Genetic differentiation of Artemia franciscana (Kellogg, 1906) in Kenyan coastal saltworks

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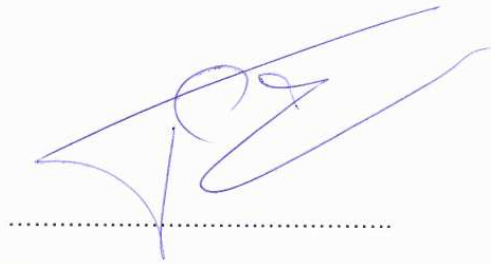
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Master's dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science in Aquaculture of Gent University, Belgium.
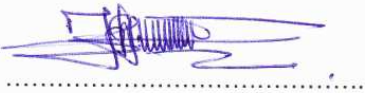
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DEDICATION

In a very special way, I dedicate this work to my lovely wife and friend, Lillian. Your words of encouragement were vital ingredients during my study period in Europe. I will forever be grateful for your unlimited support. I fully understand the challenges you experienced in my absence. Indeed, you are my true hero. And to my only child Brian, you are a wonderful son. Someday you will be able to read these words. I loved you from the beginning.

ACKNOWLEDGEMENT

LIST OF ABBREVIATIONS

&	And
^{o}C	Degrees centigrade
>	Greater than
>	Less than
µg L^{-1}	Microgram per litre
µL	Microlitre
µm	Micrometer
%	Percent
±	Plus or minus
Σ	Summation
A $_{260/280}$	Absorbance at 260 & 280 nm to assess DNA purity
AFLP	Amplified Fragment Length Polymorphism
ARC	Laboratory of Aquaculture & *Artemia* Reference Center
BADC	Belgian Agency for Development Cooperation
bp	base pair(s)
cDNA	Complementary DNA - is a single-stranded DNA
cm	Centimetres
COI	Cytochrome oxidase subunit I
DNA	Deoxyribonucleic acid
DHA	Docosaheptaenoic acid
e.g.	For instance
e.t.c	And so on
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organization of the United Nation
g	Gram
g L^{-1}	Gram per litre

GSL	Great Salt Lake
ha	Hectare
hr	Hour
H*sp*26	Heat shock protein 26
H*sp*70	Heat shock protein 70
HUFA	Highly unsaturated fatty acid(s)
ITS	Internal transcribed spacer
Ken1	Kenya *Artemia* strains collected in 2010
Ken2	Kenya *Artemia* strains collected in 1996
Ken3	Kenya *Artemia* strains collected in 2012
Kg	Kilogram
Km	Kilometer
KMFRI	Kenya Marine & Fisheries Research Institute
L	Liter
m	Meter
m^{-2}	Per meter square
m^{2}	Meter square
m^{3}	Cubic meter
m^{-3}	Per cubic meter
mg	Milligram
milliQ	PCR water
min	minutes
mL	Milliliter
mm	Millimeter
mtDNA	mitochondrial DNA
ng / µL	Nanogram per microlitre

nm	Nanometer
P	P-value
pH	Power of hydrogen
RAPD	Random Amplified Polymorphic DNA
rDNA	ribosomal DNA
RE	Restriction enzyme
RFLP	Restriction Fragment Length Polymorphism
s	Seconds
SD	Standard deviation
SFB	San Francisco Bay *Artemia* strain
sp	Species
SSCP	Single Strand Conformation Polymorphism
SW	Sea water
Taq	*Thermus aquaticus*
USA	United States of America
UV	Ultra violet
VC	Vinh Chau *Artemia* strain
VLIR – OI	Flemish Inter-universitary Council Own Initiative

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ABSTRACT

The nature of genetic divergence between the *Artemia* population native to San Francisco Bay, (SFB) USA and those from the introductions of SFB material in the Kenyan coast (Fundisha and Kensalt) 2 decades ago were investigated using the mitochondrial DNA (mtDNA) and heat shock protein 70 (H*sp*70) gene molecular markers. Control samples were obtained from Great Salt Lake (GSL), Utah, USA and Vinh Chau (VC), Vietnam, while *Artemia* cysts from Tanga, Tanzania were for additional study. The DNA was extracted from 80 single *Artemia* cysts (10 cysts per sample) using the Chelex protocol for mtDNA analysis while the Promega method was used in the H*sp*70 gene analysis. The 1,500 bp fragment of the 12S - 16S region of the mtDNA and a 1,935 bp fragment of the H*sp*70 gene were amplified through Polymerase Chain Reaction (PCR) using appropriate primers followed by Restriction Fragment Length Polymorphism (RFLP) digestion based on 6 restriction endonucleases (*Alu*I, *Hae*III, *Hinf*I, *Rsa*I, *Xba*I and *Hpa*II) for mtDNA and 4 restriction endonucleases (*Alu*I, *Sau*3A, *Hinf*I and *Rsa*I) for the H*sp*70 gene. The mtDNA analysis indicated higher haplotype diversity (0.76 ± 0.07) in *Artemia* samples from Fundisha saltworks while the rest of the samples were monomorphic. The presence of 3 haplotype genotypes in Fundisha (AAAAAA, AAAABA and AAABBA) signified a molecular evidence of a systematic genetic differentiation. However, the haplotype frequencies within Fundisha were statistically insignificant (P > 0.05). The existence of haplotypes AAAAAA and AAAABA in Fundisha, GSL and SFB cyst samples was a molecular evidence of SFB and GSL *Artemia* strains co-existing in Fundisha saltwork while the Kensalt and Tanga *Artemia* samples were purely SFB strains. The non-polymorphic DNA fingerprint observed in Kensalt *Artemia* cysts was probably caused by the non-sequential *Artemia* culture system, lack of ecological isolation and limited genetic drift. Also, the limited mtDNA fragment analysed might have excluded valuable genetic information from the study. The H*sp*70 gene RFLP fingerprint showed lack of unique gene signatures in the Kenyan *Artemia* samples suggesting that other factors besides H*sp*70 could be involved in their superior thermotolerance compared to their SFB ancestors. In conclusion, if indeed there was significant genetic differentiation between the Kenyan *Artemia* population and their SFB ancestors, then the tools used in the study were not sufficiently adequate to detect this microevolutionary divergence. Further genetical studies based on the larger mtDNA fragment using robust genetic markers are recommended. Also, evolutionary and ecological studies of the heat shock protein family and the stress response are highly encouraged.

1. INTRODUCTION

The brine shrimps *Artemia* are small crustaceans well adapted to live in stressful environmental conditions of hypersaline habitats such as salt lakes, coastal lagoons and solar saltworks found almost all over the world (Persone and Sorgeloos, 1980). The primary diet for *Artemia* is phytoplankton (Triantaphyllidis *et al.,* 1998) though they can also feed on bacteria (Toi *et al.*, 2013). Being osmotolerant animals, *Artemia* can withstand habitats whose salinity levels range between 10 - 340 g L^{-1} with fluctuating ionic composition and temperature profiles (Van Stappen, 2002). Adaptation of the genus *Artemia* to these disturbing conditions has occurred at molecular, cellular, physiological and population level making *Artemia* fit to survive and reproduce effectively in such insulting environments (Gajardo and Beardmore, 2012). The many distinctive features found in the genus *Artemia* are probably the reasons for their successful adaptations of both natural and introduced *Artemia* populations in new environments all over the world (Abatzopoulos *et al.*, 2002b). For example, besides having a short life cycle, the extremophile *Artemia* has high genetic variability (Kappas *et al.*, 2004) that makes them model animals for studying evolutionary processes such as genetic differentiation, which indeed is the focus of this thesis.

Between 1984 and 1986, a non-native *A. franciscana* was introduced along the Kenyan coast (Fundisha and Kurawa salt farms). Today, the *A. franciscana* has spread (perhaps through wind actions, water birds and human activities) and has permanently colonised the Kenyan coast. Currently, eight saltworks exist along the Kenyan coast with each having different *Artemia* inoculation policies. Since 2009, Fundisha saltworks has been re-inoculated using GSL *Artemia* strains suggesting coexistence of GSL and SFB *Artemia* strains. Kensalt farm was inoculated in 1992 and 1993 using *Artemia* nauplii hatched from Kurawa salt work's cysts suggesting that only SFB *Artemia* exist there.

To date, it is not known whether SFB and GSL *Artemia* strains coexist in Fundisha saltworks. Neither do we know the genetic microevolutionary divergences that have occurred in the Kenyan *Artemia* population compared to their SFB ancestors. The laboratory culture experiments of Mremi (2011) and Kapinga (2012) showed that Kenyan *Artemia* are superior to their original SFB inoculants in terms of reproductivity and thermotolerance at elevated temperatures. However, no information is available on their genetic architecture to support these phenotypic characteristics. In Vietnam, Kappas *et al*. (2004) discovered genetic differentiation of the Vinh Chau (VC) *Artemia* population in Vietnam compared to the original inoculated SFB strains almost 20 yrs ago. Unlike in the Kenya situation, the *Artemia* culture system in Vietnam is sequential, meaning that the VC *Artemia* populations are wiped out every monsoon season and a new inoculation is done using *Artemia* naupli hatched from

previously harvested cysts. This genetic bottleneck has favoured the selection process leading to a more locally adapted strain in the hotter Vietnam environment (Clegg *et al.*, 2001; Kappas *et al.*, 2004).

This Masters study fits within the framework of the Kenyan *Artemia* Flemish Inter-universitary Council Own Initiative (VLIR – OI) project titled '*Improvement of the living standard of rural communities through Artemia production in coastal salt works at Gongoni, Malindi, Kenya*' bringing together KMFRI and Gent University, Faculty of Bioscience Engineering, Department of Animal Production, Laboratory of Aquaculture & *Artemia* Reference Center (ARC) (Bossier *et al.*, 2010).

Through Restriction Fragment Length Polymorphism (RFLP) technique, the present laboratory based study broadly aimed to genetically characterize the Kenyan *Artemia* cysts based on the mitochondrial DNA and heat shock protein 70 (H*sp*70) genes. The *Artemia* cysts were selected from saltworks in Kenya based on their inoculation history. *Artemia* cysts from Fundisha saltwork were selected because of expected coexistence of SFB and GSL strains while Kensalt cysts appeared to be 'undisturbed' since the original SFB inoculation. The DNA was extracted from single *Artemia* cyst samples from each of the selected stations including SFB *Artemia,* VC and GSL as controls. Also, cysts from Tanga, Tanzania were analysed for additional study as these cysts were suspected to have originated from the Kenyan inoculation material. About 30 single cysts per sample were considered for DNA extraction, after which 10 best replicates were PCR amplified for mtDNA analysis. The single cyst approach was preferred not only to allow assessment of the species homogeneity of each sample but also to avoid loss of genes due to selective hatching (Van Stappen, 2008). Because of time limitations, pooled cyst samples were used to extract DNA for the H*sp*70 gene analysis. *Artemia* cysts act like gene banks that store genetic memory of historical conditions (Gajardo and Beardmore, 2012). They also contain substantial amounts of heat shock protein family compounds because cysts are the only surviving agents during stressful environmental conditions (Clegg *et. al.*, 1999; Van Stappen, 2002).

Although the original idea was to amplify the 2,973 bp ND5F – CYTBR gene fragment of the mtDNA from the single cysts, more emphasis was put on the 1,500 bp fragments of 12S - 16S mtDNA and 1,935 bp fragment of H*sp*70 gene after inconsistent PCR amplification problems encountered in the laboratory with the 2,973 bp fragment. There were problems of unspecific amplifications but this was corrected by optimizing PCR reaction conditions.

The hypothesisis of this study was that the genetic pattern of the Kenyan *Artemia* strains would be mutually polymorphic hence possessing special genetic characteristics, which enable them to survive better than original SFB inoculants in the much hotter Kenyan environment. The specific objectives of the study were:

- to detect the presence of special genetic signatures in the mtDNA and H*sp*70 gene fragments of the Kenyan *Artemia* samples and compare them with their original SFB ancestors.
- to determine the purity of *Artemia* populations in Kensalt and Fundisha saltworks
- to establish the genetic relationships between the Kenyan and Tanga *Artemia* cysts

2. LITERATURE REVIEW

2.1 Biology and ecology of *Artemia*

2.1.1 *Artemia* taxonomy

The recently updated taxonomic classification of the genus *Artemia* according to Martin and Davis (2001) is as follows:

Kingdom: Animalia
  Phylum: Arthropoda
    Subphylum: Crustacea Brünnich, 1772
      Class: Branchiopoda Latreille, 1817
        Subclass: Sarsostraca Tasch, 1969
          Order: Anostraca Sars, 1867
            Family: Artemiidae Grochowski, 1896
              Genus: *Artemia* Leach, 1819.

The genus *Artemia* contains the following species *A. franciscana, A. salina, A. urmiana, A. sinica, A. persimilis, A. tibetiana, Artemia sp* and parthenogenetic populations.

2.1.2 The morphology and life cycle of *Artemia*

The brine shrimp *Artemia* is a primitive arthropod crustacean with a segmented body growing to an adult size of about 8 - 12 mm long and about 4 mm wide (Criel and Macrae, 2002a). In addition to a thin chitin layer of exoskeleton, the body is divided into three major parts (head, thorax and abdomen) (Abatzopoulos *et al.,* 2002a). The adult *Artemia* has a pair of stalked eyes, long antennae, a digestive tract, and eleven pairs of thoracopods each holding a pair of swimming legs (Sorgeloos, 1980a, Sorgeloos *et al.*, 1986) (Figure 2.1). Whereas the adult males have a retractile penis just behind the last pair of phyllopods and large claspers, which are used for clasping during mating, females have a conical pouch called the brood sac or ovisac for storing eggs or nauplii during reproduction cycles (Figures 2.2 and 2.3) (Vos and de La Rosa, 1980; Sorgeloos *et al.*, 1986).

Depending on specific diets and perhaps environmental conditions prevailing in a given habitat, natural populations of *Artemia* may exist in different colours ranging from almost transparent to orange, green or red (Sanchez *et al.*, 2006; Dvorak *et al.*, 2012). Amat *et al.* (1991) attributed the red colouration of *Artemia* to carotenoid pigments contained in certain phytoplankton community used as food by *Artemia*. The *Artemia* nauplius is pinkish in colour and the size is about 0.4 mm with the body consisting of the head and a short thorax also called cephalothorax (Abatzopoulos *et al.,* 2002a). The nauplius has a dark eye and two

pairs of antennae on the head. The first and the second pair of antennae are used as sensory organs and swimming and / or feeding respectively (Sorgeloos *et al.*, 1986).
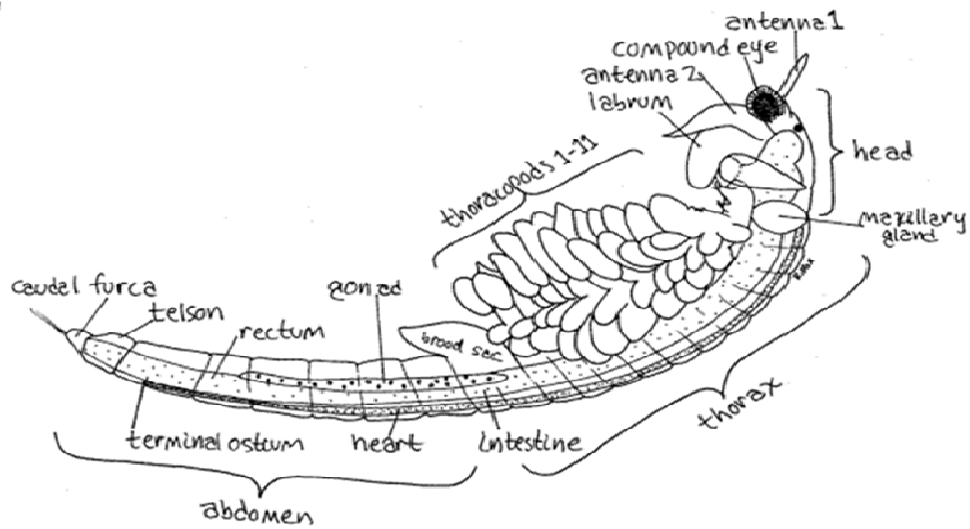
Figure 2. 1: Structural morphology of a young female *Artemia* (adapted from Fox, 2001)

Figure 2. 1: Structural morphology of an adult male and female *Artemia* species (adapted from www.NaturaMediterraneo.com)

Depending on environmental factors such as salinity and temperature, Browne *et al.* (1991) estimated the life span of *A. franciscana* to be between 2 to 4 months. This is generally applicable to all *Artemia* populations. During favourable environmental conditions (mainly conducive salinity and temperature), an ovoviviparous reproduction cycle occurs where the adult females produce the free swimming naupli (about 0.45 mm) from their brood sac into

the ambient environment (Anderson *et al.*, 1970). However, during stressing environmental conditions, an oviparous reproductive cycle prevails. Under this situation, the adult female *Artemia* produces metabolically inactive cysts that float and move about on the water surface at the mercy of water currents as the parental animals succumb to the harsh prevailing conditions (Van Stappen, 1996). At this point the cyst's metabolism are arrested by internal factors and are said to be in diapause, a state which can be broken by splitting cyst carotenoproteins (Dutrieu, 1960) or depression of internal cyst pH (Drinkwater and Crowe, 1986) to become quiescent (Lavens and Sorgeloos, 1987; Clegg and Jackson, 1998). When conducive environmental conditions return (mainly favourable salinity and temperature), the quiescent cysts get hydrated and the embryo starts internal metabolic activity, which eventually breaks the cyst and hatches to a free swimming nauplius in a process that lasts for about 20 hours depending on the *Artemia* species being observed, and the cycle is completed (Figure 2.3) (Pearson and Sorgeloos, 1980; Van Stappen, 1996).
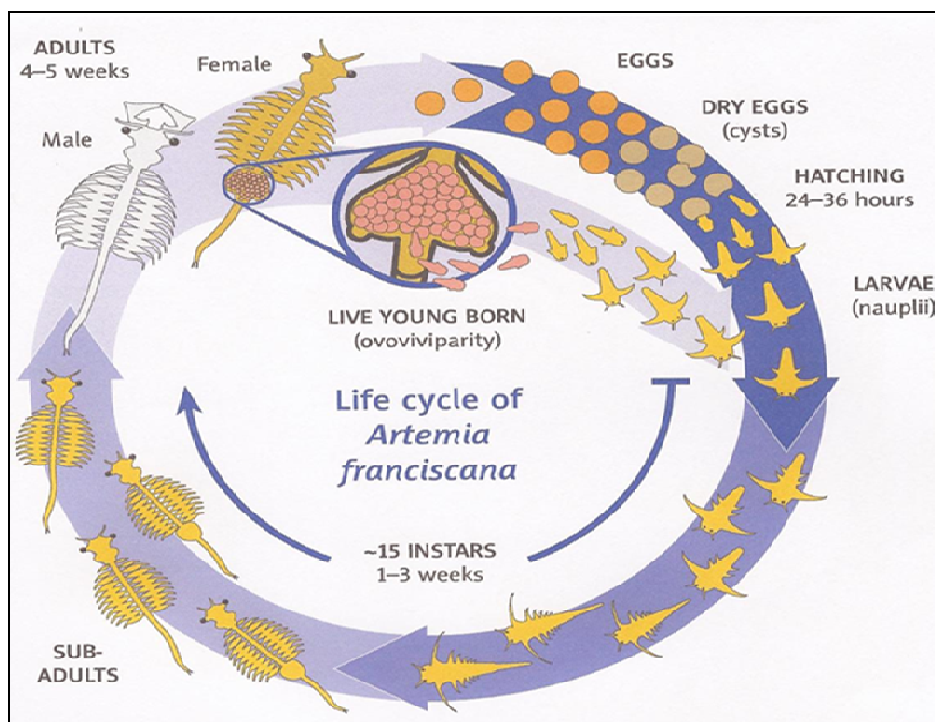
Figure 2. 2: Life cycle of *Artemia franciscana* (adapted from Madden, 2009)

2.1.3 Ecology of *Artemia*

Despite lacking anatomical defence mechanisms against predation by carnivorous animals, *Artemia* have continued to survive in natural salty waters thanks to their efficient physiological adaptations. *Artemia* produces efficient respiratory pigments to help them cope

with the low oxygen levels in hypersaline and warm environments (Croghan, 1958c). The most extensively discussed ecological aspect of the genus *Artemia* in literature is the evolution of two distinctly different paths of development, which includes the production of diapausing cysts during harsh environmental conditions and the directly developing embryos during conducive environmental conditions (Clegg *et al.*, 2004). This ecological behaviour is also common among parthenogenetic populations (Kappas *et al.*, 2004).

Many scientists have reported that the development of *Artemia* populations in their natural or introduced habitats is mostly favoured by the presence of abundant microbial community such as bacteria, protozoa and algae that form the basis of their diet, on which they non-selectively filter feed (Amat *et al.*, 1995a; Van Stappen, 1996; Toi *et al.*, 2013). Whenever the salinity levels go down, lives of *Artemia* are always threatened because carnivorous predators such as fish, other crustaceans and insects can tolerate the salinity and become abundant. (Dodson and Frey, 2001; Torrentera and Dodson, 2004). Indeed, *Artemia* enjoys the advantage of high media salinities for example above 100 g L^{-1} as the *Artemia* competitors are eliminated leading to thick monoculture densities of *Artemia* populations in their respective environments (Van Stappen, 2002). In addition, different geographical strains of *Artemia* have adapted widely to local fluctuating conditions with regard to temperature (6 - 35°C) and ionic composition of the environment (Van Stappen, 2002). At salinities above 250 g L^{-1}, *Artemia* tend to spend much energy on maintaining self regulating physiological processes, which finally fail leading to their death (Pearson and Sorgeloos, 1980; Dodson and Frey, 2001). Ovoviviparous (nauplius) reproduction is mostly dominant at low salinity levels while oviparous (cyst) production happens at salinity beyond 150 g L^{-1} (Drinkwater and Clegg, 1991). Dispersion of *Artemia* cysts is mainly accomplished by man, wind and waterfowl especially flamingos (Lenz and Browne, 1991; Gajardo *et al.*, 1992).

2.2 *Artemia* biogeography

2.2.1 Global distribution of different *Artemia* species

The fact that brine shrimp *Artemia* can inhabit a wide range of hypersaline environments (10 - 340 gL^{-1}) gives them a wide global geographical representation compared to other aquatic animals (Persoone and Sorgeloos, 1980). As Triantaphyllidis *et al.* (1998) put it; the only place where *Artemia* cannot be found is Antarctica. *Artemia* can be found in altitudes ranging from low sea level to almost 4,500 m in Tibet, from humid to subhumid and arid conditions (Vanhaecke *et al.*, 1987; Xin *et al.*, 1994). So far, discrete *Artemia* populations have been identified in about 600 natural salt lakes and saltworks spread along the coastlines of the

tropical, subtropical and temperate climatic regions and further survey efforts are still on course to identify more *Artemia* biotopes all over the world (Van Stappen, 2002).

Whereas *A. franciscana* is native in North, Central and South America (Bowen *et al.*, 1985), numerous types of *Artemia* from Europe, Africa, Asia and Australia are grouped together as parthenogenetic *Artemia* (Triantaphyllidis *et al.,* 1998; Van Stappen, 2002; Munoz *et al.,* 2009). Other *Artemia* species so far identified include: *Artemia persimilis* (Piccinelli and Prosdocimi, 1968) from the extreme south of South America (Gajardo *et al.*, 1999; Gajardo *et al.*, 2004), *Artemia salina* (Leach,1918) in the Mediterranean basin (Triantaphyllidis *et al.,* 1997b), *Artemia urmiana* (Günther, 1890) of lake Urmia, Iran (Van Stappen *et al.*, 2001; Manaffar, 2012), *Artemia sinica* (Cai, 1989) in China (Naihong *et al.*, 2000), *Artemia tibetiana* from Tibet (Abatzopoulos *et al.*, 2002a) and *Artemia sp.* in Kazakhstan (Pilla and Beardmore, 1994).

The increase in *Artemia* cyst consumption since the early 1990s due to the rapidly expanding global aquaculture hatcheries (Sorgeloos *et al.*, 2001; Dhont and Van Stappen, 2003) caused a decline in natural supply of *Artemia* cysts prompting the search for alternative sources of *Artemia* from manmade saltworks inoculations all over the world (Lavens and Sorgeloos, 2000b). Due to this initiative, several populations of non-*franciscana* strains have emerged (Persone and Sorgeloos, 1980) with inferior quality as compared to those of Great Salt Lake (GSL), which are so far regarded as "standard" in aquaculture live food nutrition (Bossier *et al.,* 2004). Indeed, *Artemia* has been inoculated in Brazil (Persoone and Sorgeloos, 1980), Australia (Geddes, 1980), Philippines (De Los Santos *et al.*, 1980), Thailand (Tarnchalanukit and Wongrat, 1987), India and Sri Lanka (Hoa *et al.*, 2007). A living example is South East Asia especially Vietnam, which is now an important supplier of high quality *Artemia* cysts in both domestic and foreign markets (Quynh and Lam, 1987; Vu Do and Nguyen Ngoc, 1987; Brands *et al.*, 1995; Baert *et al.*, 1997; Anh *et al.*, 2010).

2.2.2 The brine shrimp *Artemia* in Africa

Despite a major increase in exploration sites and knowledge of *Artemia* populations all over the world, information about *Artemia* presence in African is still scanty. Nevertheless, *Artemia* populations have been recorded from North to South of Africa. Kaiser *et al.* (2006) documented the existence of *A. salina* in South Africa while Seurat (1921) was probably the first scientist to report existence of *Artemia* in Tunisia in the North of Africa. Most recently, Naceur *et al.* (2010, 2012) identified *A. salina, A. franciscana* and *parthenogenetic* populations in Tunisia through analysis of their biological characteristics. In addition, the existence of a bisexual population of *Artemia* and *A. salina* in Morocco, Libya and Egypt has

been scientifically authenticated (Triantaphyllidis *et al.*, 1997b; El Magsodi *et al.*, 2005). In their first biogeographical work on world *Artemia* spots, Vanhaecke *et al.* (1987) acknowledged that Algeria too has climatic conditions that suit the existence of *Artemia* biotopes and listed about four *Artemia* sites in Algeria. About 8 years later, Amat *et al.* (1995a) detected congruence between the biodiversity of the Algerian *Artemia* populations with that of western Mediterranean countries.

Parthenogenetic populations of *Artemia* have been reported in the saltworks found in Mozambique and Namibia (Seaman *et al.*, 1991) while *A. parthenogenetica* and non-permanent *A. franciscana* populations have been identified in Madagascar (Triantaphyllidis *et al.*, 1998). Recently, unidentified *Artemia* cysts were found in the coastal area of Tanga in Tanzania. However, speculations point to the fact that the cysts might have originated from Kenyan coastal saltworks, which is barely 130 km away. Speculations are also high that *Artemia* cysts exist on the shore of a salt lake in West Uganda but there is no literature to support this claim. Based on the favourable climatic conditions present in most of African countries such as Mauritania, Somalia, Ethiopia, Sudan and the entire area of southern African countries, Vanhaecke *et al.* (1987) considered many African countries as potential *Artemia* habitats. However, very little research has been done in these areas hence much information is generally lacking in literature. In overall, about 130 *Artemia* sites have been recorded on the African continent (Kaiser *et al.*, 2006).

2.2.3 The brine shrimp *Artemia* in Kenya

The history of *Artemia* introduction on the Kenya coast, which occurred between 1984 -1986, was initiated by the Kenyan government through a collaborative project with state owned Kenya Marine & Fisheries Research Institute (KMFRI), funded by the Belgian Agency for Development Cooperation (BADC). The specific aim was to assess the potential of *Artemia* production in Kenyan coastal saltworks. A non-native *A. franciscana* from San Francisco Bay (SFB) was used in the first inoculation and it successfully colonised Fundisha and Kurawa farms since there was no any other *Artemia* present by then. Rasowo and Radull (1986) reported inoculation of *A. salina* in Kurawa and Fundisha saltworks in 1986. However, this might have been erroneously reported perhaps due to lack of proper nomenclature of *Artemia* at that time. Deeper consultations converge to the conclusion that it must have been *A. franciscana* from SFB (Verbal communication, B. Nyonje, KMFRI). Indeed, Kaiser *et al.* (2006) confirmed the existence of *A. franciscan*a in Kensalt, Malindi and Kurawa salt works in Kenya. In 2009 the management of Fundisha saltworks started an organized biological management of the farm after learning about the importance of *Artemia* in the salt production process. They imported *Artemia* cysts from Great Salt Lake (GSL) for the purpose of

inoculating the ponds. Before introducing the GSL strain, they discovered that the originally inoculated *Artemia* strain from SFB still existed in the ponds. Currently, the farm practice regular pond inoculations every production season using nauplii hatched from locally harvested cysts and commercial GSL *Artemia* cysts (M. Mukami - KMFRI pers. communication). The coexistence of both SFB and GSL *Artemia* strains in Fundisha salt work is a subject that can only be revealed through molecular research studies (one of the objectives of this study). According to Van Stappen (2008), coexistence of different strains or species of *Artemia* within the same site is possible just as overlapping of parthenogenetic and bisexual *Artemia* strains. However, coexistence of different bisexual species in natural populations is not yet known (Van Stappen 2002). In Kurawa saltworks, SFB *Artemia* cysts were re-inoculated into the salt ponds to boost *Artemia* population in 2009 and 2010. Since then, the management does routine inoculation of *Artemia* in the months of June, July and August using nauplii hatched from harvested *Artemia* cysts.

Between 1992 and 1993, Kensalt farm experienced a devastating algal bloom that threatened to shut down the farm forcing the management to use *Artemia* to biologically control the algal blooms. The *Artemia* population used for inoculation at Kensalt ponds was harvested from Kurawa salt works (original SFB *Artemia* strain). Today, Kensalt firm performs a routine re-inoculation program using nauplii hatched from harvested cysts every September.

In Kemu salt farm, *Artemia* was first introduced in 2008 using the GSL strain. At the time of inoculation, there was no *Artemia* population present suggesting that the current *Artemia* is purely GSL strain. An organised re-inoculation program exists where commercial GSL *Artemia* cysts are regularly hatched and the naupli are grown for 3 weeks before inoculation in ponds whose salinity is between 150 - 200 gL^{-1} (Nyonje, 2011; Verbal communication M. Mukami, KMFRI).

The *Artemia* population present in Mnarani salt works is believed to have spread from the initial inoculations that where done in Fundisha and Kurawa in the 1980s and from the neighbouring Kensalt farm in 1990s. There is no organized biological management of the salt works using *Artemia* in this farm. This artisanal farm is where the current VLIR project is currently situated.

Malindi saltworks has an interesting history because there has never been any intentional effort to inoculate *Artemia* in the ponds. Also there is no regular biological management of the ponds using *Artemia*. Therefore, it is speculated that the *Artemia* currently present in Malindi ponds must be original SFB *Artemia* inoculations made in Kurawa and Fundisha

between 1984 and 1986. *Artemia* cyst or biomass production is not the main priority in any of the salt farms in Kenya. Therefore records on cyst production are scanty with some companies such as Kurawa, Fundisha and Kensalt reporting harvests less than 10 kg of cysts in a season which are used for re-inoculation purposes when necessary. Studies of Mremi (2011) and Kapinga (2012) about the reproductive characteristics of Kenyan *A. franciscana* revealed that the original *Artemia* from SFB inoculated on the Kenyan coast almost two decades ago has successfully adapted to the local environmental conditions. They found that Kenyan *A. franciscana* (Ken1 and Ken2) had a higher number of broods per female and better survival at 33°C compared to the original SFB *Artemia* strain.

2.3 Integrated *Artemia* pond production

Depending on the seasonal patterns of each place, *Artemia* culture in salt ponds can either be permanent or seasonal if cultivation is only possible during the dry season when evaporation exceeds precipitation (Baert *et al.*, 1996; Lulijwa, 2010). In both systems the salinity of sea water (SW) is increased by the evaporation process in the saltworks and this can be combined with controlled *Artemia* production. Most seasonal ponds are about 100 m^2 wide and between 0.1 - 0.6 m deep while permanent ponds range between few to hundreds of hectares (ha) with a depth range of about 0.5 – 1.5 m (Baert *et al.*, 1996). The engineering of the saltworks is such that sea water is allowed to flow by gravity and/or pumping to other evaporation ponds from the first main pond in which sea water is pumped. During the process, the salinity of the water gradually increases as evaporation accelerates. In saltworks where *Artemia* management is done, deliberate inoculations are conducted using live nauplii stages of *Artemia* for faster colonization. At medium salinity levels (80 g L^{-1} - 140 g L^{-1}), *Artemia* individuals can be seen growing and reproducing ovoviviparously, but as salinity approaches 250 g L^{-1}, female *Artemia* produces cysts as the adults succumb to the unbearable salinity levels (Baert *et al.*, 1996).

The importance of biological management in optimizing the quality of salt produced in solar saltworks has been extensively documented (Tackaert and Sorgeloos, 1992; Magna *et al.*, 2005; Davis, 2006). Solar saltworks being man-made and artificial ecosystems, they are highly vulnerable to algal blooms that can compromise the quality of salt produced. Therefore, introduction of *Artemia* in sufficient numbers can control algal blooms, leading to improved salt production, high *Artemia* biomass and eventually greater amount of cyst production (Ahmed *et al.*, 2000). After recognizing the economic and ecological importance of *Artemia*, there have been many deliberate attempts to inoculate *Artemia* naupli in various man-made ponds and saltworks all over the world. In the process of doing this, accidental introductions have been caused by people seeding the pans with salt contaminated with

Artemia cysts (Tackaert and Sorgeloos, 1992). The inoculation of *A. franciscana* in Southern Asian countries especially Vietnam has been a big success despite the need for yearly renewed inoculation due to the seasonal monsoon rainfall, which prevents the possibility of year-round establishment of *Artemia* population, as the salinity level is lowered and *Artemia* suffers from competition and predation pressures from carnivorous animals (Kappas *et al.*, 2004).

2.4 Ecological adaptation of *A. franciscana* in different habitats

2.4.1 Phenotypic and reproductive adaptations

Meaningful adaptation can only occur after a lengthy occurrence of an evolutionary experience facilitated by forces of natural selection (Gonzalo and Beardmore, 2012). This process can lead to biological and ecological stability of an organism in any given condition after some time. According to Gajardo and Beardmore (2012), local adaptation and ecological divergence in populations can result into speciation and hence promote localised biodiversity with specific traits. The most discussed reproductive adaptation mechanism of the genus *Artemia* in literature is the existence of a short life cycle and the two distinctly different (oviparous and ovoviviparous) cycles of development (Clegg *et al.*, 2004; Kappas *et al.*, 2004). Basing on the experience with *A. franciscana* in regards to environmental stress, Clegg and Trotman (2002) declared that *Artemia* cysts are the most resistant of all animal life history stages while *Artemia* nauplii and adults stages are the best osmoregulators ever known in the animal kingdom. This observation concurs with observations by Marcarelli *et al.* (2005) that *A. franciscana* cysts act like a sedimentary cyst bank because they can remain biologically viable for more than 300 years. The cysts also have a high tolerance to ultraviolet radiation (Triantaphyllidis *et al.*, 1994; Tanguay *et al.*, 2004). Despite *A. franciscana* being a natural inhabitant of the Great Salt Lake, Utah, and in salterns located in the South San Francisco Bay (SFB), USA (Sorgeloos *et al.*, 1991), populations of this species currently exist sporadically world over thanks to dispersions by birds, wind (Persoone and Sorgeloos, 1980) and to a large extent by human intervention (Treece, 2000; Clegg *et al.*, 2004). Most experimental research has centred on *A. franciscana* because it is generally similar to all bisexual *Artemia* species (Clegg *et al.*, 2004), gets easily adapted to various tropical and subtropical environments (Hoa, 2003; Kappas *et al.*, 2004) and exhibits high phenotypic variation (Browne and Wanigasekera, 2000). Indeed this wide range of characteristics enables *A. franciscana* to colonize locations where *Artemia* are considered non-native. In Vietnam, *A. franciscana* from SFB was introduced into Vinh Chau salt fields in 1986 (Rothuis, 1987). Today Vinh Chau strain is one of the major cyst resources in the *Artemia* commercial cyst market (Hoa, 2003). Similarly, in coastal Kenya, *A. franciscana*

(SFB) has reproductively adapted to local conditions after being introduced in the area almost 20 years ago (Mremi, 2011; Kapinga, 2012).

2.4.2 Production of the heat shock proteins (H*sp*) family

Many authors agree that sudden exposure of organisms to unfamiliar environmental conditions above their tolerance limit may trigger the release of biochemical compounds such as heat shock proteins (H*sps*) in them (DuBeau *et al.*, 1998; Rahman *et al.*, 2004; Beristain *et al.*, 2010). There is much information regarding *Artemia's* ability to synthesize heat shock proteins, such as H*sp*26 and H*sp*70 (Clegg *et al.*, 2001; Clegg and Trotman, 2002; Crack *et al.*, 2002; Willsie and Clegg, 2002). *Artemia* cysts contain substantial amounts of heat shock proteins (Clegg *et. al.*, 1999) because they are the surviving agents in stressful environments (Van Stappen, 2002). The H*sp*26 is only produced at specific stages of development, triggered by harsh environmental status (Clegg *et al.*, 2004). It therefore follows that directly developing embryos of *Artemia* have limited or do not posses H*sp*26 because conditions are stable for normal reproduction. Scientific evidence has proven that the family of heat shock proteins are critical for temperature tolerance and plays a crucial role in thermal resistance (Frankenberg *et al.*, 2000; Periago *et al.*, 2002), desiccation tolerance (Bukau and Horwich 1998; Ma *et al.*, 2005) and reduces osmotic stress (DuBeau *et al.*, 1998; Todgham *et al.*, 2005). In addition, Collins and Clegg (2004) documented that H*sp*70 further assists in prevention of oxidative toxicity and damage of cells and tissues of animals including *Artemia*. It is therefore not an overstatement to claim that H*sp*70 is a compound that protects organisms against multidimensional environmental challenges. Heat shock studies of Clegg *et al.* (2001) showed clearly how *A. franciscana* from SFB has successfully adapted to the much hotter environments in Vietnam. Clegg *et al.* (2001) found that cysts produced in Vietnam contained higher levels of heat shock proteins such as artemin, p26 and H*sp*70 compared to their SFB ancestors. Thus, in addition to other factors, stress proteins could be involved in the adaptation of *A. franciscana* from SFB growing in the much hotter environments such as salt ponds in Vietnam (Clegg *et al.*, 2001) and probably Kenyan coastal areas.

In addition to the perceived protection of *Artemia* from thermal heat problems, new evidence from several studies has revealed that heat shock proteins, particularly those of the H*sp*70 family, have the capacity to produce significant adaptive immune responses against many diseases in aquaculture organisms (Morimoto *et al.*, 1994; Srivastava, 2002; Robert, 2003). These have brought new dimensions of strategies to prevent infections in other aquaculture organisms such as shrimps. Recently, Baruah *et al.* (2010) discovered that the H*sp*70 family

has protective roles against virulent Vibrios in aquaculture species. This observation was also supported by Sung *et al.* (2007).

2.5 Ecological and economic importance of *Artemia*

The use of *Artemia* nauplii as fish food began in the 1930s when scientists realised that it is an excellent ingredient for aqua feeds or starter food for fish larvae because they not only provide necessary enzymes to the primitive digestive system of larval fish but their size is relatively small for the larval fish mouth to ingest (Bengtson *et al.*, 1991; Sorgeloos *et al.*, 1991; Sorgeloos *et al.,* 2001; Lim *et al.*, 2003). Indeed many authors agree that due to its high nutritional value (EPA, DHA), *Artemia* could be one of the solutions to mass mortalities experienced in many global marine larviculture and shellfish hatcheries (Bengtson *et al.*, 1991; Merchie, 1996; Sorgeloos, 1998; Dhont and Sorgeloos, 2002). Moreover, the enhanced maturation in shrimp broodstock has been largely attributed to feeding with *Artemia* biomass (Dhont *et al.,* 1993; Naessens *et al.*, 1997; Wouter *et al.*, 2002). According to Sorgeloos *et al.* (2001) there has never been artificially formulated feed that can completely substitute *Artemia* as live prey in fish larviculture. The global estimate of the *Artemia* annual dry cyst market to feed fish and shellfish is above 2,000 tons, costing about 65 USD kg $^{-1}$ (Dhont and Sorgeloos, 2002). Shrimp hatcheries consume between 80 % – 85 % of total cyst sales mainly in China, South East Asia, Ecuador and other Latin American countries (Soltanian, 2007).

Aquatic scientists and salt production managers now agree that saltwork operations require substantial populations of *Artemia* to control the problems associated with algal blooms and to improve salt quality concurrently (Triantaphyllidis, 1995; Faruque *et al.,* 2010). Indeed nutrients from *Artemia* metabolites and decaying animals provide excellent substrates for the development of *Halobacterium* in the crystallization ponds, which promotes heat absorption and effectively accelerates evaporation in the salt ponds, hence facilitating faster and high quality salt formation (Davis, 1978; Jones *et al.*, 1981; Vanhaecke *et al.*, 1987; Sorgeloos and Tackaert, 1991; Rasowo, 1992). The metabolites and decaying animals also form mats on the pond bottom preventing water leakage from the pond bottom. Scientists consider *Artemia* as a model organism for research in various sectors due to its valuable characteristics. For example due to its filter feeding nature (Reeve, 1963), *Artemia* biomass production can be considered useful in extractive aquaculture initiatives as well as in environmental ecotoxicology studies (Nunes *et al.*, 2006). *Artemia* is also actively being used for lucrative aquaria trade purposes world over.

2.6 Genetic characterization of *Artemia* diversity

2.6.1 Evolution of molecular markers in *Artemia* diversity studies

The invention of molecular marker technologies has been a blessing to research in aquaculture genetics. According to Avise (2004), molecular techniques have succeeded not only to open the entire organisms' biological world but also to give full access to a nearly unlimited pool of their genetic variability, making generic studies simple and faster. Through successful implementation of genetic markers in various genetic studies, much information concerning intra- and inter- population polymorphism, taxonomic classification and genetic mapping is now available (Lowe *et al.*, 2004). For a long time, *Artemia* morphometric features have been used to discriminate between different populations (Naceur *et al.*, 2010). However, *Artemia* populations are similar and because of human errors, this method has been largely criticised prompting the emergence of more reliable molecular markers, which have gained popularity over recent years (Abatzopoulos *et al.*, 2002a; Gajardo *et al.*, 1998).

The first true molecular markers to be used to study the genus *Artemia* were allozymes (Kimura, 1968), but this was found to be an indirect and insensitive method of detecting variation in DNA (Botstein *et al.*, 1980). This led to the discovery of DNA based molecular makers, which are more direct to survey DNA variations (Botstein *et al.*, 1980). Moreover, DNA-based markers allow the number of mutations between different alleles to be quantified (Kimura, 1968; Bostein *et al.*, 1980; Avise, 1994, 2004). Over the years, genetic and molecular investigations have been both consistent and highly informative in the study of the development of local adaptations of the genus *Artemia* to new habitats (Bossier *et al.,* 2004; Qiu *et al.,* 2006). Today, *Artemia* phylogeny can be verified and commercial cyst samples scientifically authenticated with ease thanks to molecular techniques (Bossier *et al.,* 2004; Van Stappen, 2008). The extensive study of inter- and intra-specific diversity of *Artemia* has been made possible due to a variety of nuclear and mitochondrial DNA markers for instance ITS-1, H*sp*26, COI, 12S and 16S mtDNA (Perez *et al.*, 1994; Hou *et al.*, 2006; Murugan *et al.*, 2009) and tools such as Restriction Fragment Length Polymorphism RFLP (Bossier *et al.*, 2004; Gajardo *et al.*, 2004; Baxevanis *et al.*, 2006; Eimanifar *et al.,* 2006), Random Amplified Polymorphic DNA RAPD (Badaracco *et al.*, 1991; Sun *et al.*,1999a; Camargo *et al.*, 2002), Amplified Fragment Length Polymorphism AFLP (Triantaphyllidis *et al.*, 1997abc; Sun *et al.*, 1999b) using either single or pooled samples (individuals or cysts) (Avise, 1994; Kappas *et al.*, 2004). Other tools include microsatellites and Single Strand Conformation Polymorphism (SSCP) (Blouin *et al.*, 1996; Kubo *et al.*, 2009).

2.6.2 Use of allozymes to characterize *Artemia* diversity

The advantage of using allozymes is that they do not need primers and the protocols can be applied across species (Liu and Cordes, 2004). For nearly 40 years, allozymes have been used to determine levels of polymorphism, population structure and patterns of habitat divergence in the genus *Artemia* (Abreu-Grobois and Beardmore, 1980, 1982; Abreu-Grobois, 1987; Browne and Bowen, 1991; Kappas *et al.*, 2004). However, allozymes require a high amount of tissue and also genetic variations are limited in proteins as compared to DNA (Liu and Cordes, 2004). Nevertheless, heterozygosity of *A. franciscana* populations has been analysed using allozymes and was found to differ much more as compared to other *Artemia* species (Abreu-Grobois, 1983; Pilla, 1992; Gajardo *et al.*, 1995). Kappas *et al.* (2004) also detected considerable genetic differences between Vin Chau (VC) *Artemia* strains and San Francisco Bay *Artemia* strains (SFB) cultured at 30°C using allozyme analysis.

2.6.3 Random Amplified Polymorphic DNA (RAPD)

RAPD markers were first developed in 1990 using Polymerase Chain Reaction (PCR) technique to randomly amplify anonymous segments of nuclear DNA with an identical pair of short primers (8 –10 bp in length) (Liu and Cordes 2004). In RAPD, no knowledge of the DNA sequence for the targeted gene is required hence making the method popular for comparing the DNA of biological systems that have not been studied before (Vetriselvan and Munuswamy, 2011). However, the method has problems of reproducibility due to the use of random primers. By using the RAPD-PCR method Vetriselvan and Munuswamy (2011) observed considerable genetic differences between Kelambakkam and Vedaranyam *Artemia* strains along the South East coast of India.

2.6.4 Microsatellites

Microsatellite polymorphism depends on size differences due to varying numbers of repeat units contained by alleles at a given locus (Liu and Cordes, 2004). The advantage of microsatellites is that they are versatile due to the large number of loci involved in genetic characterization of organisms (Queller *et al.*, 1993). Munoz *et al.* (2009) characterized brine shrimp *Artemia* using microsatellite markers and found that the number of alleles in *A. franciscana* was different from that of *A. parthenogenetica*. The high population assignment power demonstrated by microsatellite loci could be useful for future studies of *Artemia* population genetics (Munoz *et al.*, 2009). However, problems caused by point mutations in the primer annealing sites may fail PCR amplification creating null alleles, which in most

cases renders the interpretation of microsatellite markers impossible (Jarne *et al.*, 1996; Dakin and Avise, 2004).

2.6.5 Amplified Fragment Length Polymorphism (AFLP)

In the AFLP technique, a restriction enzyme is used to cut DNA, followed by ligation of adaptors to sticky ends of the restriction fragments (Vos *et al.*, 1995; Liu and Cordes, 2004). A subset of the restriction fragment is then selected by using specific primers complementary to the adaptor sequence for further amplification in PCR (Vos *et al.*, 1995). AFLP markers have been mostly used for genetic variation within populations (Triantaphyllidis *et al.*, 1997a). However, Sun *et al.* (1999b) used the AFLP technique to estimate genetic relationships among bisexual *Artemia* species of the New and Old World and among parthenogenetic populations in China. They showed a clear discrimination of New from Old world *Artemia* in a dendrogram of genetic distance; they also found that parthenogenetic populations from inland salt lakes and their coastal counterparts were genetically different. Sun *et al.* (1999b) preferred AFLP because of the high polymorphism found and its sensitivity compared to other techniques e.g. RAPD, RFLP, microsattelites and allozymes.

2.6.6 Restriction Fragment Length Polymorphism (RFLP) method using mitochondrial DNA

The mtDNA Restriction Fragment Length Polymorphism (mtDNA-RFLP) technique was first put into use in the early 1990s to determine evolutionary relationships and genetic divergence of *Artemia* populations (Browne and Hoopes, 1990; Browne and Bowen, 1991; Perez *et al.*, 1994; Valverde *et al.*, 1994; William *et al.*, 2004). In the RFLP molecular technique, the targeted DNA genome size is first amplified through the PCR method before digestion by appropriate restriction enzymes. The digested product is then separated according to their size by agaorose gel electrophoresis to detect genetic diversity (Eimanifar *et al.,* 2006). The mitochondrial genome of *A. franciscana* is estimated to be 15,822 nucleotides long (Valverde *et al.*, 1994). In recent past, the analysis of mtDNA has been done in relation to the speciation of both bisexual and parthenogenetic *Artemia* populations (Perez *et al.*, 1994). Indeed, the publication of the mtDNA sequence of *A. franciscana* by Valverde *et al.* (1994) became a very important step in research on *Artemia* population structure and molecular ecology.

According to Moritz *et al.* (1987) and Vallejo *et al.* (1996), all animal mtDNA contains two rRNAs (12 S and 16 S), 22 tRNAs and 13 genes coding for polypeptides, totalling to 37 genes all of which are parts of respiratory complexes in the inner mitochondrial membrane. In fact, mtDNA is superior to nuclear DNA in the sense that nuclear DNA is inherited from

both parents and genes undergo recombination while mtDNA is maternally inherited and does not experience recombination (Wolstenholme, 1992; William *et al.*, 2004). The mtDNA is therefore highly conserved in comparison with nuclear DNA making it a robust marker for tracking animals' ancestry (Krieg *et al.*, 2000).

Studies of Gajardo *et al.* (2004) on Chilean *Artemia* populations based on mtDNA RFLP analysis revealed a higher haplotype and nucleotide diversity for *A. persimilis* compared to *A. fransiscana* population samples. Out of nine restriction enzymes they used, five produced species-specific patterns, where three restriction enzymes were polymorphic for only *A. franciscana* and two other for only *A. persimilis*. Eimanifar *et al.* (2005) conducted an optimized PCR-RFLP method on a 1,564 bp region of mtDNA using eleven restriction endonucleases to identify cyst batches of *Artemia urmiana* collected from different areas of Urmia Lake. They found that RFLP patterns of four restriction enzymes were sufficient to differentiate between the cyst samples. Eimanifar *et al.* (2006) investigated polymorphism within the rDNA gene region among floating and sinking *Artemia* cysts from different geographical sites in Lake Urmia using the RFLP method. They found nucleotide divergence within *Artemia* populations found in the main ecological zones of Lake Urmia, suggesting genetic differentiation between them. Studies of Agh *et al.* (2009) showed that bisexual *A. urmiana* are morphologically different from the parthenogenetic populations in Iran but analysis of their 1,500 bp mitochondrial rDNA fragment using RFLP confirmed that they are indeed genetically close. Most recently, Manaffar (2012) conducted an RFLP analysis of the 1,500 bp mitochondrial rDNA fragment on *A. urmiana* cysts with seven different restriction enzymes and revealed a high degree of polymorphism among cysts from different stations in Urmia Lake. Through RFLP analysis of a 1500 bp mitochondrial rDNA fragment, Bossier *et al.* (2004) developed a methodology to authenticate *Artemia* cyst samples up to the species level by using four restriction enzymes while Van Stappen *et al.* (2007) reported that the technique can reveal temporal shifts in species composition within a habitat when individual cysts are scored.

It is not an overstatement to suggest that genetic adaptations have contributed to successful colonization of *A. franciscana* to new environments. In most tropical saltworks for example, a water temperature above 30°C is common while the *A. franciscana* population in San Francisco Bay salterns rarely experiences water temperatures above 24°C during the entire growing season yet they can colonise hotter environments. Kappas *et al.* (2004) delved deep in to this subject by studying how *A. franciscana* from SFB was able to survive and colonise unfamiliar environments like Vietnam. They suspected that temperature must have had an influence in shaping the genetic architecture of *Artemia* populations making colonization

possible in Vietnam. In this regard, they investigated the microevolutionary changes that might have taken place in the Vinh Chau *Artemia* since the introduction of the *A. franciscana* from the SFB strain into Vinh Chau saltworks Vietnam in 1982. Original SFB *Artemia* was used as control. Even though their methodological approach compared genetic differences at three different levels (reproductive characters, allozyme loci and mtDNA RFLP), the mtDNA RFLP was the most relevant reference for the current study. *Artemia* cyst batches harvested successively from Vietnam and the original SFB *Artemia* strain were hatched separately at 26°C for the study. They designed primers to amplify a 2,963 bp long mtDNA target sequence before digesting the mtDNA using eight restriction endonucleases (*Alu*I, *Ava*I, *EcoR*I, *Hae*III, *Hind*III, *Hinf* I, *Rsa*I, *Xba*I) to assess genetic variation in the amplified region. Only five enzymes (*Alu*I, *Hae*III, *Hinf*I, *Rsa*I and *Xba*I) showed substantial polymorphism across strains. They further detected a reduction in haplotype gene diversity from 40.6% in SFB *Artemia* to 10.5% in the VC strains suggesting that a process of strong selective pressure to the local conditions might have eliminated unwanted genes, hence producing different signatures in the mtDNA genome of the VC strain as compared to the original inoculant *Artemia* from SFB. A clear discrimination of SFB *Artemia* from the Vinh Chau strain was shown in an UPGMA dendrogram based on mtDNA sequence divergence between the two strains (Kappas *et al.*, 2004).

The current study therefore adopted the Kappas *et al.* (2004) mtDNA analytical approach to determine genetic divergence of the Kenyan *Artemia* samples from their SFB ancestors, which were inoculated along the Kenyan coast almost 2 decades ago. Ths was expected to add more genetic information to the already known reproductive characteristics of the Kenyan *Artemia* in courtesy of Mremi (2011) and Kapinga (2012). In addition, the polymorphic analysis of the H*sp*70 gene was considered to also add more pespective on the anticipated genetic adaptation levels of the Kenyan *Artemia* populations.

3. MATERIALS AND METHODS

3.1 Production area of *Artemia* cyst samples

Although the study was purely laboratory based, it is interesting to know some information regarding the climatic conditions of the source of the main cyst samples in the study. The experimental cyst samples (see table 3.1) were harvested from the Kenyan coastal area in Malindi town located at 3° 50' 0" South, 39° 46' 0" East (Figure 3.2). The Kenyan coastline has a total length of 1,420 km with a tropical climatic condition. The air has a maximum temperature of 36°C while the average temperature is 27°C (Mremi, 2011). The area experiences a long dry season from July to September and a short dry season from January to March. The average rainfall per year is 1,016 mm, which occurs around April to June, and between October and December (Figure 3.1). A variety of phytoplankton ranging from toxic to non-toxic are present in at least some of the saltworks providing basic nutrition for *Artemia* populations present there (Mremi, 2011).
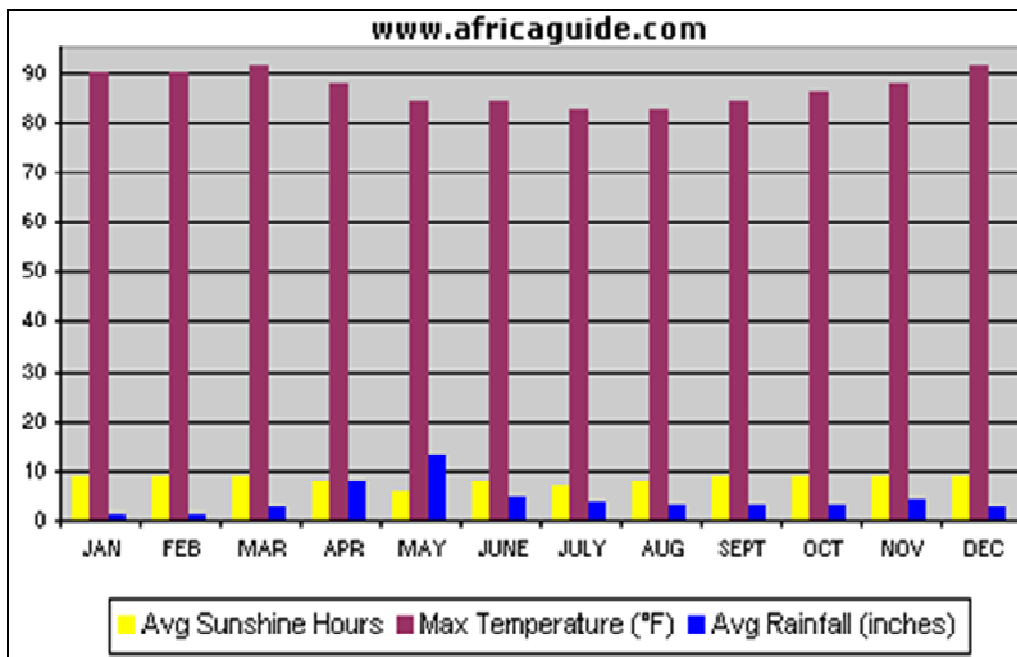
Figure 3. 1: Climatic conditions for Mombasa region in Kenya (adapted from africaguide.com)

3.2 *Artemia* cyst samples

A total of 8 different dry *Artemia* cyst samples were analysed during the study. The 8 samples included 4 samples (Fundisha, Ken1, Ken2 and Ken3) harvested between 1996 and 2012 from selected salt farms at the Kenyan coast (Figure 3.2), one sample from Great

Salt Lake, Utah state in USA (GSL), another sample from salterns located in the San Francisco Bay (SFB), one sample from Vinh Chau (VC), Vietnam, and another sample from Tanga, along the coast of Tanzania. All the *Artemia* cyst samples were available at the Laboratory of Aquaculture & *Artemia* Reference Center (ARC), Ghent University, Belgium where they were stored at 4°C in the cyst bank (Table 3.1). A total of 80 individual *Artemia* cysts, 10 from each sample were used in the study.

The selection of *Artemia* cyst samples from the Kenyan coast was based on the *Artemia* inoculation history as described by Nyonje (2011). Cysts from Kensalt farm (3,000 ha) e.g. Ken1, Ken2 and Ken3, which were actually different batches of the same *Artemia* population, were not only considered due to their 'undisturbed' nature but also because of their known reproductive characteristics thanks to studies of Mremi (2011) and Kapinga (2012). Having been harvested at different times (see table 3.1), it was expected that a genetic evolutionary trend would be detected in their respective genetic structures that would technically explain their adaptational behaviours. The much hypothesised coexistence of SFB and GSL *Artemia* strains in Fundisha saltwork (1,020 ha) was the reason for considering cysts from there. Cysts from SFB, GSL and VC were used as control while cysts from Tanga were analysed just for additional study. Of course it is believed that Tanga cysts might have originated from Kenya perhaps due to natural dispersal (birds, wind) or business contacts. For reasons explained in the introduction section, the use of cysts for DNA extraction for mtDNA and H*sp*70 analysis were preferred to individual *Artemia* adults.
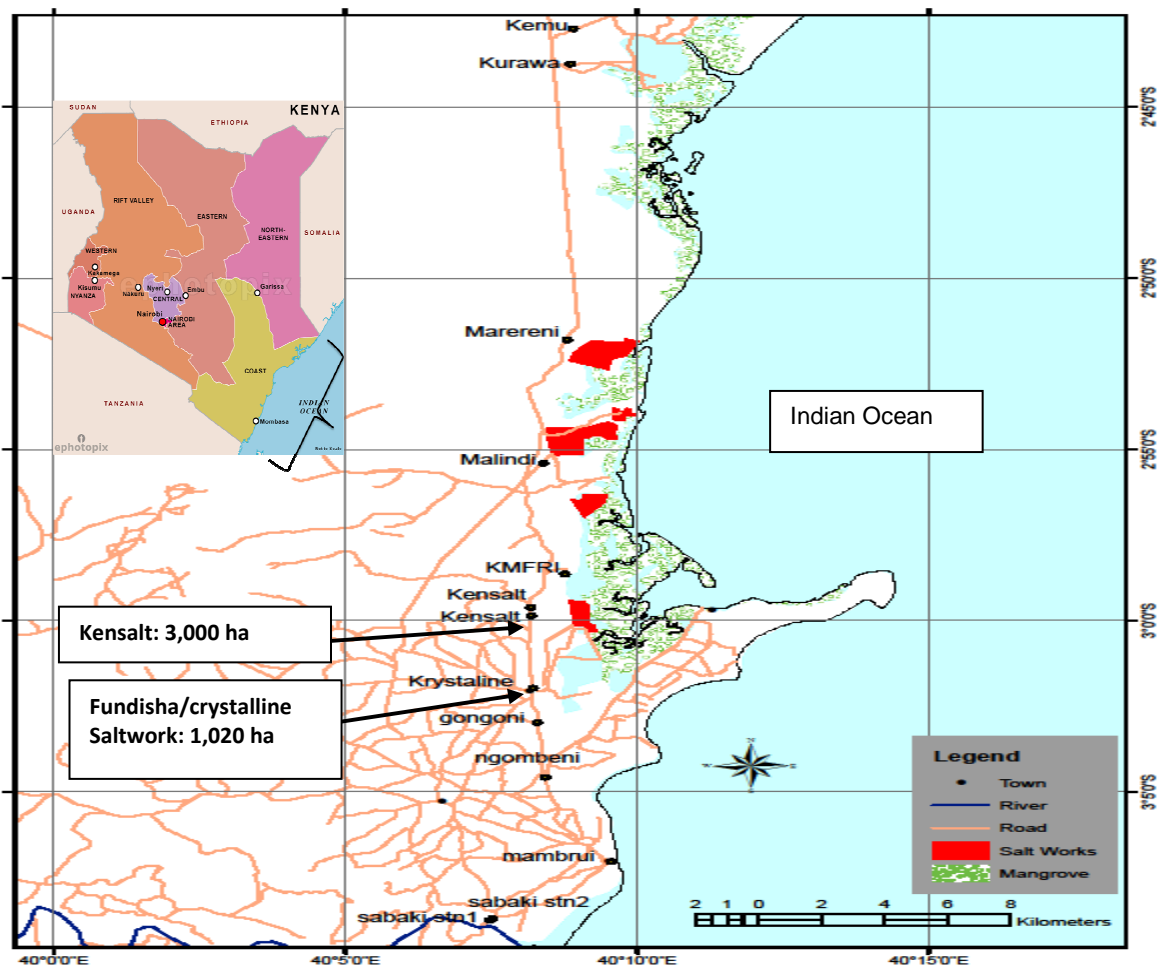
Figure 3. 2: Map of the Kenyan coast showing the location of the salt belt and a more detailed impression of the salt belt showing the individual salt producing companies in a North – South Orientation. Fundisha saltwork is also called Crystalline.

Table 3.1: *Artemia* cyst samples from different populations, harvesting year with corresponding ARC cyst bank numbers from which DNA was extracted for molecular analysis.

Source of cyst	Abbreviation	ARC cyst bank number	Year of harvest
Kensalt : Kenya	Ken2	1439	1996
Kensalt : Kenya	Ken1	1762	2010
Kensalt : Kenya	Ken3	1779	2012
Fundisha: Kenya	Fundisha	1780	2012
Tanga, Tanzania	Tanga	1773	2011
San Francisco Bay[**]	SFB	1574	2003
Vinh Chau[**]	VC	1771	2011
Great Salt Lake[**]	GSL	1768	2011

** indicates control samples

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3.2 DNA extraction

For mtDNA analysis, DNA was extracted from single cysts (10 per sample) from the 8 different samples using the Chelex method based on the protocol described by Walsh *et al.* (1991). A total of 80 individual *Artemia* cyst samples were used. One cyst was isolated using a sterile 10 µL pipette point and transferred to a sterile eppendorf (1.7 mL) where 30 µL of milliQ water (PCR water) was added and left to hydrate for 1 hr. In each eppendorf tube, the cyst was crushed using a sterile pellet pestle (Sigma-Aldrich Z35997-1EA) making sure that the inner content was released into the medium. This was done in a laminar flow to minimize any chance of contamination (Figure 3.3). To each eppendorf tube, 30 µL of well homogenized 10% Chelex slurry (Chelex-100 - Biorad, Belgium) was added and vortexed for 10 - 15 s before spinning the samples for 1 min at 13,000 rpm in a micro-centrifuge. The samples were then incubated for 20 min at 95°C, vortexed again for 10 - 15 s and further spanned at 13,000 rpm for 1 min. The samples were stored in a refrigerator at 4°C and used for PCR amplifications within one week. Fresh DNA was extracted for subsequent tests because the study lasted for about 4 months. Each time, the quantity of the extracted DNA was measured using a NanoDrop® ND-1000 machine, a microvolume spectrophotometer for measuring DNA, RNA or proteins. Similarly, the quality of extracted DNA was checked by running agarose gel electrophoresis. Only the supernatant was used for PCR reactions because chelex beads inhibit the activity of Taq-polymerase enzyme, which is a critical reagent in the PCR amplification procedure (Walsh *et al.*, 1991).

For the H*sp*70 gene analysis, the DNA was extracted from the hydrated pooled cyst samples (0.05 g) using the Wizard® Genomic DNA purification kit from Promega^{TM} based on the protocol described by Miller *et al.* (1988). After hydrating the cyst samples for 2 hrs in a 1.7 mL eppendorf tube, 600 µL chilled nucleic lysis solution was added then homogenized for 10 s by crushing with a pestle, Sigma-Aldrich Z35997-1EA, while keeping on ice. The samples were incubated for 15 - 30 min at 65°C after which 3 µL of RNAase solution was added, mixed and re-incubated for about 15 - 30 min at 37°C. The samples were cooled to room temperature before adding 200 µL of protein precipitation solution. The samples were chilled on ice for 5 min, after which they were centrifuged for 4 min at 13,000 -16,000 g. The supernatant of each sample was transferred to new eppendorf tubes filled with 600 µL of isopropanol at room temperature and mixed by gentle inversion to precipitate DNA. The samples were re-centrifuged at 13,000 - 16,000 g for 1 min before pouring away the supernatant. Then 600 µL of 70 % ethanol was added (loosen the DNA pellet and washes it) to the eppendorf and re-centrifuged for 1 min at 13,000 – 16,000 g to. The ethanol was poured away as the eppendorfs were allowed to dry in the desiccators for 5 - 10 min. At the

end, the DNA was rehydrated in 50 μL DNA rehydration solution overnight at 4°C. Again the quality of the extracted DNA was checked by running agarose gel electrophoresis before PCR amplification.
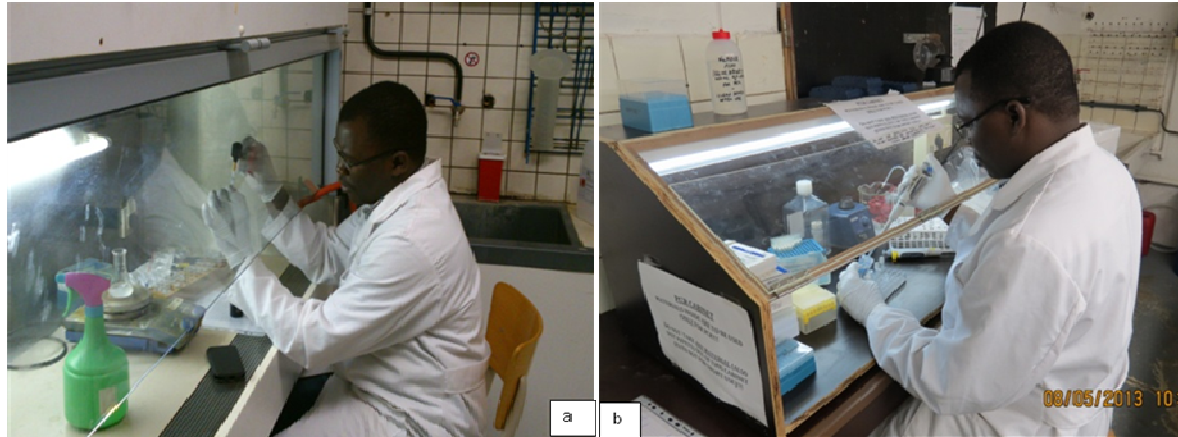
Figure 3. 3: a) Part of the Chelex DNA extraction process (crushing of single cyst in 1.7 mL eppendorf tubes in a laminar flow). b) Preparation of mastermix and DNA samples in PCR cabinet for amplification in the PCR machine

3.3 PCR amplification

3.3.1 PCR amplification of the 2, 973 bp ND5F – CYTBR mtDNA fragment

For each amplification, the total reaction volume of 25 μL consisted of 18.188 μL of PCR water, 2.5 μL 10 x Taq buffer + KCl-MgCl$_2$, 2.5 μL MgCl$_2$ (25 mM solution), 0.5 μL dNTP (10 mM each), 0.5 μL of primer 1 and 2 (work-solution), 0.063 μL BSA, 0.25 μL Taq-polymerase and 1 μL of approximately 5 - 30 ng of template DNA extract, except for the negative control tube. A combination of the forward primer (5'- ggg-atg-aga-cag-ggg-tag-ga -3'), and the reverse primer (5'- cgg-agc-agt-caa-ccg-tag-tt -3') was used for the PCR reaction based on Kappas *et al.* (2004). Due to amplification inconsistencies, the PCR conditions were slightly optimised and some trials were done as enumerated below.

- First the PCR was allowed to run for 30 cycles as prescribed by Kappas *et al.* (2004).
- The annealing temperatures were varied at 57°C, 60°C, 62°C and 63°C.
- The extension time were also varied at 2 min, 3 min 30 s, 4 min and 8 min.
- The DNA concentrations were standardized by ensuring that equal amounts of template DNA (5 ng) per reaction volume was used.

- PCR hot-start procedure was tried by introducing Taq-polymerase enzyme in the reaction volumes after the first 1 min of denaturation step at 94°C, while keeping the samples in the PCR machine.

- Fresh sensitive reagents such as dNTP mix and Taq-polymerase were ordered

Finally some positive amplifications occurred after the samples were heated at 94°C for 2 minutes to activate the Taq polymerase enzyme, then followed by 35 amplification cycles in which each of the cycle consisted of: 1) a denaturing step at 94°C for 1 min to disrupt hydrogen bonds and melt DNA to produce single strands; 2) an annealing phase at 60°C for 1 min to allow annealing of primers as the polymerase binds to the template DNA; 3) an elongation phase at 72°C for 4 min to synthesise a new strand by DNA polymerase and the final cycle of elongation step at 72°C for 10 minutes to fully extend any incomplete fragments of DNA.

3.3.2 PCR amplification of the 1,500 bp 12S - 16S mtDNA fragment

The double stranded DNA amplification was performed in 50 µL reaction volumes containing a mixture of 36.375 µL PCR water, 5 µL 10 x Taq buffer + KCl-MgCl$_2$, 5 µL MgCl$_2$ (25 mM solution), 1 µL dNTP (10 mM each), 1 µL of primer 1 and 2 (work-solution), 0.125 µL BSA, 0.5 µL Taq-polymerase and 2 µL of approximately 5 - 30 ng of template DNA extract, except for the negative control tube. The DNA samples were assayed for PCR amplification of the 1,500 bp mtDNA fragment between the 12S - 16S region as described by Valverde *et al.* (1994). A combination of the forward primer 12S - SP (5'-cta-gga-tta-gat-acc-cta-3'), and the reverse primer 16S - SP (5'- ccg-gtc-tga-act-cag-atc-3') was used for the PCR reaction according to Bossier *et al.* (2004). The BioRad PCR equipment was programmed such that the first cycle of PCR reaction heated the mixture to 94°C for 2 minutes to activate the Taq polymerase enzyme. This was followed by 34 cycles of: 1) a denaturing step at 94°C for 1 min to disrupt hydrogen bonds and melt DNA to produce single strands; 2) an annealing phase at 52°C for 45 seconds to allow annealing of primers as the polymerase binds to the template DNA; 3) an elongation phase at 72°C for 2 min to synthesise a new strand by DNA polymerase and the final cycle of elongation step at 72°C for 4 minutes to fully extend any incomplete fragments of DNA.

3.3.3 PCR amplification of the H*sp*70 gene

The PCR reaction mixture of 25 µL contained 16.32 µL PCR water, 4.8 µL 10 x GoTaq buffer + KCl-MgCl$_2$, 1.2 µL MgCl$_2$ (25 mM solution), 0.48 µL dNTP (10 mM each), 0.48 µL of primer 1 and 2 (work-solution), 0.24 µL GoTaq-polymerase and 1 µL of approximately 150 ng of template DNA extract except for the negative control tube. The DNA samples were

assayed for PCR amplification of the 1,935 bp H*sp* 70 gene fragment (Baruah *et al.*, 2010). A combination of the forward primer H*sp*70$_{forward}$ (5'-cac-cat-ggc-aaa-ggc-acc-agc-aat-agg-3') and the reverse primer H*sp*70$_{reverse}$ (5'-ata-gtt-ggg-cca-ctg-cct-gtt-cca-g-3') were used for the PCR reaction (Baruah *et al.*, 2010). The PCR conditions were modified from Baruah *et al.* (2010) as follows: denaturation step at 94°C for 5 min followed by 35 cycles of 95°C for 1 min, annealing at 63°C for 1 min and elongation at 72°C for 4 min followed by a final extension step for 10 min at 72°C. Amplification of the appropriate 1,935 bp fragment was verified by electrophoresis gel analysis.

3.4 Checking PCR products on agarose gel-electrophoresis

The amplified PCR products were verified for the appropriate fragment size by agarose gel-electrophoresis. To do this, 0.5 g of agarose was dissolved in 50 mL (1 % agarose) of TAE 0.5 x in a 250 ml erlenmeyer by heating in a microwave and shaking every 30 seconds until the agarose dissolved completely. Before cooling down the solution to below 60°C, 1 μL of GelRed was added to enable the visualization of the DNA under UV light. The gel solution was poured gently in a tank with a toothcomb inserted to create wells for loading and left to solidify for about 15 - 20 minutes. The electrophoresis was done in a tank carrying a 0.5X TAE buffer of pH 8.3. At this pH, the DNA building blocks get deprotonated and thus are attracted to the cathode side during electrophoresis (Lind *et al.*, 2006). Using about 10 μL of loading buffer, 5 - 7 spots were lined up on a small sheet of parafilm where 2 spots were designated for loading with 3 μL of the ladder (100 bp promega Ladder). In each of the remaining spots, 5 μL of PCR products were added and mixed before loading into the wells of the gel using a micropipette fitted with a loading pipette point. Electrophoresis was done at 75 V and the DNA fragments were allowed to migrate through the gel for 1 hr. Finally, the DNA fragments in the gel were visualized under UV light hood and a photograph of the image was taken with a digital camera (Canon power shot G10).

3.5 Restriction digestion - RFLP procedure

3.5.1 RFLP of the 1500 bp 12S - 16S mtDNA fragment

The amplified mtDNA fragments were screened for polymorphism using six restriction endonucleases (*Alu*I, *Hae*III, *Hinf*I, *Rsa*I, *Xba*I and *Hpa*II) based on previous studies (Bossier *et al.*, 2004; Kappas *et al.*, 2004). The reactions were done according the manufacturer's instructions (Table 3.2). For each reaction tube, the total reaction volume of 23.5 μL consisted of 16 μL PCR water, 2 μL Tango buffer, 0.5 μL of enzyme and 5 μL of PCR amplified DNA product. Digested products were electrophoretically separated on 2 % agarose gel in a 1 X TAE buffer solution and stained with 1 μL of GelRed. A high voltage of

100 V was used to push the digested DNA fragments through the solidified 2 % agarose gel for 1 hour. A DNA ladder (100 bp promega ladder) was loaded as reference. A UV transilluminator was used to visualise the fragments and photographed with a digital camera (Canon power shot G10). Due to limited capacity of electrophoresis tank wells (60), only 7 best PCR replicate products in each of the 8 sample were digested with restriction enzymes totalling to 56 lines plus 4 lines reserved for the ladder.

Table 3.2: The list and recognition sequences of the restriction enzymes used in the study including incubation and activation temperatures as described by the manufacturer; N = C, G, T or A.

Enzyme	Recognition sequence	Incubation temperature	Inactivation temperature
*Alu*I	5'...A G C T...3' 3'...T C G A...5'	37° C	65° C / 20minutes
*Hae*III	5'...G G C C...3' 3'...C C G G...5'	37° C	80° C / 20minutes
*Hinf*I	5'...G A N T C...3' 3'...C T N A G...5'	37° C	65° C / 20minutes
*Rsa*I	5'...G T A C...3' 3'...C A T G...5'	37° C	80° C / 20minutes
*Xba*I	5'...T C T A G A...3' 3'...A G A T C T...5'	37° C	65° C / 20minutes
*Hpa*II	5'...C C G G...3' 3'...G G C C...5'	37° C	80° C / 20minutes

3.5.2 RFLP of the 2, 973 bp ND5F – CYTBR mtDNA fragment

Due to limited quantity of PCR products of the 2,973 bp mtDNA fragment, only two polymorphic restriction endonucleases (*Hae*III, *Hpa*II), also used in the 1500 bp 12S -16S fragment, were employed to assess variation in the amplified region. The reaction conditions were similar to 3.5.1 above.

3.5.3 The RFLP of the H*sp*70 Gene

Restriction enzymes were selected considering the presence of the number of cleavage sites in the 1,935 bp fragment of the *Artemia* franciscana nucleotide sequence (cDNA) of Hsp70, which is available in the NCBI database. Four restriction enzymes (*Sau*3A, *Rsa*I, *Alu*I and *Hinf*I) with recognition sequences GATC, GTAC, AGCT and GAATC respectively were selected to digest the Hsp70 gene. In each reaction tube, a total reaction volume of 23.5 µL contained 16 µL PCR water, 2 µL Tango buffer, 0.5 µL of enzyme and 5 µL of PCR amplified

DNA product. Restriction digestion was done according to the manufacturing company recommendations (Table 3.2). The incubation temperature was $37^{o}C$ while inactivation temperature was $65^{o}C$ for 20 min for Sau3A enzyme. Digested products were electrophoretically separated on 2 % agarose gel in a 1X TAE buffer solution and stained with 1 µL of GelRed. A high voltage of 100V was used to push the digested DNA fragments through the solidified 2 % agarose gel for 1 hour. A DNA ladder (1kb promega ladder) was loaded as reference. A UV transilluminator was used to visualise the fragments and photographed with a digital camera (Canon power shot G10)

3.6 Data analysis

The NanoDrop® ND - 1000 automatically recorded the quantity of DNA in each sample, whose purity was verified by the $A_{260/280}$ indexes. This index shows the ratio of absorbance at 260 nm and 280 nm and it assess the purity of DNA in samples. The DNA samples whose $A_{260/280}$ ratios fall between 1.7 and 2.0 are generally accepted as pure (Glasel, 1995).

Only the RFLP restriction patterns for the 1,500 bp 12S -16S mtDNA fragments were manually scored. The fragments, whose size was less than 100 bp were not considered because of technical inconsistencies. Unique endonuclease restriction patterns were identified by using specific letters. Each cyst replicate was assigned a multi-letter code that described its composite mtDNA genotype haplotype. For each sample, the haplotype frequency (hf) was manually calculated by counting the identical haplotypes and dividing by the total replicates per sample. The mean haplotype frequency was calculated by adding all the haplotype frequency in each haplotype then dividing by the total number of samples. The haplotype diversity within samples was manually calculated using the formula below (Nei, 1978, 1987; Nei and Tajima, 1981).

$$H = \frac{N}{N-1} \left[1 - \sum x^2 \right]$$

Where
- H = haplotype diversity
- N = Sample size
- x = haplotype frequency

The non-parametric Wilcoxon signed rank one sample t-test from the SPLUS (Sportifire 2 + 8.2) statistical programme was used to test significant difference among the sample's

haplotype frequencies at P = 0.05 level of significance. The cluster dendrogram for samples was drawn using PyElph 1.4 software (Pavel and Vasile, 2012), which automatically extracts information from the RFLP gel images. Within the six steps that gradually leads to the desired results, a band position tolerance value of 4% was used to compensate for misalignment of homologous bands due to technical imperfections (Rim *et al.*, 2012). Finally the dendrogram was produced on the basis of the unweighted average pair group method (UPGMA). The dendrogram showed genetic distances between samples. For the RFLP of the H*sp*70 gene, the sizes of the DNA fragments were only estimated by comparison with a 1kb ladder. No further data processing was done whatsoever for the H*sp*70 RFLP marker.

4. RESULTS

4.1 Molecular analysis of the mtDNA fragments

4.1.1 DNA quality

The DNA extraction was successful because all the 10 replicates in every sample produced DNA. Only three replicates per sample are shown (Figure 4.1). Based on $A_{260/280}$ index, some samples showed high quality of DNA (Tanga, SFB, GSL and VC) while others had low quality (Fundisha, Ken1, Ken2 and Ken3) (Table 4.1). Values of $A_{260/280}$ indexes between 1.7 and 2.0 indicate the presence of pure DNA (Glasel, 1995).
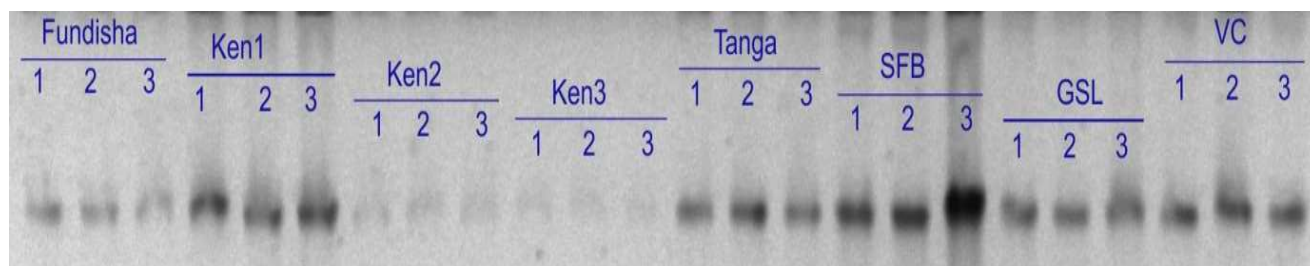
Figure 4.1: Example of agarose gel for un-amplified DNA fragments from single cysts from each of the 8 samples. The DNA was extracted from 10 individual cysts per sample (only 3 are shown). For abbreviations of samples, see table 3.1

Table 4.1: Average quantity of DNA extracted from individual cysts from each sample including ARC code. The DNA quantity was measured using a NanoDrop® ND-1000 machine. Values are mean ± SE. For abbreviations of samples, see table 3.1

Sample	ARC code	DNA (ng /µL)	$A_{260/280}$
Fundisha	1780	9.55 ± 0.69	2.12 ± 0.03
Ken1	1762	15.01 ± 0.43	1.59 ± 0.12
Ken2	1439	7.51 ± 0.45	3.04 ± 0.32
Ken3	1779	7.53 ± 0.63	2.99 ± 0.22
Tanga	1773	14.38 ± 0.70	1.99 ± 0.10
GSL	1768	27.99 ± 1.95	1.87 ± 0.02
SFB	1574	37.25 ± 1.26	1.92 ± 0.01
VC	1771	28.80 ± 1.75	2.01 ± 0.03

4.1.2 PCR amplification

4.1.2.1 The 2, 973 bp ND5F – CYTBR mtDNA fragment

Out of the 10 replicates in each sample examined, only a few replicates were positively amplified (Figure 4.2). This was due to PCR amplification inconsistencies of the 2,973 bp mtDNA fragment. Samples from Fundisha and Ken3 failed to amplify absolutely (Figure 4.2). Consequently, the idea to continue with this fragment was abandoned and much focus was dedicated to the 1,500 bp fragment instead.
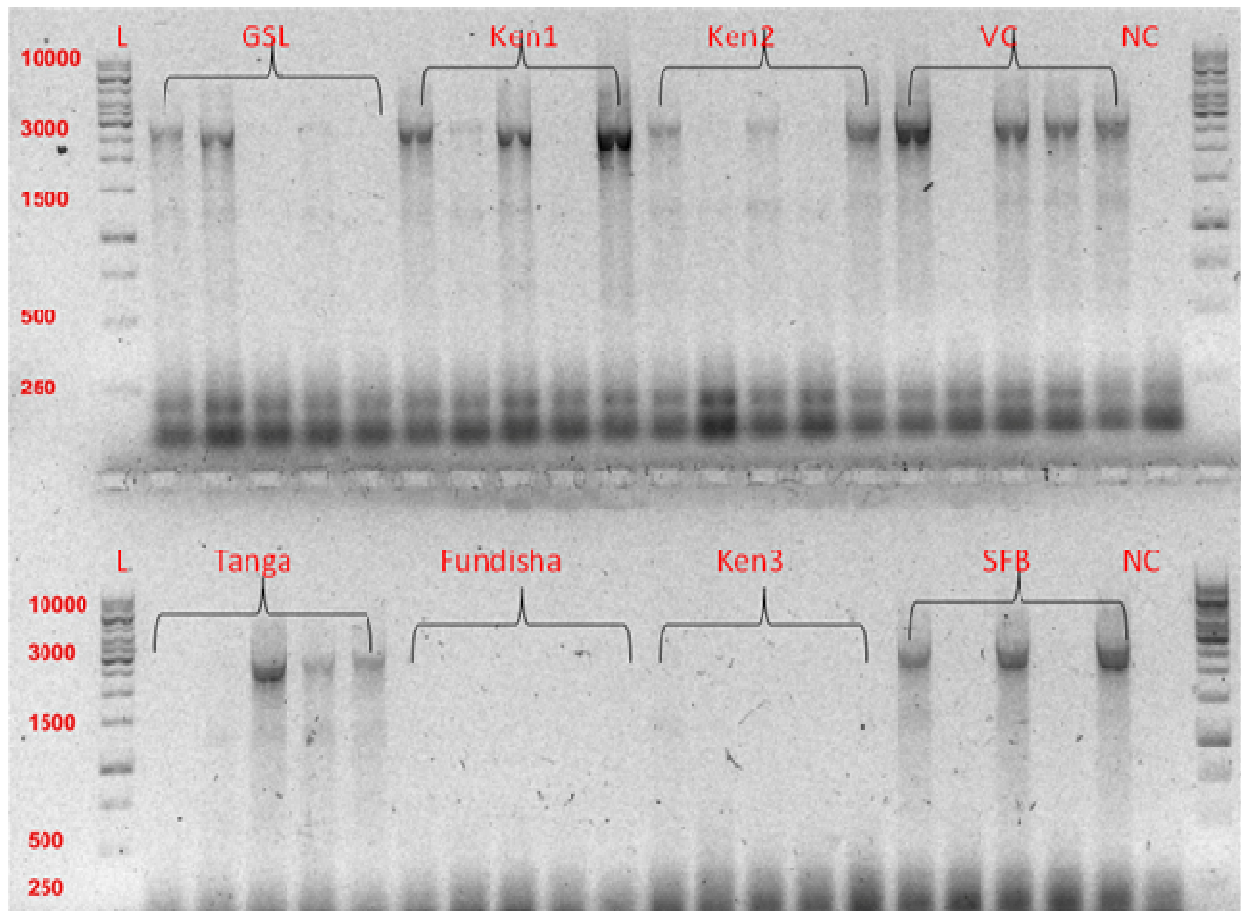
Figure 4.2: Example of agarose gel showing the inconsistent PCR-amplification of the 2,973 bp mtDNA fragment for single cyst replicates per sample. L: 10,000 bp ladder; NC: Negative control. For abbreviations of samples, see table 3.1

4.1.2.2 The 1,500 bp 12S -16S mtDNA fragment

The primer combinations produced identical 1500 bp fragments in all the 10 replicates in every sample analysed. Only one replicate per sample is shown (Figure 4.3)
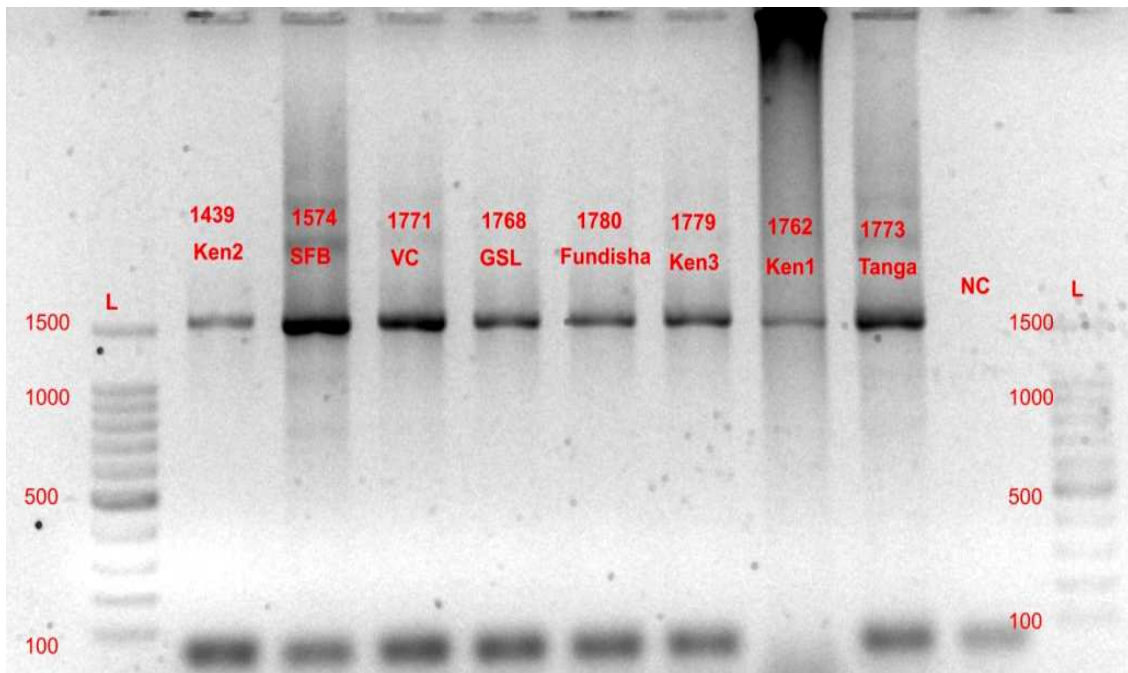
Figure 4.3: Example of agarose gel for PCR-amplified 1500 bp 12S – 16S mtDNA fragment for a single cyst per sample. L: 1500 bp ladder; NC: Negative control. For abbreviations of samples, see table 3.1

4.1.3 RFLP analysis of mtDNA

4.1.3.1 The 2, 973 bp ND5F – CYTBR mtDNA fragment

Restriction digestion was done on the few replicates, which amplified the 2,973 bp mtDNA fragment, using *Hae*III and *Hpa*II restriction endonucleases. This is because both *Hae*III and *Hpa*II recorded polymorphism in the 1,500 bp fragment (refer to 4.1.3.2 below). All the samples produced two fragments of approximately 2,400 bp and 500 bp when digested with *Hae*III (Figure 4.4). The *Hpa*II digestion produced two fragments each 1,500 bp except one replicate of GSL, which showed 2 fragments of approximately 1,550 and 1,500 bp (Figure 4.4).
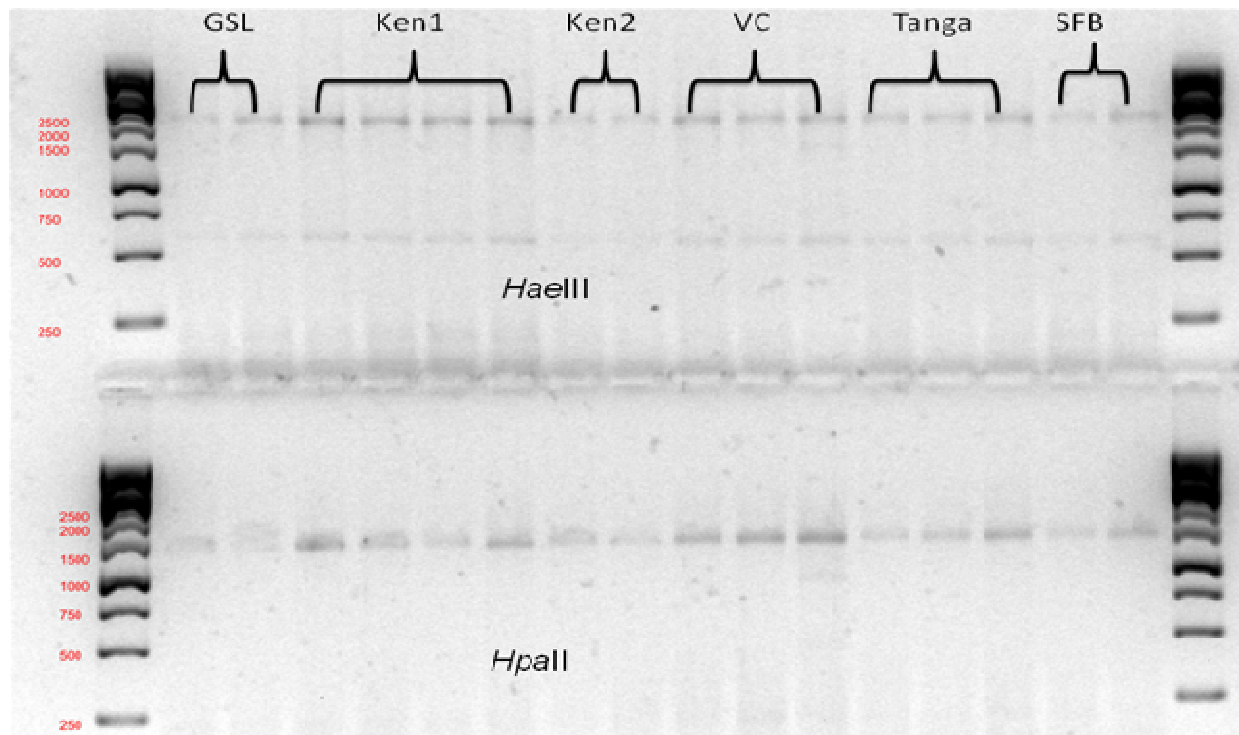
Figure 4.4: Agarose restriction fragment profile for RFLP of 2, 973 bp mtDNA fragment for a single cyst per sample using *Hae*III and *Hpa*II restriction enzymes. L: 10,000 bp ladder. For abbreviations of samples, see table 3.1

4.1.3.2 The 1500 bp 12S -16S mtDNA fragment

Seven cysts per sample were analyzed through electrophoresis. The enzymes *Hae*III and *Hpa*II detected polymorphism only in the Fundisha sample (Figure 4.5). The enzymes *Alu*I, *Xba*I, *Hinf*I and *Rsa*I detected a lack of polymorphism across all samples (Appendix). These enzymes displayed 4, 2, 2 and 4 fragments respectively (Appendix). In total, approximately 1,216 fragments were surveyed in the 12S - 16S mtDNA target sequence. Homologies of fragment patterns were established through side by side visual comparisons.

Three composite haplotypes were identified in the mtDNA target sequence. Cyst samples from Fundisha generated most haplotypes (three) while the rest of the samples were monomorphic (Table 4.2). The most common haplotype was AAAAAA, being detected in all the sample populations except GSL. Within Fundisha cysts this same haplotype attained the highest frequency (0.4286) among the three haplotypes displayed. There was one private haplotype in Fundisha (AAABBA) while one haplotype (AAAABA) was only shared between Fundisha and GSL (Table 4.2). The highest haplotype diversity (h) was recorded in Fundisha cyst samples (0.76 ± 0.07). This was higher than that of the originally inoculated SFB.
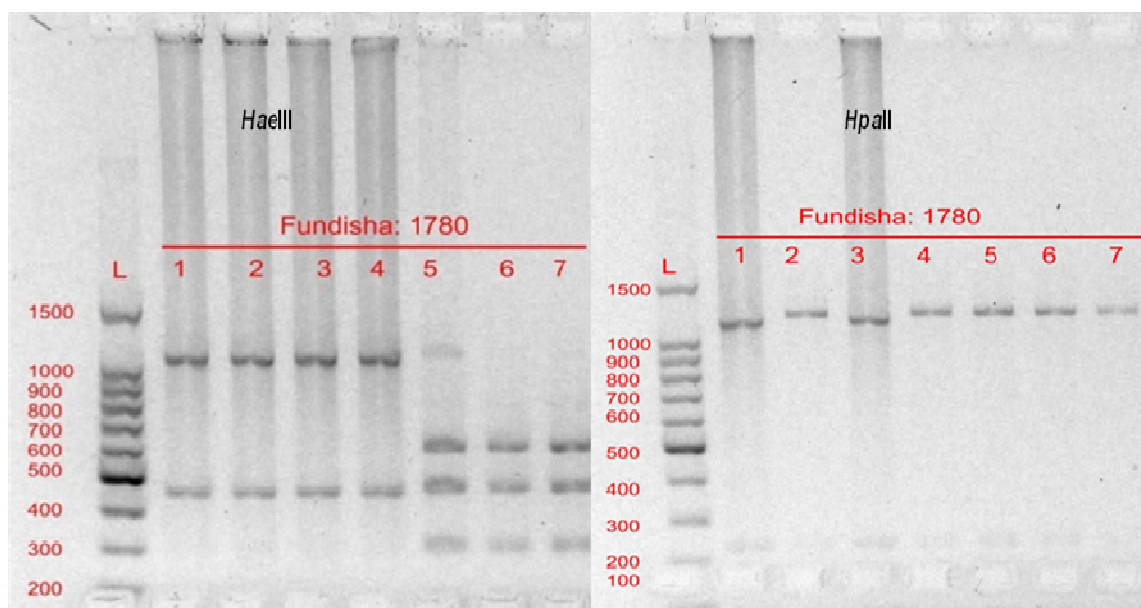
Figure 4.5: Example of agarose restriction fragment profile for the polymorphic *Hae*III and *Hpa*II enzymes on Fundisha individual cyst samples. PCR-amplified 1500 bp of 12S – 16S mtDNA fragment for 7 single cyst replicates per sample.

Table 4.2: Haplotype genotype frequencies, mean haplotype frequency (mhf), sample size, number of haplotypes (nh) and haplotype diversity (h) in samples. Haplotype genotypes are denoted with capital letters, each one corresponding to the restriction pattern obtained by a restriction enzyme in the following order; *Alu*I, *Xba*I, *Hinf*I, *Hpa*II, *Hae*III and *Rsa*I

Haplotype	Haplotype genotype	Samples								
		Fundisha	Ken1	Ken2	Ken3	Tanga	GSL	SFB	VC	mhf
H_1	AAAAAA	0.4286	1.0000	1.0000	1.0000	1.0000	0	1.0000	1.0000	0.80
H_2	AAAABA	0.2857	0	0	0	0	1.0000	0	0	0.16
H_3	AAABBA	0.2857	0	0	0	0	0	0	0	0.04
S. size		7	7	7	7	7	7	7	7	
nh		3	1	1	1	1	1	1	1	
h ± SD		0.76 ± 0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	

The dendogram, which was produced on the basis of the unweighted average pair group method (UPGMA), showed two major groups (GSL and SFB) while the Fundisha cyst samples appeared to be intermediate. The genetic distance between the samples was automatically computed by the software (Figure 4.6).
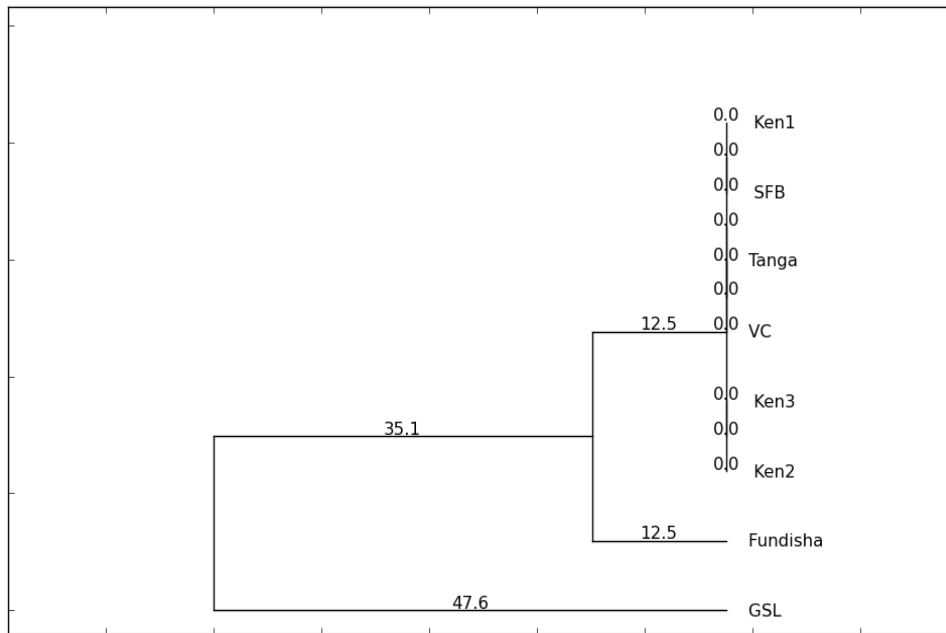
Figure 4.6: UPGMA dendrogram of Nei's genetic distance for 8 *Artemia franciscana* population samples. The values on the horizontal lines stand for Neis genetic distances in percentage. For abbreviations of samples, see table 3.1

4.2 Molecular analysis of the H*sp*70 gene fragment

The 1,935 bp H*sp*70 gene fragment, which was obtained from pooled *Artemia* cysts was successfully amplified, and after digestion with the four restriction enzymes (*Sau*3A, *Rsa*I, *Alu*I and *Hinf*I), a non-polymorphic pattern was found (Figure 4.8). Considering each enzyme, no genetic variations among the samples were observed in the H*sp*70 gene analysis except for the GSL samples, which was digested differently in 3 restriction enzymes (*Sau*3A, *Alu*I and *Hinf*I) (Figure 4.7). Interestingly, even restriction enzymes such as *Alu*I and *Sau*3A with 7 and 6 cleavage sites respectively on the 1,935 bp H*sp*70 gene fragment did not register any polymorphism in any of the samples. The *Artemia* cyst samples from Fundisha were poorly digested in all the restriction enzymes (Figure 4.7).
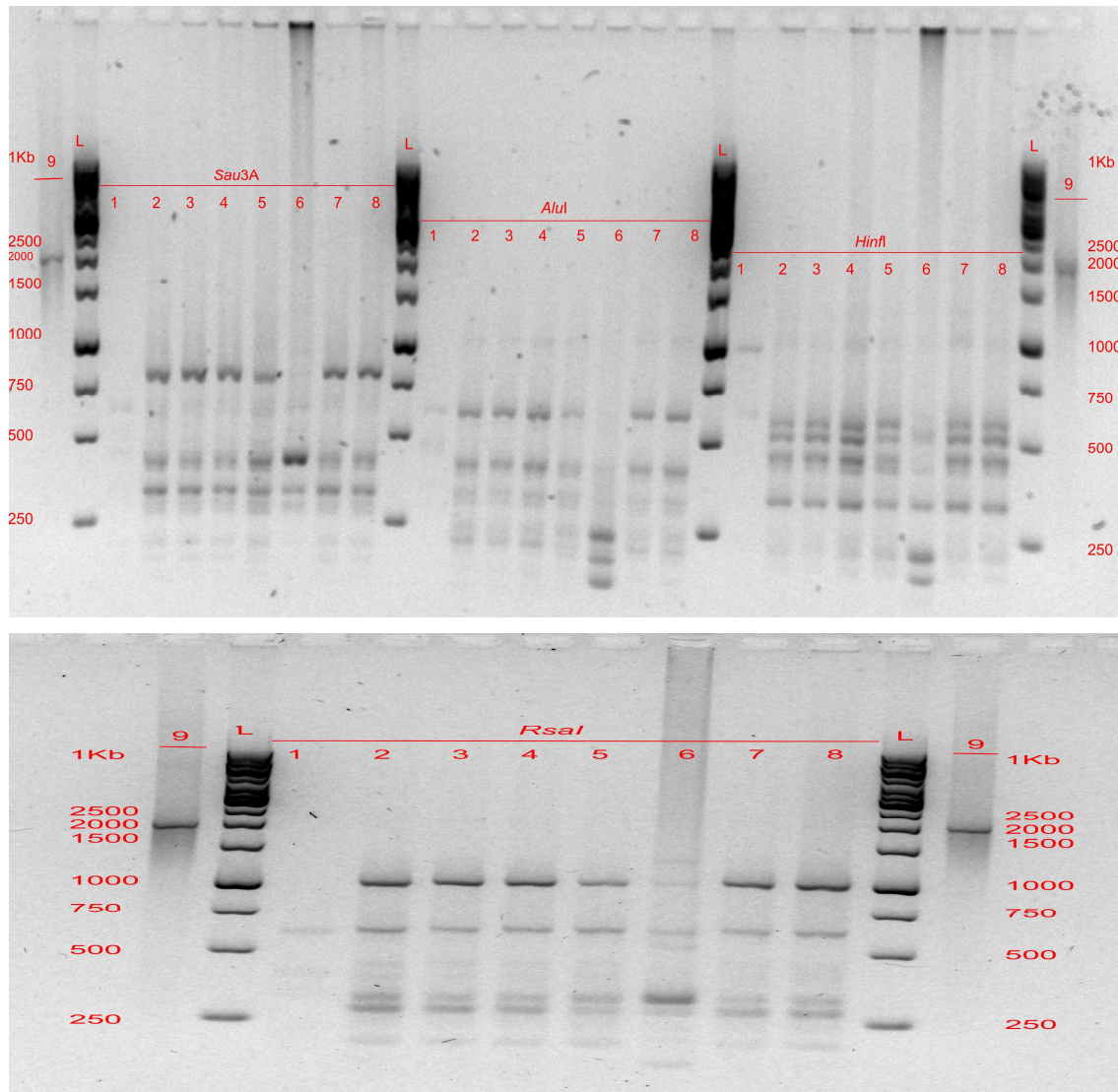
Figure 4.7: Agarose restriction fragment profile for the enzymes: *Sau*3A, *Alu*I, *Hinf*I and *Rsa*I. The PCR fragment was generated using DNA extracted from pooled Artemia cysts. L: 1Kb ladder, Lanes 1: Fundisha, 2: Ken1, 3: Ken2, 4: Ken3, 5: Tanga, 6: GSL, 7: SFB, 8: VC, 9: Undigested PCR product (2, 000 bp) control. For abbreviations of samples, see table 3.1

5. DISCUSSION

5.1 DNA quality and PCR amplification

The quantity and quality of DNA extracted varied among the cyst samples. All cyst samples from the Kenyan coast produced poor quality of DNA according to the $A_{260/280}$ indexes (Table 4.1). This could be related to harvesting, processing and storage conditions of the cysts before shipment to ARC. Pure DNA samples are expected to have $A_{260/280}$ ratios between 1.7 and 2.0 but abnormal $A_{260/280}$ ratios may result from contamination by proteins or reagents such as phenol during the DNA extraction process (Glasel, 1995). It is however understood that best DNA quality can be obtained from adult *Artemia* samples. However, preference was given to *Artemia* cysts because they act like gene banks that store the historical genetic information (Gajardo and Beardmore, 2012). Moreover, the single cyst approach was preferred in the mtDNA analysis to allow assessment of the species homogeneity of each sample and also to avoid genes loss due to selective hatching (Van Stappen, 2008).

Despite several attempts to optimise PCR conditions and to standardize template DNA for amplification of the 2,973 bp fragment of mtDNA, only few replicates were positively amplified. It was not possible to determine the exact cause of the amplification failures in some of the replicates. The amplification of the 1,500 bp 12S -16S mtDNA fragment was successful in all the replicates analysed and therefore the main RFLP procedure was based on the 1,500 bp fragments.

5.2 Genetic divergence of Kenyan *Artemia* populations from their SFB ancestors

5.2.1 Lack of genetic differentiation in Kenyan Kensalt *Artemia* populations

The present study analysed mitochondrial DNA (Bossier *et al.*, 2004; Ruiz *et al.*, 2008) using the RFLP tool to detect polymorphism in *A. franciscana* using DNA extracted from individual cyst samples harvested from Kenya while cysts from SFB, GSL, VC were controls. According to the results of this study, the RFLP fingerprints based on the 2,973 and 1,500 bp fragments of mtDNA did not show any genetic polymorphism in the *Artemia* cyst samples from Kensalt salt works including the control samples (VC, SFB and GSL). The RFLP pattern obtained from the VC - *Hae*III digestion of the 2,973 bp fragment (Figure 4.4) showed similar results to the findings of Kappas *et al.* (2004), which also recorded 2 fragments of approximately 2,400 bp and 500 bp long. This was probably the point of connection between the current study and that of Kappas *et al.* (2004).

The absence of genetic distance as shown by the UPGMA dendrogram further indicated lack of genetic differentiation between the samples analysed in this study. In view of this, lack of *Artemia* genetic variability within the populations should be a major concern for Kenyan aquaculture scientists because maintaining genetic variability is important in any successful inoculation process in Kensalt saltworks. However, the current study might not have sufficiently assessed the intra-population diversity per sample due to limited tools applied. In the absence of genetic diversity, all the *Artemia* populations may be threatened at the slightest provocation of any genetic bottleneck. Despite the presence of ecological processes such as migration, researchers have reported that even though water bird, wind and human driven cyst dispersal can cause high *Artemia* population heterogeneity in the habitat, generally limited effective gene flow is observed (Gajardo *et al*., 1995; Munoz *et al*., 2009; Hajirostamloo, 2009). This argument is supported by the Naihong *et al*. (2000) general hypothesis that effective gene flow is much slower compared to the process of dispersal. This is because of the low amount of *Artemia* cysts transported by birds (Green *et al*., 2005). Moreover, the different ecological conditions (temperature, salinity and food availability) can prevent *Artemia* nauplii survival even if the cysts manage to hatch in the natural conditions. In addition, Beristain *et al*. (2010) reported that the process of assortative mating can prevent intercrossing even among coexisting *Artemia* species and this reduces chances of speciation.

The absence of genetic polymorphism within samples could partly be attributed to the limited 1,500 bp fragment of mtDNA analysed. Palumbi (1996) reported that mitochondrial rDNA polymorphism being common at the genus or species level, it should be possible to differentiate samples at the species or sub-species level. Kappas *et al*. (2004) used a larger mtDNA fragment of 2,973 bp and detected significant genetic polymorphism within the *A. franciscana* introduced in Vietnam almost 2 decades ago. Inoculation in Vietnam and Kenya happened almost at the same time and the fact that climatic conditions are approximately the same; one would expect the Kenyan *Artemia* population to have undergone substantial genetic differentiation as well. The larger fragment has high chance of showing detailed microevolutionary changes that might not be detected in a limited DNA fragment such as 1,500 bp, which was used in the current study.

The range of environmental conditions and indeed the climatic conditions are critical factors that may influence the *Artemia* population patterns (Evjemo and Olsen, 1999). This may also have a bearing on the *Artemia* genetic expression in their different habitats (Lenz and Browne, 1991; Van Stappen, 2002). In Kenya, integrated salt - *Artemia* culture is a continuous process where *Artemia* flourishes year round, while in other places such as

Vietnam, the saltworks are predictably sequential, which favours a faster evolution of VC *Artemia* strain. Manaffar (2012) reported that genetic drift in the presence of limited gene flow facilitates the speciation process. Therefore, the absence of periodical genetic bottlenecks in the Kenyan situation suggests that the natural selection process is responsible for gene loss. However, natural selection is a slow process and a substantial amount of time may be needed to cause meaningful genetic divergence (Gonzalo and Beardmore, 2012). According to Lenz (1987), permanence and seasonality of the environment are key instruments driving considerable genetic differentiation of *Artemia* in their biological communities. Michels *et al.*, (2001) documented the effects of permanence and seasonality of the habitat on population genetic structure of local populations of the cladoceran *Daphnia ambigua*. In particular they observed that the zooplankton in temporary pools require specific adaptations to deal with variable and often extreme local environmental conditions. This leads to very specific biota with definite genetic structures (Wiggins *et al.*, 1980). It is therefore prudent to argue that the monomorphic genetic structure of the Kensalt *Artemia* could be related to the permanence of the culture system as currently practiced. Interestingly, Wear and Haslett (1986) and Wear *et al.* (1986) attributed the exclusive ovoviviparity of the New Zealand *A. franciscana* population (inoculated in 1950s) to the genetic differentiation caused by constant year-round salinity and temperature conditions. A factor such as high UV radiation has also been linked to driving genetic evolutionary changes in *Artemia* populations since it increases mutation rates (Hebert *et al.*, 2002). Food availability under the form of primary production has an influence on *Artemia* population dynamics and perhaps their genetic expression (Manaffar, 2012). Research interests should be focused on ecological aspects including specific food items such as phytoplankton and bacteria community that form the basis of Kenyan Kensalt *Artemia* environments.

Finally, the RFLP technique may not have been robust enough to detect intra-specific polymorphism as eluded by Bossier *et al.* (2004) and Avise (2004). Using the mtDNA-RFLP tool to discriminate between populations below the species level may only work for some species but not others (Bossier *et al.*, 2004). Similarly, Avise (2004) insisted that an informative genetic tool should be able to reveal inter- and intra-specific genetic variations existing in any given *Artemia* populations. Therefore, if indeed there was genetic differentiation between Kenyan (Kensalt) *Artemia* and the original SFB material, then the tools used in the study (mtDNA-RFLP fragment and the Restriction enzymes) were not sufficiently adequate to detect this differentiation.

The presence of a common haplotype in Kensalt saltworks and SFB *Artemia* cyst samples confirmed the historical information that only SFB *Artemia* strain is present in the Kensalt farm. Studies of Bossier *et al.* (2004) to authenticate global *Artemia* cyst samples also ranked the Kenyan Kensalt *Artemia* samples with the *A. franciscana* group. This observation has further confirmed that the reporting of Rasowo and Radull (1986) about the inoculation of *A. salina* in Fundisha salt works in Kenya was erroneous.

The fact that the single haplotype identified in Tanga samples was similar to Kensalt and Fundisha samples can only confirm the speculations that the *Artemia* populations currently present in the castal zones of Tanga, Tanzania might have originated from Kenya or SFB. This must have occurred through human inoculations (Treece, 2000) because Kensalt farm is operating another salt farm in Tanga, also called Kensalt (verbal communication B. Nyonje and M. Mukami, KFMRI). It is also possible that waterbirds could transfer cysts from the Kenyan side to the Tanzanian side given that Tanga is only 130 km away from the Kenyan coastline.

5.2.2 Genetic differentiation in the Kenyan Fundisha *Artemia* populations

Based on the RFLP fingerprint pattern and the number of haplotype genotypes obtained in this study, only the *Artemia* population in Fundisha saltworks was polymorphic. This was illustrated by the occurrence of 3 haplotype genotypes. However, there was no significant difference between the haplotype genotype frequencies in Fundisha saltworks ($P > 0.05$). The presence of a private haplotype (AAABBA) in Fundisha cyst samples could be an indication of a systematic genetic differentiation within the *Artemia* populations in Fundisha, pointing to a possibility of molecular evidence of an existing subpopulation and genetic divergence from their SFB ancestors. According to Manaffar (2012), subpopulations are established through adaptive mtDNA mutations. Then further mutations occur at the nuclear DNA level too and this allows physiological adaptation before causing final speciation of the organism in question (Mishmar *et al.*, 2006). Hajirostamloo and Pourrabbi (2011) reported that *Artemia* strain identification based on genetic composition is important in understanding their molecular ecology. The population- specific haplotype identified in Fundisha saltwork may become useful in monitoring the geographic expansion of the *Artemia* populations along the Kenyan coast. However, further studies using more robust genetic tools like AFLP and microsatellites are needed as the results of this study may not be absolutely conclusive. Fundisha's haplotype gene diversity (0.76) and the one of the original SFB (0.0) may suggest genetic divergence between the two samples. If this observation was true, one would ask why the genetic structure of Fundisha *Artemia* would diverge but not Kensalt *Artemia.* More habitat ecological studies could be helpful to understand and unravel such

unique scientific observations. It is important to recall that Fundisha saltwork is smaller in size (1,020 ha) than Kensalt saltwork (3,000 ha). This observation contrasted with that of Kappas *et al.* (2004), who noted that the locally adapted VC strain had lost haplotypes (haplotype diversity 0.11) compared to the original SFB (0.406). However, it is important to remember that Kappas *et al.* (2004) observation was based on analysis of a much larger mtDNA fragment (2,973 bp) but the current study focused on a limited 1,500 bp mtDNA fragment. According to Kappas *et al.* (2004), limited variation of the mtDNA diversity can be caused by mutation drift equilibrium rather than by a long process of selection. Therefore, further studies using more robust molecular tools including nuclear DNA analysis and sequencing, which can detect fine mitochondrial haplotype differences, are recommended to authenticate the occurrence of the present study.

The fact that the haplotype AAAABA was shared between Fundisha and GSL cysts samples while haplotype AAAAAA was also shared by Fundisha and SFB could be molecular evidence that both SFB and GSL *Artemia* strains co-exist in Fundisha saltwork (consult table 4.2). The inoculation history indicated that both SFB and GSL *Artemia* strains were introduced in Fundisha salt work in mid 1980s and 2009 respectively. Having been introduced in 2009, the GSL populations have since then been regularly re-inoculated in the saltwork and have co-existed with their SFB counterparts in the new Kenyan environment. This finding is consistent with the studies of Van Stappen (2002), who documented that coexistence of different *Artemia* strains or species within the same site is a common scientific possibility. The dominant *Artemia* strain and level of cross breeding between the two strains could be an interesting future study as that was beyond the scope of the current study. SFB and GSL being basically same species (*A. franciscana*), they can freely interbreed to produce viable off springs.

5.3 *Artemia* H*sp*70 gene differentiation

Based on the literature reports on the importance of the heat shock protein family, there is no doubt that the family of stress proteins plays significant roles in the heat resistance of a wide variety of organisms (Feder and Hofman, 1999; Clegg *et al.*, 1999, 2001). The H*sp*70 RFLP fingerprint pattern did not show variation, which suggested that, the Hsp70 gene was similar in all the *Artemia* populations analysed. This observation was similar to that of Feder and Hofmann (1999) who reported that little variation in the H*sp* could be due to the fact that the heat shock protein is evolutionary and functionally conserved. Similar results were also provisionally observed by Clegg and Gajardo (2009) for three stress proteins (p26, artemin and H*sp*27). The result of this study was rather surprising because studies of Kapinga (2012) and Mremi (2011) suggested that the Kenyan (Kensalt) *Artemia* populations were

superior to their SFB ancestors in terms of thermal tolerance. It was then expected that unique H*sp*70 gene signatures would be identified in the Kenyan *Artemia* samples to explain the reproductive characteristics at high temperature observed by the previous authors. Factors other than the H*sp*70 profile and temperature could be involved in the observed adaptations in the Kenyan case. Indeed, Browne and Wanigesekera (2000) reported that animals in the culture ponds may respond to many other different stresses in addition to temperature and salinity.

In *Artemia* studies, Clegg *et al.* (2001) proved that organism's thermal tolerance is directly proportional to the stress protein content. Nevertheless, the current study did not focus to quantify the H*sp*70 gene in cyst samples analysed as was the case in Clegg *et al.* (2001). Future studies should therefore focus on the more quantitative technological ways of H*sp* 70 analysis such as western blot by chemiluminescence techniques (Schutz-Geschwender *et al.*, 2004). If H*sp*70 polymorphism exists in the samples analysed, then it may be assumed that the RFLP tool was not powerful enough to detect any special signatures in this case. However, the GSL and Fundisha *Artemia* samples showed a different RFLP fingerprint from the rest (Figure 4.7). This was similar to mtDNA analysis, which separated GSL from the SFB strains. Clegg *et al.* (1999) believed that the increase in thermal resistance of the VC cysts compared to the original SFB inoculants involved an elevated level of H*sp*26. However, *Artemia* cysts also contain substantial amounts of other compounds e.g. trehalose (Clegg and Conte, 1980), which are known for their stabilizing properties (Crowe *et al.*, 1992).

6. Conclusions and recommendations

Based on the results of the current study, the mtDNA sequence analysis has provided some diagnostic power in comparing SFB, GSL and Kenyan *Artemia* strains. Even though the genetic differentiation of the Kenyan Fundisha *Artemia* population from its SFB ancestors is not statistically significant, the presence of a private haplotype genotype (AAABBA) discovered in Fundisha saltwork could be the beginning of a long term micro-evolutionary process, which could lead to eventual geographic differentiation and progressive speciation of *A. franciscana* in the new Kenyan environment. It may also help to explore and monitor future geographic expansion of the Fundisha saltworks *Artemia* population. The genetic structure of the Kenyan Kensalt *Artemia* population was absolutely monomorphic, similar to its original SFB inoculants, suggesting that no other *Artemia* strain or species contamination has occurred there apart from SFB. In addition, the similarity of the mtDNA genetic pattern of the Kensalt *Artemia* population and the one currently observed in Tanga, Tanzania, proved that the Tanga *Artemia* population must have originated from Kenyan Kensalt saltworks or SFB. Furthermore, this study found molecular evidence of co-existence of SFB and GSL *Artemia* strains in Fundisha saltworks. The absence of unique H*sp* 70 gene signatures in the RFLP fingerprint suggested that other factors other than the H*sp*70 family could be involved in the much cited thermotolerance superiority of the Kenyan *Artemia* populations compared to their SFB ancestors. Finally, the study concludes that if indeed there was significant mtDNA and H*sp*70 genetic differentiation between Kenyan *Artemia* and the original SFB material, then the tools used in the current study were not sufficiently adequate to detect this micro-evolutionary differentiation.

The study recommends that a similar study should be done on *Artemia* cyst samples using more robust molecular markers targeting larger mtDNA fragment and probably other mitochondrial regions such as the D-loop. Integration of *Artemia* lifespan traits with more ecological information and the molecular studies should provide a more comprehensive interpretation as far as genetic characterization of Kenyan *Artemia* population is concerned. More emphasis should be exerted on the evolutionary and ecological aspects of the heat shock protein family and stress response rather than on the technical cellular and molecular aspects (Clegg *et al*., 1999). A more quantitative approach such as western blot by chemouminescence method to study the H*sp* family would be more relevant than the current qualitative RFLP technique.

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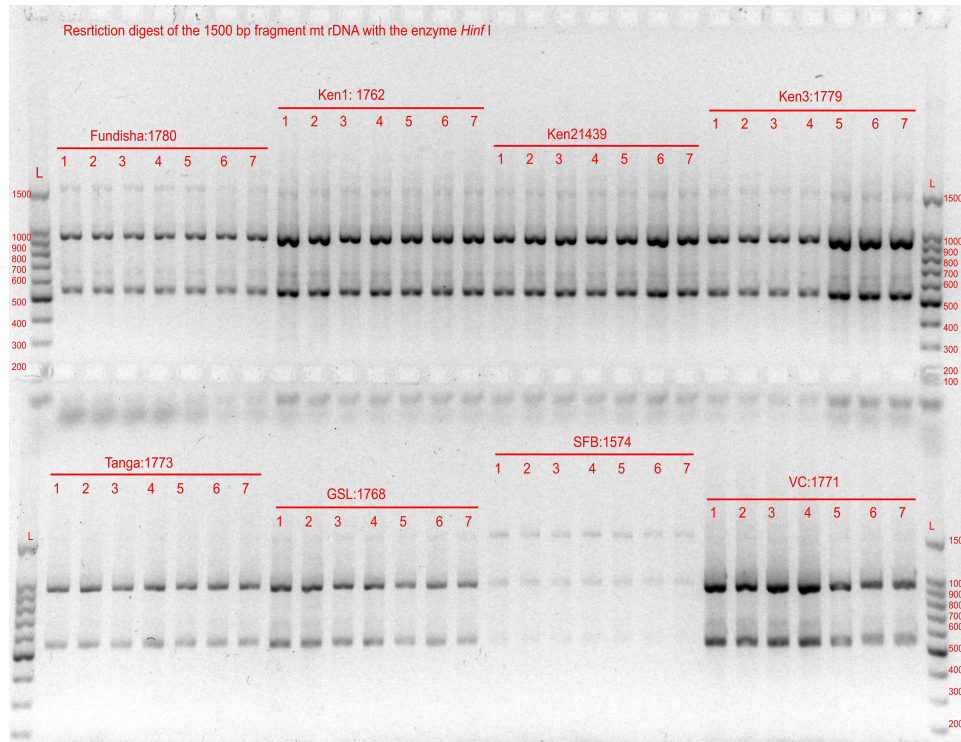
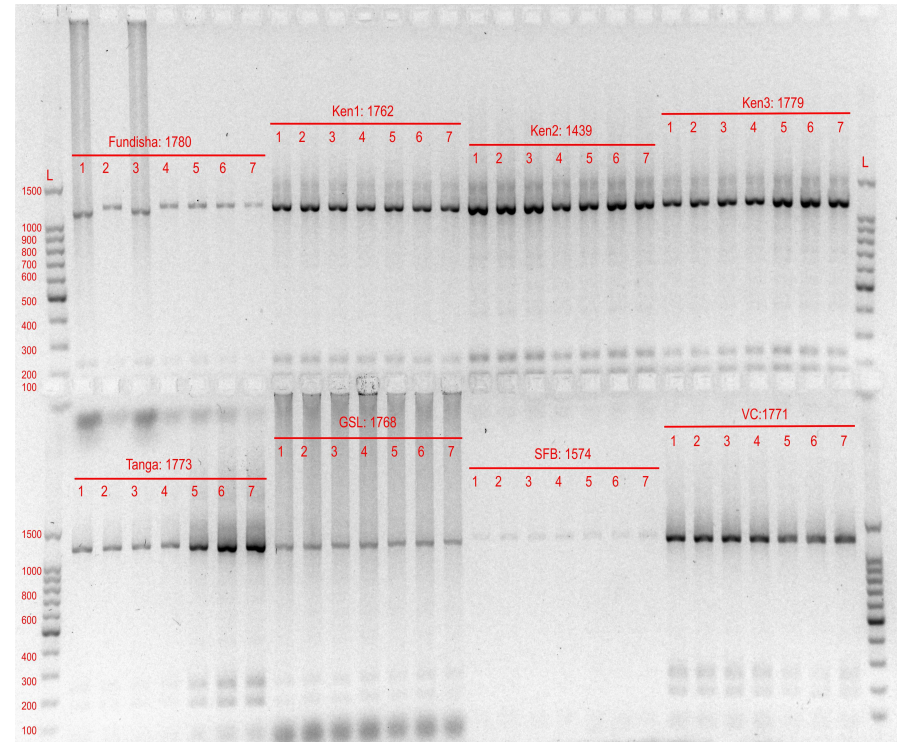## 8. APPENDIX

Appendix 1: Gel for *Alu*I digestion
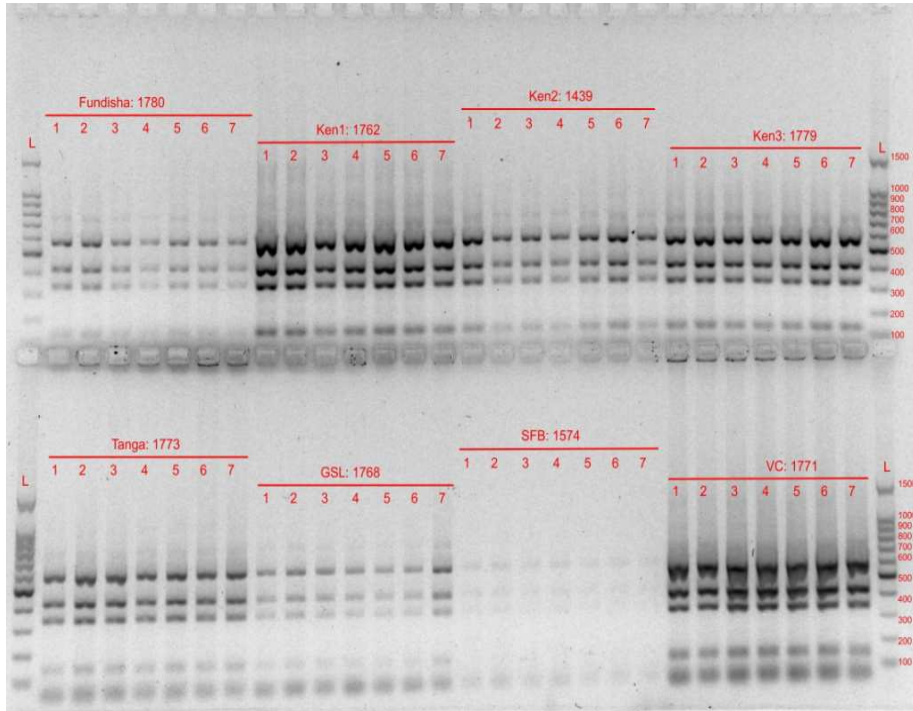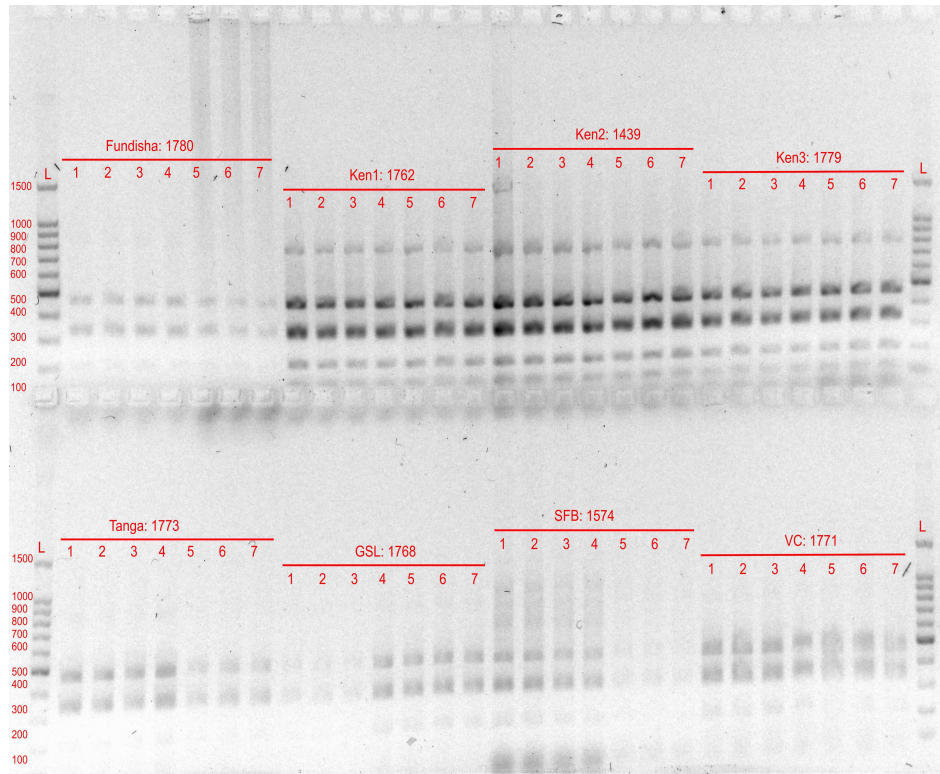
Appendix 2: Gel for *Hae*III digestion
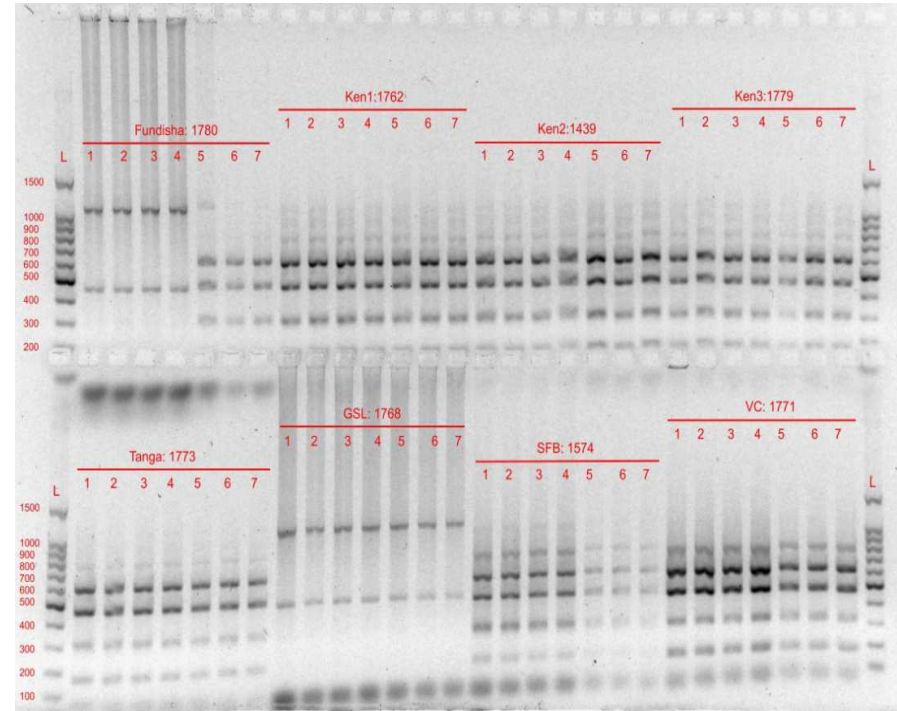
Appendix 3: Gel for *Hinf*I digestion

Resrtiction digest of the 1500 bp fragment mt rDNA with the enzyme *Hinf* I

Appendix 4: Gel for *Hpa*II digestion

Appendix 5: Gel for *Rsa*I digestion

Appendix 6: Gel for *Hpa*II digestion