Journal of the Egyptian Society of Parasitology, Vol.44, No.1, April 2014
J. Egypt. Soc. Parasitol. (JESP), 44(1), 2014: 197 - 204

SECONDRAY STRUCTURE AND SEQUENCEOFITS2-rDNA OF THE EGYPTIAN MALARIA VECTORANOPHELES PHAROENSIS (THEOBALD)

By

NAHLA M. WASSIM

Molecular Biology Unit, Faculty of Science, Suez University, Egypt

Abstract

Out of the twelve Anophelines present in Egypt, only five species known to be malaria vectors. *Anopheles (An.) pharoensis* proved to be the important vector all over Egypt, especially in the Delta. *Anopheles sergenti* proved to be the primary vector in the Oases of the Western Desert, *An. multicolor* in Faiyoum, *An. stephensi* in the Red Sea Coast, and *An. superpictus* in Sinai. Genomic DNA was isolated from single adult mosquito of *An. pharoensis* (Sahel Sudanese form), PCR was performed to amplify ITS2 region of rDNA using specific primers for 5.8S and 28S rDNA genes. The amplicons were purified, directly sequenced and aligned to the sequence of the same region of *An. gambiae*, using clustalw2. The length of ITS2-rDNA of *An. pharoensis* was 411bp. The GC content of the ITS2 reported 53% is consistent with spacer base composition in *Anopheles* species. The similarity between the two species was 52% and genetic distance was 0.46.Variable simple sequence repeats (SSRs) are found at low frequency. The secondary structure of rDNA-ITS2was predicted by MFOLD and was -192; 60 to-195.32 kilocalories/mole.

Key words: *Anopheles* Mosquitoes, Genetic Variations, ITS2-rDNA, Egypt

Introduction

Anopheles pharoensis has been divided into at least two species which are widespread in Africa (Miles *et al,* 1983). The form (or species) found in East and South of Africa is zoophilic and does not act as a vector. The Sahel Sudanese form that covers an area extending from the Atlantic Coast to the Valley of the Nile and to the Middle East displays mixed, opportunistic behavior (Zahar, 1989; Mouchet *et al*, 2008). Out of twelve Anopheline mosquitoes present in Egypt, only five species are known to be malaria vectors. *An. pharoensis* proved to be the important vector all over Egypt, especially in the Delta (Zahar, 1975). *An sergenti* proved to be the primary vector in the Oases of the Western desert, while *An. multicolor, An. stephensi* and *An. superpictus* plays a predominant role in Al-Faiyoum, the red sea coast and in the Sinai respectively. Also, they are known to be malaria vectors in other countries.

Despite all the official efforts, malaria is still consider one of the health problems may threatening public health in Egypt (El Said and Farid, 1982; El Said *et al*, 1983; Mikhail *et al*, 2009). An evaluation of the clinical and parasitic status of malaria as a cause of fever among patients admitted to the Almaza Military fever hospital in Cairo only revealed the presence of thirty six malarial cases (El Bahnasawy *et al*, 2010). Besides, El Bahnasawy *et al*. (2011) recorded *An. multicolor*, *An. sergentii*, and *An. algeri-ensis* in Toshka Project, and added that *A. sergentii* is a malaria-vector and *A. multicolor* is a suspected vector. Conse-quently, the endemicity of the chloro-quine resistant *Plasmodium falciparum* on the Egyptian-Sudanese border pave the way for the malignant malaria trans-mission particularly among the travelers returning back from Sudan.

The mosquito genus *Anopheles* (443 formally named species) contains all the vectors of human malaria parasites. Because many of the primary vectors belong to cryptic species complexes, it is necessary to have accurate phylogenetic reconstructions and species diagnostic tools. The evolution, molecular biology and biochemistry of rDNA have been the subject of intense study since it was characterized. The rDNA is a multicopy gene family that exists as one or more tandem arrays of many transcriptional units per cell, where concerted evolution rapidly spreads mutations to all members of the gene family, even if arrays are located on the different chromosomes (Dover, 1982; Gerbi, 1985; Tautz *et al*, 1988). *Anopheles* has the least amount of the repetitive DNA of any mosquitoes analyzed to date (Black and Rai, 1988). In mosquitoes, each rDNA transcriptional unit is composed of an external trans-cribed spacer, an 18S subunit, an internal transcribed spacer 1 (ITS1), a 5.8S subunit, an ITS2, and a 28S subunit. The rDNA units within an array are linked to each other by an intergenic spacer (IGS). The transcribed spacers are thought to contain conserved structures important in forming the mature ribosomal amplicon (Wesson *et al*, 1992; Paskewitz *et al*, 1993). The rDNA sequence is a valuable source of information because the functional regions that produce the ribosomes are highly conserved but the transcribed and non-transcribed spacers have the high interspecific and low intraspecific variability, making them useful for explaining relationships of recently diverged species and also useful as a basis for polymerase chain reaction (PCR) identification of morphologically similar species. As such, ITS1and ITS2 were used extensively in phylogenetic reconstruction of closely related and cryptic species complexes, as well as in the development of diagnostic species-specific PCR-based markers. However, because PCR can amplify all sequences of ITS present within the genome, variation among ITS sequences within individuals or species could result in accurate phylogenies and markers for species diagnostics. Consequently, the identifying and quantifying levels of intra-genomic and intraspecific variations among ITS sequences are of real importance (Li and Wilkerson, 2007).

The aim of this study was to examine ITS2-rDNA sequences from multiple individuals of *An. pharoensis* and aligned with *An. gambiae* (Genbank, accession No. AY423072) to investigate the ITS2 genotype of the main malaria vector in Egypt and to evaluate its relation to *Anopheles* evolution.

Materials and Methods

This study of ITS2- rDNA variation conducted using individuals of *An. pharoensis* colony from the Egyptian Medical Insect Institute, Dokki, Giza, Egypt and reared under the insectary conditions (temp. 27 °C and 12 hr light–dark) till adult stage and kept at -80 °C until processing for DNA extraction. Genomic DNA isolated from single adult mosquito using Wizard Genomic DNA Purification Kit (Promega, cat #A1620, USA).The Wizard Genomic DNA purification Kit (Promega) used with some modifications described by (Vidigal *et al*, 2000; Wassim, 2005). The DNA concentration determined using UV spectrophotometer at a wave length= 260nm (Sambrook, 1989). The ITS2 region of rDNA was amplified using 5.8S primer (GTGGATCCTGTGGAACTGCAGGACA CATG) and 28S primer (GTG TCGACATGCTTAAATTTAGGGGGTA) (Wesson *et al*, 1992). Genomic DNA of single mosquito used for each reaction and PCR amplification, performed in 25µl using the following profile 5 min at 93°C, 35cycles (1 min at 93°C, 2 min at 58°C, 1min at 72°C), and 7 min at 72°C. The PCR products checked on 2% agarose gel electrophoresis with the ethedium bromide. The amplicons were excised and purified for sequencing analysis using QUIA quick gel

extraction kit (Promega). The ampicons were directly sequenced and aligned to the sequence of ITS2-rDNA of *An. gambiae* available in Genbnak (Aceesion no. AY423072) and published by Garros *et al*. (2005) using (http://www. ebi.ac.uk /Tools/ msa/clustalw2/) then the expected secondary structure and free energy have been obtained.

Results

The ITS2 is referred to as the extent region between the 5.8S and 28S genes (coding regions) of rDNA from indivi-duals for the examined species (Figure 1). Using the above primers, we were able to identify the length of ITS2-rDNA of *An. pharoensis* (411) bp (Fig. 2), and study the genotype in comparison with *An. gambiae* (Garros, 2005).The length difference of ITS2-rDNA was 81bp between the two species. The sequence alignment of ITS2-rDNA of *An. pharoensis* and *An. gambiae* showed rate of point mutations between the two species occurred at different variable nucleotide positions. There were 61 indels account-ing for both; (30 insertions and 21 deletions) and base substitutions (61 Transitions/103 Transversions). The GC content of the ITS2 reported here (53%) is consistent with spacer base composi-tion in *Anopheles* species. The similarity between the two species was 52% and genetic distance was 0.46.

ITS2-rDNA of *An. pharoensis* contained sequences tandem repeated, where an array of repeats located in different locations. Each repeat sequence was~ 3-4bp long. Some of the repeat are more common than others, the most common simple repeats were the trinucleotides; CGC, GCC, CTG, GGG, CGT, GCG, GGC, GCT, GTG, CTC, CGG and the tetranucleotides were CTCG, CTGC and GTGG (Tab. 1) less frequenting. The examination of the frequency of base composition of these repeats in *An. pharoensis* showed that 41% of these repeats involved guanine followed by cytosine 33% and thymine15%. These repeats may under-line the relatively large degree of the sequence divergence between the Anopheline species. The genetic distance separating the two species was 0.46

The secondary structure of rDNA-ITS2 was predicted by MFOLD (Zuker *et al*, 1999). The minimum free energies were 192.60 to -195.32 kilocalories for *An. pharoensis*. The structures of the two variants of *An. pharoensis* have the significantly high energy and lower stability. The stem and loop were clear in the presumptiveITS2 (Fritz *et al*, 1994) as in (Fig. 3). *An. pharoensis* had not identical base pairing between certain regions where sequence homology had already been differed. Most of the inter specific variability observed occurred outside the domains of the specific sequence homology of coding regions. The inter specific variability added to stem length, maintained a stem base pair, or occurred in a loop or other region of unpaired sequence.

Discussion

Unambiguous identification of *Anophe-les* malaria vector species is essential for the study of an array of factors that affect control and disease transmission. The phenomenon of lack of correspondence between the morphological similarity and similarity in the bionomics, vector potential, or insecticide resistance is well documented in the *Anopheles* mosquitoes (White, 1996). There is a growing body of evidence to suggest that within Anopheline taxa, genetic variation can be related to the adaptation of mosquito species to their environment (Coluzzi *et al*, 1979). *An. pharoensis* prefers the fresh water habitats (Marglit and Tahori, 1973; El Said *et al*, 1983; Mikhail *et al*, 2009) in Egypt. The genotypes have been associated with different ecotypes and geographical distribution, providing evi-dence of barriers to gene flow between populations of the species (Favia *et al*, 1997). The genotypes appear to relate to behavioral characteristics which have impor-

tant implications in malaria trans-mission (Gakhar *et al*, 2013). The present results revealed that the length ITS2 of *An. pharoensis* is 411bp and come quit together with the published sequences and unpublished Gen Bank submissions which show that most *Anopheles* ITS2s are ranged between 300 and 600 bp (Paskewitz *et al*, 1993; Fritz 1994; Wilkerson *et al*, 2004; Black *et al*, 2006; Oshaghi *et al*, 2013).

In the present study, direct sequence of ITS2-rDNA of *An. pharoensis* does not explain the intragenomic variations could not be expect the location of the rDNA arrays in *An. pharoensis*. The rDNA arrays are usually on chromosomes associated with sex determination. Kumar and Rai (1990) mapped dozens of species of mosquitoes and found rDNA loci on the autosomes of Culicine mosquitoes and on X and Y chromosomes of *Anopheles*. X and Y chromosomes in *Anopheles* are only partially homologous and X chromosome variants do occur (Rafael *et al*, 2003). Multiple rDNA locations are not unusual, for example, there are 5 in humans (Gonzalez and Sylvester, 2001) and at least 2 in *Drosophila hydei* (Hennig *et al*, 1975) and grasshoppers (White *et al*, 1982). Similar explanations were considered for other *Anopheles* mosquitoes by Onyabe and Conn (1999); Beebe *et al*. (2000) and Wilkerson *et al*. (2004). Physical mapping using in situ hybridization is needed to confirm the location of rDNA loci in the *An. pharoensis* species (Sahel Sudanese form).

The mutation rates are higher than homogenization rates at ITS2 than other parts of the rDNA gene array, is probably very common (Harris and Crandall, 2000). Molecular events such as the insertions, deletions, duplication and an equal crossing-over in sub repeat regions can generate spacer length heterogeneity. The results came quit to the above conclusion whereas the ITS2 sequence divergence between the two species has been maximal and occur at 61 indels (30insertions and 21 deletions). The point mutations were 51 transitions and 103 transversions. Homogeneity theoretically decrease as mutation rate and the repeat number increase. The observation on the degree of homogenization among family members in a species can indicate at which levels of heterogeneity is gene-rated. Many of the genomic charac-teristics that have been described as conducive to faster rates of the homo-genization and fixation were more characteristics of Anopheline mosquetoes than they are of Aedine species (Levinson and Gutman, 1987)

The inter-specifically variable simple sequence repeats (SSRs) are found at low frequency in ITS2 of *An. pharoensis*. Sixty one indels in regions of SSRs and simple - base repeat motifs account for most of the sequence variation observed and suggest their role as a major cause of divergence in the evolution of this spacer .The use of SSRs in Systematics has yet to be fully modeled. The process of SSM is more likely to be a major factor in the initial expansion of the short repeat motifs, which are subsequently pre-disposed to further expansion by an equal crossing over. A rapid rate of fixation of such mutations in tenderly repeated genes may subsequently distinguish closely and even distantly related invertebrates (Hancock *et al*, 1988). The observation of the addition SSRs at the tips of the secondary structure stems in 28S variable regions. Slippage mechanism or non-homologous exchange between chromosomes in these areas has resulted in the addition of SSRs with high self- similarity (Hassouna *et al*, 1984). These SSM are hot spots of slipped strand miss pairing events and these events in concert with unequal crossing-over can account for wide spread simple rDNA sequences.

The secondary structure model of *An. pharoensis* is similar to the models proposed for *Anopheles* mosquitoes. The evidence that slippage synthesis is a major mechanism generating variability observed between related species may be due to some extent

constrained by the secondary structure. Also, compensatory base change in the homologous regions and the hetero genoas highly variable areas, which tend toward GC rich simple sequences added to the length. This observation is based on evidences from secondary structure stem maintenance the complexity of the predicted structure was based on stems formed from base pairing between homologous domains (Levinson and Gutman, 1987). Under realistic rates of gene conversion, unequal exchange and transposition, fixation of mutant copy in a multigene family proceeds without a large variance at any given generation. This small variance is due to the much slower rates at which chromosomes distribute at each generation, so variance affected by many factors including this phenomena and the mechanism used to explain it have been termed the "concerted evolution" and "molecular drive" respectively (Dover, 1982).

Conclusion

The outcome results indicated that the comparison of ITS2-rDNA sequences and studying the secondary structures can be very useful for the analyzing of the uncharacterized vectors of malaria in Egypt. Phenomenon of lack of the correspondence between similarity in the bionomics, vector potential, or insecticide resistance is needed. Only by knowing the genetic identity of the vector under investigation can this particular part of an experimental design be accounted for furthermore, the subsequent studies or control efforts.

Next research should be focused on the use of molecular markers to identify other *Anopheles* mosquitoes in Egypt and the neighboring countries.

References

Beebe, NW, Cooper, RD, Foley, DH, Ellis, JT, 2000: Populations of the south-west Pacific malaria vector *Anopheles farauti* s.s. revealed by ribosomal DNA transcribed spacer polymerphisms. Heredity 84:244-53.

Black, WB, Rai KS, 1988: Genome evolution in mosquitoes: Interspecific and intra-specific variation in repetitive DNA amounts and organization. Genet. Res. 51:185-96.

Black, W, Brelsfoard, CL, Fritz, GN, Rodriguez, R, 2006: Sequence analysis of the rDNA internal transcribed spacer 2 and polymerase chain reaction identification of *Anopheles fluminensis* (Diptera: Culicidae: *Anopheles*) in Bolivia. J. Med. Entomol. 43, 3:460-6.

Bresfoard, C, McLain, DK, Rai, KS, 1989: Patterns of variation in the rRNAcistron within and among world populations of a mosquito *Aedes albopictus* (Skuse). Genetics 121:539-50**.**

Coluzzi, MA, Sabatini, V, Petrarca, MA, Di, D, 1979: Chromosomal differentiation and adaptation to human environment in the *Anopheles gambiae* complex. Trans. R. Soc. Trop. Med. Hyg. 73:483-97.

Dover, G, 1982: Molecular drive: a cohesive mode of species evolution. Nature 299, 5879: 111-7.

El Bahnasawy, MM, Dabbous, HK, Morsy, TA, 2010: Imported malaria as a threat to Egypt. J. Egypt. Soc. Parasitol. 40, 3:773-88.

El-Bahnasawy, MM, Saleh, NM, Khalil, M F, Morsy, TA, 2011: The impact of three anopheline mosquito species in Toshka, on the introduction of chloroquine resistant *P. falciparum* to Egypt. J. Egypt. Soc. Parasitol. 41, 3: 573-92.

El Said, S, Farid, H, 1982: Experimental transmission of *Plasmodium vivax* by *Anopheles multicolor* under the laboratory conditions and the effect of temperature on the sporogonic conditions and sporogonic cycle. J. Egypt. Pub. Hlth. Assoc. 57:512-40**.**

El Said, S, El Sawaf, B, Gebril, M, Kena- wy, MA, Azzab, BA, 1983: Bionomics of *Anopheles* in the Fayium Governorate, Egypt in relation to transmission and control of Malaria. J. Egypt. Pub. Hlth. Assoc. 58, 3/4: 12-9.

Favia, G, Della Torre, A, Yoko, BM, Branfrancotti, A, Sagnon, NF, *et al*, 1997: Molecular Identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. Insect Mol. Biol. 6, 4:377-83.

Fritz, GN, Conn, J, Cockburn, A, Seaw-right, J, 1994: Sequence analysis of the rDNA internal transcribed spacer 2 of populations of *Anopheles nunestovari* (Diptera: Culicidae). Mol. Biol. Evol. 11: 406-16.

Gakhar, SK, Sharma, R, Sharma A, 2013: Population genetic Liston (Diptera: Culicidae) structure of malaria vector *Anopheles stephensi*. Indian J. Exp. Biol. 51:273-9**.**

Garros, C, Harbachin, SRE, Manguin, S, 2005: Morphological assessment and molecular phylogenetics of the funestus and Minimus Groups of (Cellia). J. Med. Entomol. 42:522-536.

Gerbi, SA, 1985: Evolution of ribosomal. In: RJ, Mac-Intyre (ed.), Molecular Evolutionary Genetics, Plenum, New York.

Gonzalez, IL, Sylvester, JE, 2001: Human rDNA: Evolutionary patterns within the genes and tandem arrays derived from multiple chromosomes. Genomics 73:255-63.

Hancock, JM, Tautz, D, Dover, GA, 1988: Evolution of the secondary structures and compensatory mutations of the ribosomal RNAs of *Drosophila melanogaster*. Mol. Biol. Evol. Evol. 5:393-414**.**

Harris, DJ, Crandall, KA, 2000: Intra-genomic variation within ITS1 and ITS2 of fresh water crayfishes (Decapoda: Cambaridae): implica-tions for phylogenetic and microsatellite studies. Mol. Biol. Evol. 17: 284-91.

Hassouna, NB, Michot, BA, Chellerie, JP, 1984: The complete nucleotide sequence of mouse 28S rRNA gene: Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. Nucl. Acids Res. 12:3563-83.

Hennig, W, Link, B, Leoncini, O, 1975: The location of nucleolus organizer regions in Drosophila hydei. Chromosoma 51:57-63.

Kumar, A, Rai, KS, 1990a: Chromosomal localization and copy number of 18S+28S ribosomal RNA genes in Evolution are diver-se mousquites (Diptera: Culicidae). Heredit-ies 113:227-89.

Levinson, G, Gutman, GA, 1987: Slipped strand mispairing: a major mechanism for DNA sequence evolution. Mol. Biol. Evol. 4, 3:203-21.

Li, C, Wilkerson, RC, 2007: Intragenomicro-DNA ITS2 variation in the neotropical *Anopheles* (*Nyssorhynchus*) *albitarsis* complex (Diptera: Culicidae). J. Heredity 98, 1: 51-9.

Marglit, J, Tahori, AS, 1973: The mosquito fauna of Sinai. J. Med. Entomol.10:89-96**.**

Miles, SJ, Green, CA, Hunt, RH, **1983:** Genetic observations on the taxon *Anopheles* (Cellia) *pharoensis* Theobald (Diptera: Culici-dae). J. Trop. Med. Hyg. 86:153-7.

Mikhail, MW, Al-Bursheed, KM, Abd El-Halim, AS, Morsy, TA, 2009: Studies on mosquito borne diseases in Egypt and Qatar. J. Egypt. Soc. Parasitol. 39, 3:745-56.

Mouchet, J, Carnevale, P, Manguin, S, 2008: Biodiversity of Malaria in the World. Esther, UK: John Libbey Eurotext.

Onyabe, DY, Conn, JE, 1999: Intragenomic heterogeneity of a ribosomal. DNA spacer (ITS2) varies regionally in the neo-tropical malaria vector *Anopheles nuneztovari* (Dip-tera: Culicidae). Insect Mol. Biol. 8:435-42**.**

Oshaghi, MA, Sedaghat, MM, Vatandoost, H, 2003: Molecular characterization of the *Anopheles maculipennis* complex in the Isla-mic Republic of Iran. East. Mediterr. Hlth. J. 9, 4:659-66.

Paskewitz, SM, Wesson, DM, Collins, FH, 1993: The internal transcribed spacers of ribosomal DNA in +ve members of the *Anopheles gambiae* species complex. Ins. Mol. Biol. 2:247-57.

Rafael, MS, Tadei, WP, Pimentel, SM, 2003: Location of Ribosomal Genes in the Chromosomes of *Anopheles darlingi* and *Anopheles nuneztovari* (Diptera, Culicidae) from the Brazilian Amazon. Mem. Inst. Oswaldo Cruz, Rio de Janeiro 98, 5:629-35.

Sambrook, J, Fritsch, EF, Maniatis, T, 1989: Molecular cloning: A laboratory Manual, 2nd ed. Cold Spring Harbor Labora-tory, Cold Spring Harbor, NY.

Tautz, D, Hancock, JM, Webb, DA, Tautz, C, Dover, GA, 1988: Complete sequences of the rDNA genes of *Drosophila melanogaster*. Mol. Biol. Evol. 5:366-76.

Vidigal, TH, Kissinger, JC, Caldeira, RL, Pires, EC, Monteiro, E, *et al*, 2000: Phylo-gentic relationship among Brazilian *Biom-phalaria* species (Mollusca: Planorbidae) based upon analysis of ribosomal ITS2 sequences. Parasitol. 121, 6:611-20.

Wassim, NM, 2005: Sequence Analysis of the Second Internal Transcribed spacer region of Ribosomal DN (ITS2-rDNA) of *Anopheles multicolor* (Cambouliu) populations from different isolated geographical areas of Egypt. Egypt. Ger. Soc. Zool. 46, E:161-72, Cairo, Egypt.

Wesson, DM, Porter, CH, Collins, FH, 1992: Sequence and secondary structure comparisons

of ITS rDNA in mosquitoes (Diptera: Culicidae). Mol. Phylogenet. Evol. 1:253-69.

White, NJ, 1996: The treatment of malaria. Engl. J. Med. 335:800-6.

White, MJ, Dennis, ES, Honeycutt, RL, Contreras, N, 1982: Cytogenetics of the parthenogenic grasshopper Warramabavirgo and its bisexual relatives. IX. The ribosomal RNA cistrons. Chromosoma 85:181-99.

Wilkerson, RC, Reinert, J, Cong, LI, 2004: Ribosomal DNA ITS2 sequence differentiate six species in the *Anopheles crucians* complex (Diptera: Culicidae). J. Med. Entomol. 41, 3: 391-400.

Zahar, AR, 1975: Review of the ecology of malaria vectors in the WHO eastern Mediterranean Region. Bull WHO. 50:427-40.

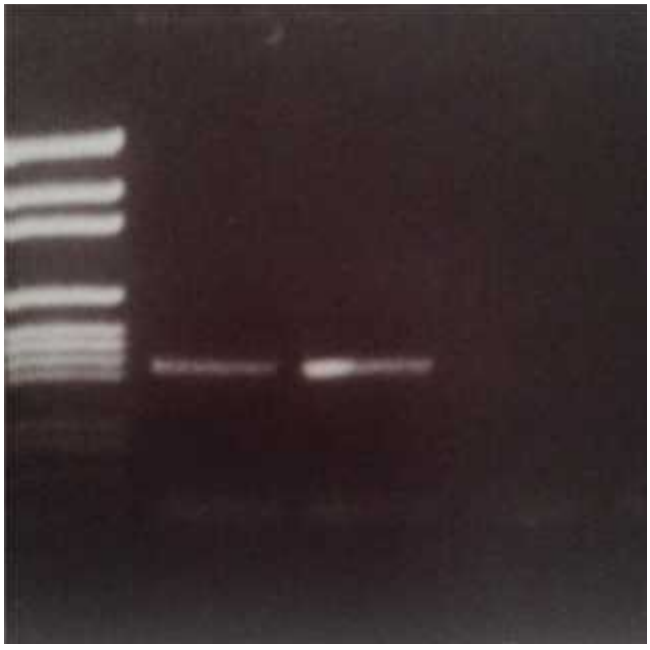
Zahar, AR, 1989: Vector Bionomics in the Epidemiology and Control of Malaria. Part II-WHO European region and WHO Eastern Mediterranean Region. Vol. II. Section II. VBC/90, 2:226-32.

Zuker, M, Mathews, DH, Turner, DH, 1999: Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide in RNA biochemistry and biotechnology. In: Barciszewski, J, Clark, BFC, editors. NATO-ASI Series. Dordrecht (Netherland): Kluwer Academic Publishers.

Table 1: simple sequence Repeats (SSRs) in ITS2-rDNA of *An. pharoensis* and *An. gambiae*

SSRs	*An. gambiae*	*An. pharoensis*
CGC	3	11
GGG	7	2
GCC	6	7
CTG	4	14
GTG	8	4
CTC	3	6
GGC	12	8
GCG	7	9
CGT	10	5
CGG	5	4
GCT	9	11
GTGG	4	3
CTCG	4	6
CGCG	2	3

Fig.1: PCR amplifications of ITS2- rDNA of *An.pharoensis* (lane 2, 3) & PEGM-DNA marker (lane 1)



```
An.pharonsis       -------TGGCTTT-----CCGCCAGCTGCAGGAAAGTTT-------------TCACCAC 35
An.gambiae         GCGCATCGGACGTTTAATCCCGACCGATGCACACATTCTTGAGTGCCTACTAATTACCAA 60
                          *.* **     ***.*.*.**** ..*:  **             * ****.

An.pharonsis       G--CTCAT--------CCGCTCGGGCCTGCTGCCAG---ACCCTGGTGTGCCACATTCGC 82
An.gambiae         AGTCTCATTTAGTTAACTACAGTGGCCGTCCGCGAAGGTGCCCGGGT---CATCCGACGC 117
                   .  *****        * .*:  ****  * ** *.   .*** ***   *.:*. :***

An.pharonsis       ACTGTGACTGTCTGCTGTGTATACT-CCTCGAATTGTCCGC-TCT-CGCTTTC-CGGTCA 138
An.gambiae         ACTG-GGCGGTC-GCTGTGCATAATGACGTGCTTGGTCCCCGTCTGCGGGTCCTCGGGCG 175
                   **** *.* *** ****** ***.* .*  *.:* **** * *** **  * * *** *.

An.pharonsis       TATAGCGCAATTCCATCTCGCGACCTTTGACCGGAATGTGCCG-AATGCTGGCATCACAT 197
An.gambiae         TTGAAAGTGG-ACACTCTCGAG-CGTATGTTGGATGCGTTTCGTGTTGGTGGTGTTTGAT 233
                   *: *..* .. :*..*****.* * *:**:  *.:. **  ** .:** *** .* : **

An.pharonsis       GCGAATCATCTGTGGCTGACTGCCTTGGTGCACTGT-----CTCTGGCGTCGTCGGGGGC 252
An.gambiae         GCGTAGGGCTTGTGG-TGTGTGTCAAGCCGCATGGTTCGAACTAATGCTACGTCGTTCCC 292
                   ***:*  .  ***** **: ** *::*  ***  **     **.: ** :*****    *

An.pharonsis       TTTGGATCGTTCGGGTCAAAGGTCGCTAGTATGGAATTGCCATTGATGACAGGAAAGGCA 312
An.gambiae         GATGG--CCACCGGCAGTCTACTCTCCAGGCTAAAGTCGGCT----CGTCTAGGGATTCG 346
                    :***  * : *** : :.:. ** * ** .*..*.* * *:     *:*:.*..*  *.

An.pharonsis       TTGAGCTAACTTGATGGATTAAACAGCGTCCCATTGTCAGTTTGTGGCACACAAACATTG 372
An.gambiae         GAAAGCTAAGTCGCTGTAACTCATGTGGGCCCATACACGG--CGTTGCGCTACCACGCT- 403
                    :.****** * *.** *: :.* .  * *****: :*.*   ** **.*:...**. *

An.pharonsis       AACGCTGGCCCTGACATATTTAGATTCTCGCAAAACATCTCGCATCGGAAAACACCATTT 432
An.gambiae         -AAGTTAGCCCT-ACATAT------ACAAGCATCAACCCACGGCACGGGCGTAGCTGTAA 455
                    *.* *.***** ******      :*:.***:.*.. *:** .:***...:..* .*::

An.pharonsis       TGCCCACAACGCG-------CGTGCAG-CCCCATGGGAAAA----- 465
An.gambiae         TACTTACGTCTCGGTTATACCACGTAGGCCTCAAGTGATGTGTGAC 501
                   *.*  **.:* **       *. * ** ** **:* **:.:
```

Fig. 2: Sequence alignment of the ITS2-rDNA and flanking 5.8S and 28S *gene*s of *An. pharoensis* and *An. gambiae*. Asterisks indicate identical nucleotide positions. Alignment generated by CLUSTALW 2

Fig.3: Secondary Structure of ITS2-rDNA of *An. pharoensis*