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RESEARCH ARTICLE

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

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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 two decades ago were investigated using the mitochondrial DNA (mtDNA) and heat shock protein 70 (Hsp70) gene molecular markers. The DNA was extracted from 80 single Artemia cysts using the Chelex protocol. The 1,500 bp fragment of the 12S - 16S region of the mtDNA and a 1,935 bp fragment of the Hsp70 gene were amplified through Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) digestion using appropriate endonucleases. The mtDNA analysis indicated higher haplotype diversity (0.76 ± 0.07) in Artemia from Fundisha saltworks while the rest of the samples were monomorphic. A private haplotype (AAABBA) in Fundisha samples confirmed a molecular evidence of a systematic genetic differentiation albeit in an insignificant manner ($P > 0.05$). There was molecular evidence of coexistence of SFB and GSL Artemia strains in Fundisha saltworks. The monomorphic DNA fingerprint in Kensalt Artemia cysts was probably caused by non-sequential Artemia culture system and limited mtDNA fragment size analysed. The Hsp70 gene RFLP fingerprint did not show any unique gene signatures in the Kenyan Artemia samples suggesting that other factors other than Hsp70 were involved in their superior thermotolerance. Further genetical studies based on the larger mtDNA fragment using robust genetic markers are recommended. Ecological studies of the heat shock protein family and the stress response would be more relevant than the qualitative RFLP technique.

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Introduction

The brine shrimps Artemia are small crustaceans adapted to live in stressful environmental conditions of hypersaline habitats such as salt lakes, coastal lagoons and solar saltworks, where they feed primarily on phytoplankton and bacteria (Persone and Sorgeloos, 1980; 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). Artemia adaptation to these 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). 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 paper. The most discussed reproductive adaptation mechanism of the genus Artemia is the existence of two distinctly short cycles of development (Clegg et al., 2004; Kappas et al., 2004). During favourable environmental

conditions, an ovoviviparous reproduction cycle occurs where the adult females produce the free swimming naupli (Anderson et al., 1970). However, during stressing environmental conditions, an oviparous reproductive cycle prevails and the adult female Artemia produces metabolically inactive cysts as the parental animals dies (Dutrieu, 1960; Van Stappen, 1996). When conducive environmental conditions return, the cysts hatch into free swimming nauplii in a process that lasts for about 20 hours, thus completing the cycle (Pearson and Sorgeloos, 1980; Van Stappen, 1996).

The ability of the brine shrimp Artemia to inhabit hypersaline environments gives them a wide global geographical representation (Persoone and Sorgeloos, 1980). In fact, as Triantaphyllidis et al. (1998) put it; the only place where Artemia cannot be found is Antarctica. So far, discrete Artemia populations have been identified in about 600 natural salt lakes and saltworks and further survey efforts are still on course to identify more Artemia biotopes all over the world (Van Stappen, 2002).

For a long time, Artemia morphometric features have been used to discriminate between different populations despite many human errors (Naceur et al., 2010). Today, Artemia phylogeny can be easily verified and cyst samples scientifically authenticated thanks to molecular techniques (Bossier et al., 2004; Van Stappen, 2008). According to Avise (2004), molecular techniques provide full access to unlimited pool of organism's genetic variability. 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, Hsp26, COI, 12S and 16S mtDNA (Perez et al., 1994; Hou et al., 2006) and tools such as Restriction Fragment Length Polymorphism (RFLP) (Bossier et al., 2004; Gajardo et al., 2004; Eimanifar et al., 2006), Random Amplified Polymorphic DNA (RAPD) (Sun et al.,1999a; Camargo et al., 2002), Amplified Fragment Length Polymorphism (AFLP) (Sun et al., 1999b) using either single or pooled individuals or cysts samples (Kappas et al., 2004). Other tools include microsatellites and Single Strand Conformation Polymorphism (SSCP) (Blouin et al., 1996).

In the RFLP molecular technique, the targeted DNA genome size is first PCR amplified before digestion with restriction enzymes. The digested product is then separated according to their size by agaorose gel electrophoresis (Eimanifar et al., 2006). The mitochondrial genome of A. franciscana is estimated to be 15,822 nucleotides long (Valverde et al., 1994). The mtDNA is highly conserved compared to nuclear DNA, making it a robust marker for tracking animals' ancestry (Krieg et al., 2000). Eimanifar et al. (2006) found genetic nucleotide divergence within Artemia populations found in different ecological zones of Lake Urmia using the RFLP method. Agh et al. (2009) showed that bisexual A. urmiana and parthenogenetic populations in Iran are genetically close based on RFLP of their 1,500 bp mtDNA fragment. Manaffar (2012) also conducted an RFLP analysis of the 1,500 bp mtDNA fragment on A. urmiana cysts and detected high polymorphism among cysts from different stations in Urmia Lake. Through RFLP analysis of a 1,500 bp mitochondrial rDNA fragment, Bossier et al. (2004) developed a methodology to authenticate Artemia cyst samples. Kappas et al. (2004) investigated how A. franciscana native to SFB colonised unfamiliar Vietnam environments through RFLP technique based on the 2,963 bp long mtDNA target sequence. Unique genetic signatures were observed in the mtDNA genome of the Vietnam Artemia strain suggesting a process of strong selective pressure in them (Kappas et al., 2004).

There is much information regarding Artemia's ability to synthesize heat shock proteins, such as Hsp26 and Hsp70 (Clegg et al., 2001; Crack et al., 2002; Willsie and Clegg, 2002). Artemia cysts contain substantial amounts of heat shock proteins because they are the surviving agents in stressful environments (Clegg et al., 1999; Van Stappen, 2002). Scientific evidence has proven that the family of heat shock proteins are critical for thermal resistance (Frankenberg et al., 2000), desiccation tolerance (Ma et al., 2005) and reduces osmotic stress (DuBeau et al., 1998; Todgham et al., 2005). Therefore Hsp70 protects organisms against multidimensional environmental challenges. Clegg et al. (2001) found that Artemia cysts produced in hotter environments contain higher amounts of heat shock proteins such as artemin, p26 and Hsp70. Therefore, the 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.

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 permanently colonised the Kenyan coast, where about eight saltworks exist today. Since 2009, Fundisha saltworks has been re-inoculated using GSL Artemia strains suggesting coexistence of GSL and SFB Artemia strains. This is a subject that can only be revealed through molecular studies. 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. To date, it is not known whether SFB and GSL Artemia strains coexist in Fundisha saltworks. Neither do we know the genetic micro-evolutionary divergences that have occurred in the Kenyan Artemia population. Artemia population have also been discovered in Tanga region (Tanzania). However, no scientific information is available about them. The

polymorphic analysis of the 1,935 bpHsp70 gene was considered to add more perspective on the much anticipated genetic adaptation levels of the Kenyan Artemia populations. The hypothesis of this study was that the genetic pattern of the Kenyan Artemia strains would be mutually polymorphic. The present laboratory based study aimed to genetically characterize the Kenyan Artemia cysts based on the mitochondrial DNA and heat shock protein 70 (Hsp70) genes. The study also determined the purity of Artemia populations in Kensalt and Fundisha saltworks and established the genetic relationships between the Kenyan and Tanga (Tanzania) Artemia cysts.

Materials and methods

Source of Artemia cyst samples and study area

A total of 80 individual Artemia cysts, 10 replicates from each of 8 samples were used in 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, located at located at 3° 50' 0" South, 39° 46' 0" East (Study area Fig. 1). One sample from Great Salt Lake, Utah state in USA (GSL), another sample from San Francisco Bay (SFB) USA and one sample from Vinh Chau (VC), Vietnam, were used as controls. Artemia samples from Tanga (Tanzania) were also analysed as additional study. 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. The selection criteria for the Kenyan Artemia cyst followed Nyonje (2011) report. Cysts from Kensalt farm (Ken1, Ken2, Ken3), which were different batches of the same population, were considered because of their known reproductive characteristics (Mremi, 2011; Kapinga 2012) while the much hypothesised coexistence of SFB and GSL Artemia strains in Fundisha saltwork was the reason for considering cysts from there. The use of cysts for DNA extraction for mtDNA and Hsp70 analysis were preferred to individual Artemia adults to prevent loss of genetic information due to selective hatching (Van stappen 2008).

DNA extraction

DNA was extracted from single Artemia cysts using the Chelex method (Walsh et al., 1991). The 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) before adding 30 µL of well homogenized 10% Chelex slurry (Chelex-100 - Biorad, Belgium). The samples were vortexed for 10 - 15 s before spinning for 1 min at 13,000 rpm in a micro-centrifuge. The samples were incubated for 20 min at 95°C, vortexed again for 10 - 15 s and further spanned at 13,000 rpm for 1 min. The quantity of the extracted DNA was measured using a NanoDrop® ND-1000 machine while the quality of the DNA was verified through agarose gel electrophoresis (Lind et al., 2006).

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

The double stranded DNA amplification was performed in 50 µL reaction volumes each 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 PCR amplified for the 1,500 bp mtDNA fragment between the 12S - 16S region (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 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; 2) an annealing phase at 52°C for 45 seconds; 3) an elongation phase at 72°C for 2 min and the final elongation cycle step at 72°C for 4 minutes.

PCR amplification of the Hsp70 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 Hsp 70 gene fragment (Baruah et al., 2010). A combination of the forward primer $Hsp70_{forward}$ (5'-cac-cat-ggc-aaa-ggc-acc-agc-aat-agg-3') and the reverse primer $Hsp70_{reverse}$ (5'-ata-gtt-ggg-cca-ctg-cct-gtt-cca-g-3') were used (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.

Restriction digestion: RFLP procedure

The amplified 1,500 bp mtDNA fragments were screened for polymorphism using six restriction endonucleases (AluI, HaeIII, HinfI, RsaI, XbaI and HpaII) (Bossier et al., 2004; Kappas et al., 2004). The reactions were done according the manufacturer's instructions (see Tab. 1). For each reaction tube, a 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 voltage of 100 V was used to push the digested DNA fragments through the solidified agarose gel for 1 h. A 100 bp promega DNA ladder was loaded as reference. A UV transilluminator was used to visualise the fragments and photographed with a digital camera (Canon power shot G10).

For the Hsp 70 gene, restriction enzymes were selected based on the number of cleavage sites in the 1,935 bp fragment of the Artemia franciscana nucleotide sequence (cDNA). Four restriction enzymes (Sau3A, Rsal, AluI and HinfI) with recognition sequences GATC, GTAC, AGCT and GAATC respectively were used. 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. The incubation temperature was 37°C while inactivation temperature was 65°C for 20 min for Sau3A enzyme. Gel-electrophoresis was as explained above but 1kb promega DNA ladder was loaded as reference. The homologies of fragment patterns were established through side by side visual comparisons for both mtDNA and Hsp70 gene.

Data analysis

The RFLP restriction pattern fragments were manually scored. Fragments less than 100 bp were neglected 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 (Nei, 1978). The mean haplotype frequency was calculated by adding all the haplotype frequency in each haplotype then dividing by the total number of samples (Nei, 1987). The haplotype diversity within samples was calculated based on Nei and Tajima's (1981) formula.

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

Where: H = haplotype diversity; N = Sample size; x = haplotype frequency

The non-parametric Wilcoxon signed rank one sample t-test of 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) based on the unweighted average pair group method (UPGMA). For the RFLP of the Hsp70 gene, the sizes of the fragments were only estimated by comparison with a 1kb ladder. No further data processing was done whatsoever for the Hsp70 RFLP marker.

Results

Based on A $_{260 / 280}$ index, some samples showed high quality DNA (Tanga, SFB, GSL and VC) while others had low quality (Fundisha, Ken1, Ken2 and Ken3) (Table 2). Values of A $_{260 / 280}$ indexes between 1.7 and 2.0 indicate the presence of pure DNA (Glasel, 1995).

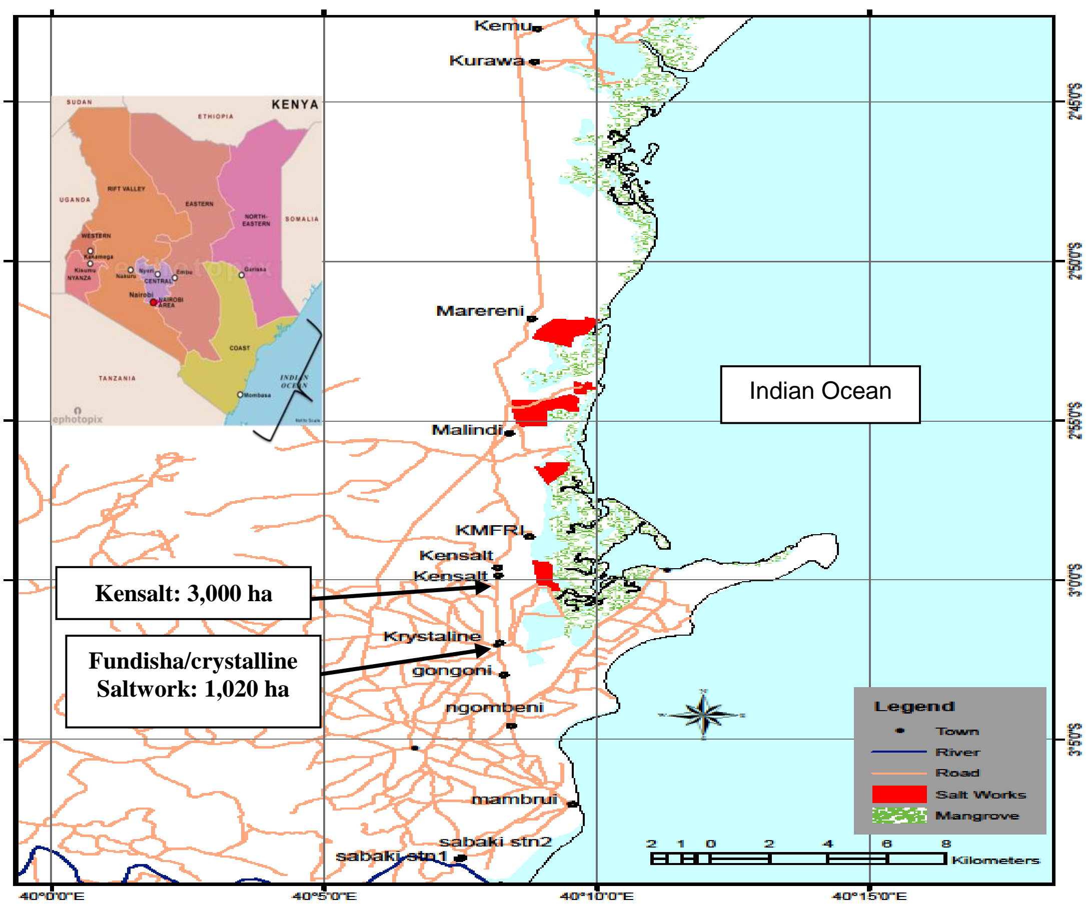


Fig. 1: Study area - map of the Kenyan coast showing the location of the salt belt and a more detailed impression of the salt belt showing the individual salt producing companies in a North – South Orientation. Fundisha saltwork is also called Crystalline

Table 1: 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
AluI	5'...A G C T...3' 3'...T C G A...5'	37° C	65° C / 20minutes
HaeIII	5'...G G C C...3' 3'...C C G G...5'	37° C	80° C / 20minutes
HinfI	5'...G A N T C...3' 3'...C T N A G...5'	37° C	65° C / 20minutes
RsalI	5'...G T A C...3' 3'...C A T G...5'	37° C	80° C / 20minutes
Xbal	5'...T C T A G A...3' 3'...A G A T C T...5'	37° C	65° C / 20minutes
HpaII	5'...C C G G...3' 3'...G G C C...5'	37° C	80° C / 20minutes

Table 2: Average quantity of DNA extracted from individual cysts from each sample including Artemia Reference Centre (ARC) code. The DNA quantity was measured using a NanoDrop® ND-1000 machine. Values are mean ± SE.

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

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

The primer combinations produced identical 1,500 bp fragments in all the 10 replicates in every sample analysed. Only one replicate per sample is shown (Fig. 2)

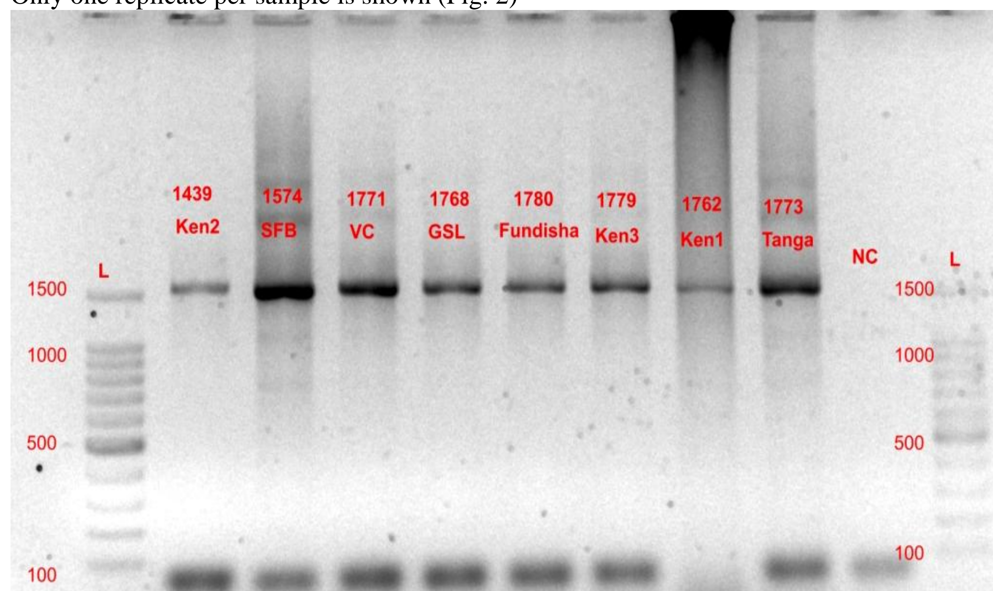


Fig. 2: Example of agarose gel for PCR-amplified 1500 bp 12S – 16S mtDNA fragment for a single cyst per sample. L: 1500 bp ladder; NC: Negative control.

RFLP analysis of the mtDNA

The enzymes HaeIII and HpaII detected polymorphism only in the Fundisha sample (Fig. 3). The enzymes AluI, XbaI, HinfI and RsaI were monomorphic across all samples (results not shown). In total, approximately 1,216 fragments were surveyed in the 1,500 bp 12S - 16S mtDNA target sequence. A total of three composite haplotypes were identified in the mtDNA target sequence. All the three haplotypes were present in Fundisha Artemia samples while the rest of the samples were monomorphic (Table 3). The most common haplotype was AAAAAA, being detected in all the sample populations except GSL. This haplotype attained the highest frequency (0.4286) within Fundisha samples. A private haplotype (AAABBA) was discovered in Fundisha sample while the haplotype (AAAABA) was only shared between Fundisha and GSL (Table 3). The highest haplotype diversity (h) was recorded in Fundisha cyst samples (0.76 ± 0.07).

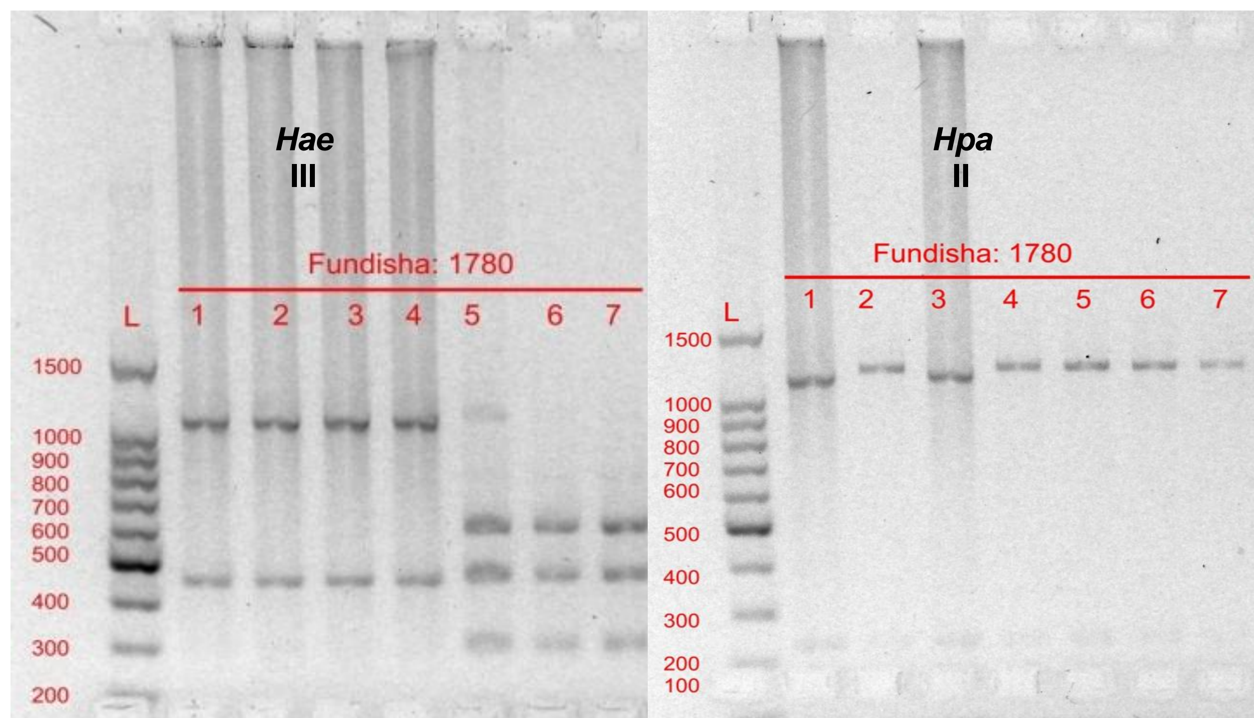
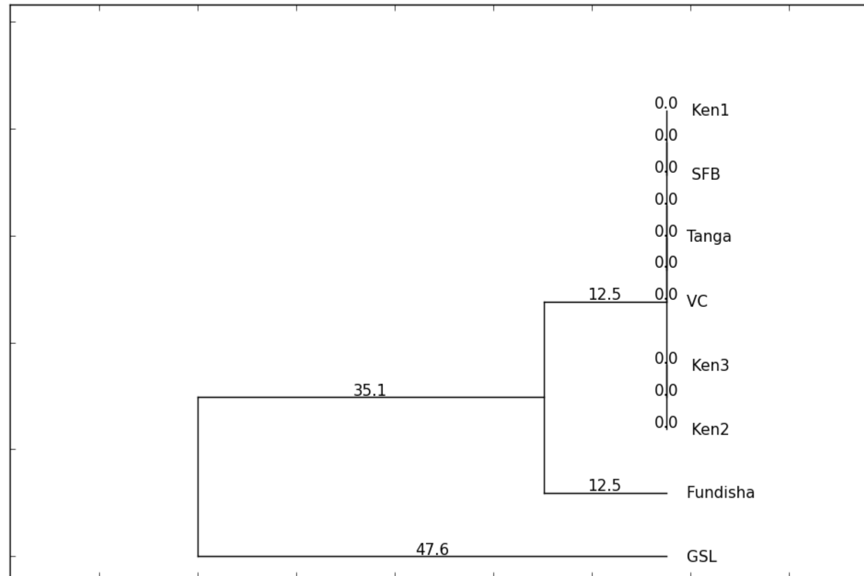


Figure 3: Example of agarose restriction fragment profile for the polymorphic HaeIII and HpaII enzymes on Fundisha individual cyst samples. PCR-amplified 1500 bp of 12S – 16S mtDNA fragment for 7 single cyst replicates per sample.

Table 3: 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; AluI, Xbal, HinfI, HpaII, HaeIII and RsaI

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 showed two major groups (GSL and SFB) while the Fundisha cyst samples appeared to be intermediate (Fig.4).



Figur 4: 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.

Molecular analysis of the Hsp70 gene fragment

The 1,935 bp Hsp70 gene fragment produced a non-polymorphic pattern in all the enzymes. Only the RFLP pattern of enzyme Rsal is shown (Fig. 5). Interestingly, even restriction enzymes such as AluI and Sau3A with 7 and 6 cleavage sites respectively on the 1,935 bp Hsp70 gene fragment did not show any polymorphism in any of the samples.

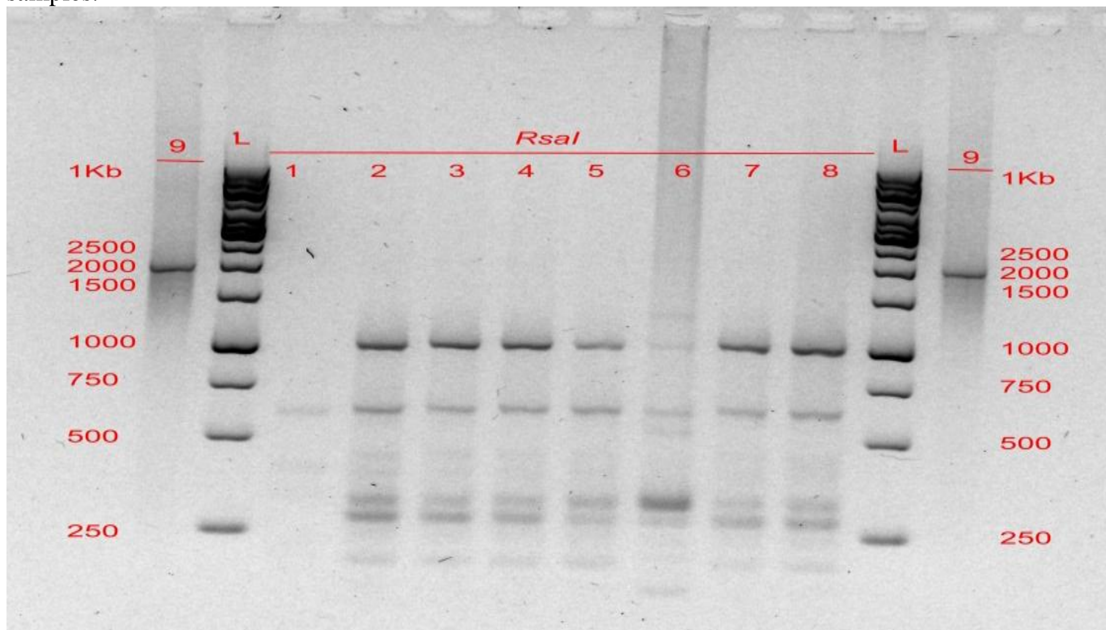


Figure 5: Agarose restriction fragment profile for the enzymes: Sau3A, AluI, HinfI and Rsal. The PCR fragment was generated using DNA extracted from pooled Artemia cysts. L: 1Kb ladder, Lanes 1: Fundisha, 2: Ken1, 3: Ken2, 4: Ken3, 5: Tanga, 6: GSL, 7: SFB, 8: VC, 9: Undigested PCR product (2, 000 bp) control.

Discussion

The present study analysed mitochondrial DNA using the RFLP tool to detect polymorphism in the Kenyan A. franciscana using DNA extracted from individual cysts. The monomorhic DNA fingerprints corresponded with zero

genetic distance as shown by the UPGMA dendrogram (Fig. 4), indicating lack of genetic differentiation between and among the Artemia samples. Lack of genetic diversity is risky in times of genetic bottleneck as the entire population may perish. Ecological processes such as migration can cause high Artemia population heterogeneity in the habitat but limited effective gene flow is observed (Hajirostamloo, 2009) because effective gene flow is much slower compared to the process of dispersal (Naihong et al., 2000). The process of assortative mating can prevent intercrossing even among coexisting Artemia species to reduce chances of speciation (Beristain et al., 2010). The absence of genetic polymorphism within samples could have been due to the limited 1,500 bp fragment of mtDNA analysed. 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. Since Kenya and Vietnam share similar Artemia inoculation history, one would have expected similar genetic evolutions. However, a larger fragment has high chances of showing detailed microevolutionary changes that might not be detected in a limited DNA fragment.

The environmental conditions are critical factors that may influence the Artemia population patterns and genetic expressions (Evjemo and Olsen, 1999; Van Stappen, 2002). In Kenya, integrated salt - Artemia culture is a continuous process where Artemia flourishes year round. In Vietnam, the saltworks are predictably sequential and this favoured a faster evolution of VC Artemia strain (Kappas et al., 2004). This conforms to Manaffar's (2012) observation 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 only natural selection process is responsible for gene loss. Natural selection requires long time to cause meaningful genetic divergence (Gajardo and Beardmore, 2012). Permanence and seasonality of the environment are key instruments driving considerable genetic differentiation of Artemia leading to specific biota with definite genetic structures (Lenz, 1987). However, the exclusive ovoviviparity of the New Zealand A. franciscana population (inoculated in 1950s) was due to genetic differentiation caused by constant year-round salinity and temperature conditions (Wear and Haslett, 1986). The mutations caused by high UV radiation have also been linked to genetic evolutionary changes in Artemia populations (Hebert et al., 2002).

The current study might not have sufficiently assessed the samples intra-population diversity due to limited the RFLP technique, which is inferior to detect intra-specific polymorphism (Bossier et al., 2004; Avise, 2004). The fact that the single haplotype identified in Tanga samples was similar to Kensalt and Fundisha samples suggested they are genetically close. This provides evidence that Artemia in tanga region was introduced by Kensalt management who own saltworks there.

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 albeit in an insignificant manner ($P > 0.05$). Therefore, if indeed there was significant genetic differentiation between Kenyan (Kensalt) Artemia and their SFB ancestors, then the tool used was not sufficiently adequate to detect this micro-evolutionary divergence. Nevertheless, the private haplotype (AAABBA) in Fundisha cyst samples suggested a systematic genetic differentiation thus molecular evidence of an existing subpopulation and genetic divergence from their SFB ancestors. 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 superior genetic tools like AFLP and microsatellites are needed to authenticate this finding. There was molecular evidence of co-existence of both SFB and GSL Artemia strains in Fundisha saltwork, conforming to Nyonje (2011) report. 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 lack of genetic variation in the Hsp70 RFLP fingerprint pattern suggested that the samples analysed had the same Hsp70 gene structure. Feder and Hofmann (1999) reported that little variation in the Hsp70 gene could be due to the fact that it is evolutionary and functionally conserved. Based on Kapinga (2012) and Mremi (2011) findings, it was hypothesised that the Kenyan Artemia posses unique Hsp70 gene signatures. Having rejected this hypothesis, it means that factors other than the Hsp70 gene are responsible for the observed adaptations (thermotolerance). Future studies should focus on more quantitative Hsp70 analysis such as western blot by chemiluminescence techniques (Schutz-Geschwender et al., 2004).

Conclusions and recommendations

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 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 Kenyan environment. It may also help to explore and monitor future expansion of the Artemia population. The Kenyan Kensalt Artemia population is not contaminated by other Artemia strains while there is co-existence of SFB and GSL Artemia strains in Fundisha saltworks. Other factors other than the Hsp70 family could be involved in the much cited thermotolerance superiority of the Kenyan Artemia populations. More robust molecular markers targeting larger mtDNA fragment should be considered concurrently with Hsp70 quantitative technique.

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References

Agh, N., Bossier, P., Abatzopoulos, T.J., Beardmore, J.A., Van Stappen, G., Mohammadyari, A., Rahimian, H. and Sorgeloos, P. (2009): Morphometric and preliminary genetic characteristics of Artemia populations from Iran. International Review of Hydrobiology, **94** (2) 194 – 207.

Anderson, E., Lochhead, J.H., Lochhead, M.S. and Huebner, E. (1970): The origin and structure of the tertiary envelop in the thick-shelled eggs of the brine shrimp Artemia. Journal of Ultra structure Research, **32**: 497 - 525.

Avise, J.C. (1994): Molecular markers, natural history and evolution. Chapman and Hall, New York, pp 541.

Avise, J.C. (2004): Molecular markers, natural history and evolution, 4th edition. Kluwer Academic Publishers, pp 511.

Baruah, K., Ranjan, J., Sorgeloos, P. and Bossier, P. (2010): Efficacy of heterologous and homologous heat shock protein 70s as protective agents to Artemia franciscana challenged with Vibrio campbellii. Fish & Shellfish Immunology, **29**: 733 – 739.

Beristain, P., Gajardo, P. and Bossier, P. (2010): Species-specific RFLP pattern in the heat shock protein 26 genes (Hsp26): a single-locus tool for species identification and experimental testing of habitat-induced isolation in the New World Artemia species. Molecular Ecology Resources, **10**: 229 – 231.

Blouin, M.S., Parsons, M., Lacaille, V. and Lotz, S. (1996): Use of microsatellite loci to classify individuals by relatedness. Molecular Ecology, **5** (3): 393 – 401.

Bossier, P., Xiaomei, W., Catania, F., Dooms, S., Van Stappen, G., Naessens, E. and Sorgeloos, P. (2004): An RFLP database for authentication of commercial cyst samples of the brine shrimp Artemia spp. (International Study on Artemia LXX). Aquaculture, **231**: 93 -112.

Camargo, W., Sorgeloos, P. and Bossier, P. (2002): Preliminary genetic data on some Caribbean Artemia franciscana strains based on RAPD's. Hydrobiologia, **468**: 245 - 249.

Clegg, J.S., Hoa, N.V. and Sorgeloos, P. (2001): Thermal tolerance and heat shock proteins in encysted embryos of Artemia from widely different thermal habitats; Hydrobiologia, **466:** 221 – 229.

Clegg, J.S., Tanguay, J.A. and Reyes, R.C. (2004): Habitat diversity and adaptation to environmental stress in encysted embryos of the crustacean Artemia; Journal of Bioscience, **29**: 489 – 501.

Clegg, J.S., Willsie, J.K. and Jackson, S.A. (1999): Adaptive significance of a small heat shock/α-crystallin protein in encysted embryos of the brine shrimp, Artemia franciscana. American Zoologist, **39**: 836 – 847.

Crack, J.A., Mansour, M., Sun, Y. and MacRae, T.H. (2002): Functional analysis of a small heat shock / alpha-crystallin protein from Artemia franciscana, Oligomerization and thermotolerance. European Journal of Biochemistry, **269:** 1 - 10.

DuBeau, S.F., Pan, F., Tremblay, G.C., Bradley, T.M. (1998): Thermal shock of salmon in vivo induces the heat shock protein Hsp70 and confers protection against osmotic shock. Aquaculture, **168**: 311- 323.

Dutrieu, J. (1960): Observations biochimiques et physiologiques sur le development d' Artemia salina Leach. Arch. Zool. Exp. Gen. **99**: 1-134.

Eimanifar, A., Rezvani, S. and Carapetian, J. (2006): Genetic differentiation of Artemia urmiana from various ecological populations of Urmia Lake assessed by PCR amplified RFLP analysis. Journal of Experimental Marine Biology and Ecology, **333**: (2-13) 275 - 285.

Evjemo, J.O., Olsen, Y. (1999): Effect of food concentration on the growth and production rate of Artemia franciscana feeding on algae. Journal of Experimental Marine Biology and Ecology, **242**: (2) 273 – 296.

Feder, M.E. and Hofmann, G.E. (1999): Heat-shock proteins, molecular chaperones and the stress response: evolutionary and ecological physiology. Annual Review of Physiology, **61**: 243 – 282.

Frankenberg, M.M., Jackson, S.A. and Clegg, J.S. (2000): The heat shock response of adult Artemia franciscana. Journal of Theoretical Biology, **25**: 481- 490.

Gajardo, G.M. and Beardmore, J.A. (2012): The brine shrimp Artemia: adapted to critical life conditions. Frontiers in Physiology / Fractal Physiology, Volume **3** Article 185.

Gajardo, G., Crespo, J., Triantafyllidis, A., Tzika, A., Baxevanis, A.D., Kappas, I. and Abatzopoulos, T.J. (2004): Species identification of Chilean Artemia population based on mitochondrial DNA RFLP analysis. Journal of Biogeography, **31**: 547 – 555.

Glasel, J.A. (1995): Validity of nucleic acid purities monitored by $A_{260/280}$ absorbance ratios. Biotechniques, **18**: 62 - 63.

Hajirostamloo, M. (2009): Genetic differentiation of Artemia parthenogenetica from Various ecological population of Iran. International Journal of Biological and Life Sciences, **5**: 3 – 9.

Hajirostamloo, M. and Pourrabbi, R. (2011): Genetic differentiation of Artemia franciscana in a new environment (Iran). World Journal of Zoology, **6** (1): 16 – 21.

Hebert, P.D.N., Remigio, E.A., Colbourne, J.K., Taylor, D.J. and Wilson, C.C. (2002): Accelerated molecular evolution in halophilic crustaceans. Evolution **56**: 909 – 926.

Hou, L., Bi, X., Zou, X., He, C., Yang, L., Qu, R. and Liu, Z. (2006): Molecular systematics of biosexual Artemia populations. Aquaculture research, **37**: 671 – 680.

Kapinga, I. (2012): Characterization of Artemia from solar salt works in Kenya. Msc thesis Ghent University, Belgium pp 1 - 60.

Kappas, I., Abatzopoulos, T.J., Van Hoa, N., Sorgeloos, P., Beardmore, J. (2004): Genetic and reproductive differentiation of Artemia franciscana in a new environment. Marine Biology, **146**: 103 -117.

Krieg, F., Triantafyllidis, A. and Guyomard, R. (2000): Mitochondrial DNA variation in European populations of Silurus glanis. Journal of Fish Biology, **56**: 713 - 724.

Lenz, P. H. (1987): Ecological studies on Artemia: a review. In: Sorgeloos, P., Bengtson, D. A., Decleir, W. and Jaspers, E (Eds), Artemia research and its publications, Universa press, Wetteren, Belgium, pp 5-18.

Lind, M.C., Bera, P.P., Richardson, N.A., Wheeler, S.E. and Schaefer, F.H (2006): The deprotonated guanine-cytosine base pair. Proceedings of the National Academy of Science of USA (PNAS), **103** (20): 7554 –7559.

Ma, X.C., Jamil, K., MacRae, T.H., Clegg, J.S., Russell, J.M., Villeneuve, T.S., Euloth, M., Sun, Y., Crowe, J.H., Tablin, F. and Oliver, A.E. (2005): A small stress protein acts synergistically with trehalose to confer desiccation tolerance on mammalian cells. Cryobiology, **51**: 15 – 28.

Manaffar, R. (2012): Genetic diversity of Artemia populations in Lake Urmia, Iran. phD thesis, Ghent University, Belgium pp 160.

Mremi, P.A. (2011): Pond production of Artemia in a solar salt work in Kenya. Msc thesis, Ghent University, Belgium. pp 1 - 92.

Munoz, J., Green, A.J., Figuerola, J., Amat, F. and Rico, C. (2009): Characterization of polymorphic microsatellite markers in the brine shrimp Artemia. Molecular Ecology Resources, **9:** 547 – 550.

Naceur, H. B., Jenhani, B.R. and Romdhane, M.S. (2010): Biological Characterization of the New Invasive Brine Shrimp Artemia franciscana in Tunisia: Sabkhet Halk El-Menzel International Journal of Biological and Life Sciences, **6**: 3 – 24.

Naihong, X., Audenaert, E., Vanoverbeke, J., Brendonck, L., Sorgeloos, P. and Meester, L. D. (2000): Low among population genetic differentiation in Chinese bisexual Artemia populations. Heredity, **84**: 238 – 243.

Nei, M. (1978): Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, **89**: 583 – 590.

Nei, M. (1987): Molecular evolutionary genetics. Columbia University Press, New York: pp 512.

Nei, M. and Tajima, F (1981): DNA polymorphism detectable by restriction endonucleases. Genetics, **97**:145 – 163.

Nyonje, B. M. (2011): A report on survey of Artemia populations in saltworks in Magarini – Kenya, KMFRI documentaries pp 7(unpublished report).

Pavel, A.B. and Vasile, C.L. (2012): PyElph – A software tool for gel images analysis and phylogenetics. BMC Bioinformatics, **13**: 9.

Perez, M.L., Valverde, J.R., Batuecas, B., Amat-Domenech, F., Marco, R. and Garesse, R. (1994): Speciation in the Artemia genus: mitochondrial DNA analyses of bisexual and parthenogenetic brine shrimp. Journal of Molecular Evolution, **38**: 156 – 168.

Persoone, G. and Sorgeloos, P. (1980): General aspects of the ecology and biogeography of Artemia. Vol. 3, Ecology Culturing Use in Aquaculture, pp 3-24.

Schutz-Geschwender, A., Zhang, Y., Holt, T., McDermitt, D. and Olive D.M. (2004): Quantitative, two-color western blot detection with Infrared fluorescence. LI-COR Biosciences, Lincoln, Nebraska 68504. Doc. No. 979-07702. pp 1 – 7.

Sun, Y., Zhong, Y., Song, W.Q., Zhang, R.S., Chen, R.Y. and Sorgeloos, P. (1999a): Detection of genetic relationships among four Artemia species using randomly amplified polymorphic DNA (RAPD). International Journal of Salt Lake Research, **8**: 139 –147.

Sun, Y., Song, W.Q., Zhong, Y.C., Zhang, R.S., Abatzopoulos, T.J. and Chen, R.Y. (1999b): Diversity and genetic differentiation in Artemia species and populations detected by AFLP markers International Journal of Salt Lake Research **8**: 341–350.

Todgham, A.E., Schulte, P.M., Iwama, G.K. (2005): Cross-tolerance in the tide pool sculpin: the role of heat shock proteins. Physiological and Biochemical Zoology, **78**: 133–144.

Toi, H.T., Boeckx, P., Sorgeloos, P., Bossier, P. and Van Stappen, G. (2013): Bacteria contribute to Artemia nutrition in algae-limited conditions: A laboratory study. Aquaculture, **388 - 391**: 1 – 7.

Triantaphylidis, G.V., Abatzopoulos, T.J. and Sorgeloos, P. (1998): Review of the biogeography of the genus Artemia (Crustacea, Anostraca). Journal of Biogeography, **25**: 213 – 226.

Valverde, J.R., Batuecas, B., Moratilla, C., Marco, R. and Garesse, R. (1994): The complete mitochondrial DNA sequence of the crustacean Artemia franciscana. Journal of Molecular Evolution **39**: 400 - 408.

Van Stappen, G. (1996): Artemia: Use of cysts. In: Lavens, P. and Sorgeloos, P. (Eds), Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Curriculum vitae 223 Paper No. 361, 295 pp.

Van Stappen, G. (2002): Zoogeography, In Abatzopolous, T. H. J., Beardmore, J. A., Clegg, J. S. and Sorgeloos, P. (Eds.), Artemia basic and applied biology. Kluwer Acadamic Publishers, Dordrecht/London/Boston, pp 171 – 215.

Van Stappen, G. (2008): Artemia biodiversity in Central and Eastern Asia. PhD thesis, Ghent University, Belgium pp 270.

Van Stappen, G., Fayazi, G. and Sorgeloos, P. (2001): International study on Artemia LXIII. Field study of the Artemia urmiana (Günther, 1890) population in Lake Urmiah, Iran. Hydrobiologia, **466**: 133 - 143.

Walsh, S.P., Metzger, D.A. and Higuchi, R. (1991): Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques, **10** (4): 506 - 513.

Wear, R.G. and Haslett, S.J. (1986): Effects of temperature and salinity on the biology of Artemia franciscana from Lake Grassmere, New Zealand. I. Growth and mortality. Journal of Experimental Marine Biology and Ecology, **98**: 153 – 166.

Willsie, J.K. and Clegg, J.S. (2002): Small heat shock protein p26 associates with nuclear lamins and Hsp70 in nuclei and nuclear matrix fractions from stressed cells; Journal of Cell Biochemistry, **84:** 601– 614.