Holothuria fuscogilva*	929	929	96	0	99.22	601	EU848241.1
Holo_A03	*Holothuria fuscogilva*	*Holothuria fuscogilva*	893	893	95	0	98.43	601	EU848241.1
Holo_A04	*Holothuria fuscogilva*	*Holothuria fuscogilva*	902	902	96	0	98.44	601	EU848241.1
Holo_A05	*Holothuria fuscogilva*	*Holothuria fuscogilva*	549	549	97	1e-151	93.26	601	EU848241.1
Holo_A06	*Holothuria fuscogilva*	*Holothuria nobilis*	863	863	97	0	96.92	636	EU848246.1
Holo_B01	*Holothuria fuscogilva*	*Holothuria fuscogilva*	900	900	97	0	98.63	601	EU848241.1

Holo_B02	*Holothuria fuscogilva*	*Holothuria fuscogilva*	885	885	99	0	98.41	601	EU848241.1
Holo_A07	*Holothuria fuscogilva*	*Holothuria fuscogilva*	929	929	96	0	99.41	601	EU848241.1
Holo_B03	*Holothuria fuscogilva*	*Holothuria fuscogilva*	893	893	96	0	98.80	601	EU848241.1
Holo_B04	*Holothuria fuscogilva*	*Holothuria fuscogilva*	924	924	95	0	99.03	601	EU848241.1
Holo_B05	*Holothuria fuscogilva*	*Holothuria fuscogilva*	933	933	96	0	99.23	601	EU848241.1
Holo_B06	*Holothuria fuscogilva*	*Holothuria fuscogilva*	918	918	96	0	99.03	601	EU848241.1

Appendix 3

Phylogenetic relationship among *H. fuscogilva* sampled in south coast Kenya.The relationship was paraphyletic among the species.



Phylogenetic relationships analysis using Neighbour--joining methods based on *H. fuscogilva* data. *Holothuria whitmaei* (AY176840.1) was used as the out-group. The numbers above the branches are bootstrap support values of Neighbor-Joining posterior probabilities (PPs) of 100%

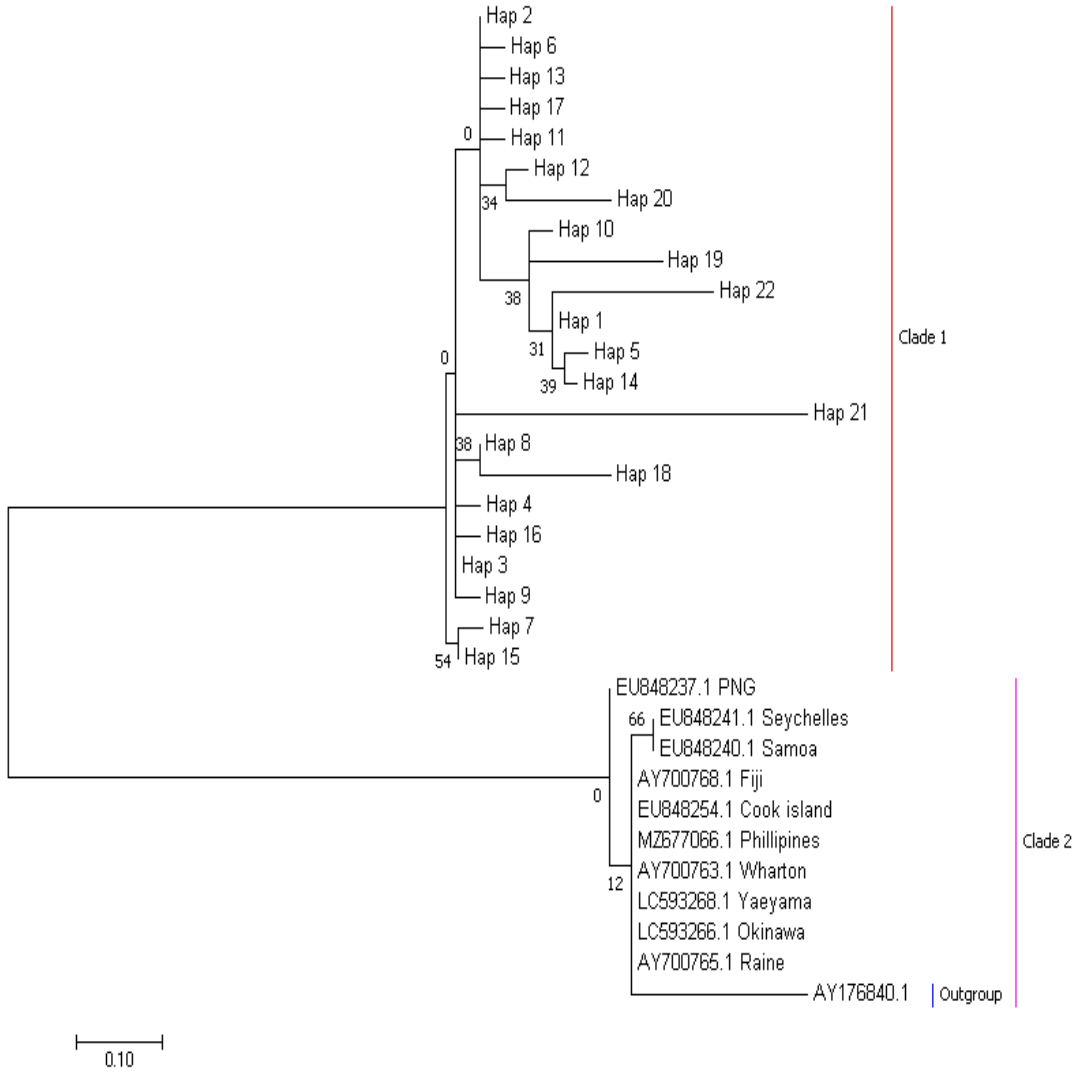
Appendix 4

The COI sequences used for phylogenetic analyses of *Holothuria fuscogilva* were from various geographical locations. They range from Pacific Ocean, Atlantic Ocean and Indian Ocean samples as accessed from GenBank.

Species	**Origin**	**GenBank Accession**	**Reference**
Holothuria fuscogilva	Seychelles	EU848241.1	Uthicke et al., (2010)
	Fiji	AY700768	Uthicke et al., (2004)
	Wharton	AY700763	Uthicke et al., (2004)
	Samoa	EU848240.1	Uthicke et al., (2010)
	Yaeyama(Japan)	LC593266.1	Tanita et al., (2021)
	Okinawa(Japan)	LC593260.1	Tanita et al., (2021)
	Raine	AY700761.1	Uthicke et al., 2004
	Raine	AY700765.1	Uthicke et al., 2004
	Philippines	MZ677066.1	Nguyen(Unpublished)
	Cook islands	EU848254.1	Uthicke et al., 2004
Holothuria whitmaei	Australia	AY176840.1	Uthicke and Benzie (2003)

Appendix 5

Phylogenetic relationship as illustrated by Maximum-likelihod tree



Phylogenetic tree of Holothuria species as inferred using the Maximum-likelihood method were based on Tamura-Nei model with 1000 bootstraps supports. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar at the bottoms shows substitutions per site.

Appendix 6

DNA Extraction process that were used for acquiring genetic materials used commercial Qiagen Kit (DNeasy 96) for Animal Tissues with the following steps;

1. Prerequisites that were done before using the Qiagen kit for extraction included;

- Buffer AL was premixed with ethanol before using, with addition of 90 ml of absolute ethanol to the bottle containing the 86 ml Buffer AL and shaken thoroughly. The bottle was then labelled to indicate that ethanol has been added and ready for use.
- Buffer AW1 and Buffer AW2 were dispensed to falcon tubes with the appropriate amount calculated based on the samples extracted to prevent contamination and were labelled as working solutions.
- Preheat the heat plate or water bath at 56°C for use in step 4.

2. Procedure

1. The *H. fuscogilva* samples stored in ethanol was removed using forceps and then excess ethanol were removed. The tissue was then cut with 10 mg tissue weighed and then cut into smaller pieces in an eppendorf to enable efficient lysis.
2. The eppendorf tube with tissue was then added 20 µl Proteinase K and then added 180 µl Buffer ATL per sample and mix thoroughly by vortexing.
3. Incubate the contents at 56°C until the samples are completely lysed. Vortexing occasionally in between the incubation to disperse the tissue. Moreover, the caps of the micro centrifuge tubes were ensured that they were properly sealed during the incubation. Lysis was complete after 2 hours. Once the lysate was homogeneous with vigorous shaking in between the incubation, Step 4 then proceeded.

4. Carefully remove the caps, and add 200 µl of AL buffer and mix thoroughly by vortexing for 15s and then add 200ul ethanol (96–100%) to each sample and then mixed again by vortexing.
5. Afterwards pipet the mixture from step 4 into a spin mini-column and then place them into 2ml collection micro centrifuge tubes, properly seal them with the caps provided. , centrifuge the collection micro centrifuge tubes at 8000rpm for 1 min.Once the centrifuge is complete the flow thorough and collection was discarded
6. Carefully take the spin column into a new collection tube and then add 500 µl Buffer AW1 to each sample. Proceed to centrifuge them at 1min at 8000rpm and also discard the flow thorough from it together with the collection tube.
7. Remove the spin column carefully into a new collection tube and then add 500 µl Buffer AW2 to each sample. Proceed to centrifuge them at 3min at 14,000rpm and also discard the flow thorough from it together with the collection tube. The spin column was removed carefully to ensure there was no carry-over of ethanol that would tamper with the DNA.
8. To elute the DNA, addition of 70 µl of Buffer AE was done to each sample, with the pipet placed on the middle of the Spin membrane to ensure maximum elution. In addition the first few samples had a repeat of elution as way to increase yield. The samples were then incubated for 1 min at room temperature and centrifuged for 4 min at 6000 rpm.
9. After DNA was extracted, each sample was then quantified using a NanoDrop ND-1000 spectrophotometer and electrophoresis and those with successful DNA bands were stored at –20 ºC until it was later used in the downstream processes.