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ITS 1 AND 2 BASED CHARACTERIZATION OF ALLOPATRIC POPULATIONS OF *ARMIGERES (ARMIGERES) SUBALBATUS* (DIPTERA: CULICIDAE) ASSOCIATED WITH FILARIAL TRANSMISSION

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A B S T R A C T

Armigeres subalbatus is known to be the vector for parasites of many human diseases like malaria, Japanese encephalitis, filariasis etc. In India, the molecular nature of *Ar. subalabtus* species is poorly understood. We studied ITS2 molecular characterization of two allopatric populations of this species. Methods: The ITS1 and 2 spacer regions of the two populations A and B of *Ar. subalbatus* were amplified and sequenced. Restriction digests were also generated using three restriction enzymes *Hae*III, PstI and *Hpa*II, however, only *Hae*III proved to be useful for distinguishing the two populations by ITS1, while none was found useful for ITS2 sequence. Results: The correlation of indel bias with intron length was also analyzed. Both ITS1 and 2 sequences showed positive correlation with intron length. The variation in intron length is mainly due to presence of highly repetitive sequences, which results in higher base pair length of introns. Both interspersed and tandem repeats were analyzed but none of the type of repeat was found to be common in both the sequences of populations A and B. In addition, secondary structures were also analyzed and only two portions were found to be similar in population A and B of both the sequences. Conclusion: An improved understanding of the mosquito population genetics is needed for insight into the population dynamics and dispersal, which can aid in understanding the epidemiology of disease transmission and control of the vector. From this comparative data, it is evident that detectable changes in the genome can prove useful as first indicators that a monotypic population actually consists of two or more genotypes.

Keywords: *Ar. subalbatus* populations, ITS1, ITS2, secondary structure analysis.

INTRODUCTION

Armigeres subalbatus is commonly found close to human dwellings, especially in sub-urban areas with poor sanitation that contain polluted water such as septic tanks (Rajavel, 1992).This species has been known to be a vector of Japanese encephalitis virus (Das *et al*., 1983), filarial worm *Wuchereria bancrofti* (Das *et al*., 1983) and the dog heartworm *Dirofilaria immitis* (Cheong *et al*., 1981). It bites especially in the day and also at night (Das *et al*., 1971; Ghosh and Hati, 1980; Das *et al*., 1983). The classification based on morphological features poses problems in many groups because of their small size, and a number of minor variations which arise in response to environment. In order to upgrade taxonomic parameters, the use of molecular entomological protocols has yielded valuable results (Xiang and Kochar 1991; Kambhampati 1995; Tang *et al.,* 1996). However, it has been realized that results obtained by application of a single technique are not sufficient because of complexity of the genome of populations, and as each parameter has its own limitations. Therefore, genetic analysis of species using a combination of different techniques has become desirable (Narang *et al*., 1993 a,b; Munstermann, 1994; Reinert *et al*., 1997; Chaudhry *et al*., 2006). Recently, DNA based techniques have been applied and there appears to be good agreement between the chromosomal and DNA-based genotypes (Favia *et al*., 1997). With the advances made in molecular biology, molecular genetics and molecular taxonomy are gaining more and more significance in terms of their utility in

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applied cytogenetic. Like most other insect groups, in mosquitoes too, studies have been carried out using sequences of nuclear ribosomal DNA (r DNA) genes, internal transcribed spacers (ITS) of rDNA, mitochondrial DNA and various randomly amplified polymorphic DNA (RAPD) segments, and restriction fragment length polymorphism (RFLP) markers (Black et al., 1989; Collins et al., 1990; Cooper et al., 1991; Kambhampati and Rai 1991a, b; Porter and Collins 1991; Ballinger-Crabtree et al., 1992; Apostol et al., 1994; Severson et al., 1994; Mutebi et al., 1997; Torres et al., 2000; Ayres et al., 2002; Van Bortel et al., 2002; Kengne et al., 2003). These studies have proved a useful source of DNA diagnostics of species. Analysis of these and other hypervariable regions is known to provide information about inter- and intra-specific genetic relatedness of mosquitoes of interest. The noncoding sequences forming ITS1 and 2 have been found to evolve at a relatively faster rate than the coding sequences, therefore these sequences are considered as ideal sources for the analysis of phylogenetic relationships between members of a species complex (Coleman and Heminway, 2007).

The explosion of research into the molecular genetics of mosquito vectors has dramatically altered the direction of, and thus interest in, mosquito population genetics. Once the sequence characteristics are used, further information regarding species relatedness and intra-species variations could then be obtained by studying the functional folding patterns of ITS1 and 2 based rRNA secondary structure (Wesson et al., 1992; Severini et al., 1996; Joseph et al., 1999; Dassanayake et al., 2008). Studies on the sequences of ITS1 and 2 have revealed that ITS2 has more important role for correct and efficient processing and maturation of 26S rRNA ribosomal units. It has also been observed that during the course of evolution, insertions and deletions (indels) have considerably influenced secondary structures which have an important role in efficient functioning of rDNA clusters. Motivated by the advances made in the field of population genetics of mosquitoes, the present work was planned to differentiate two allopatric populations of *Ar. subalbatus* by using molecular techniques and bioinformatics tools.

MATERIAL AND METHODS

Mosquito collection: Larvae of population A were collected from breeding and resting sites from the rural areas around Hamirpur, Himachal Pradesh in north India and reared in the laboratory up to adult stage. The adults of population B were procured from an urban area in south India (Malaria Research Centre, Goa).

Preservation of samples: Freshly hatched adults were stored in separate eppendorf tubes at –20°C for DNA extraction.

Morphological identification: Morphological identification was carried out under a microscope using morphological key by Wattal and Kalra (1967).

DNA Extraction: DNA was extracted by following the protocol of Ausubel et al. (1999) and the one previously standardized in this laboratory (Chaudhry and Sharma, 2006).

ITS1 and ITS2 amplification: PCR master mix was prepared by mixing 10X PCR buffer, dNTP mix (100mM each), $MgCl_2$, Taq polymerase (3units/µl), double distilled water and template DNA. The specific forward and reverse primers (FP, RP) used in the process were: ITS1-FP-5'-CCTTTGTACACACCGCCCGT-3', RP-5'-GTTCA TGTGTCCTGCAGTTCAC-3'; ITS2-FP-5'-TGTGAACTGCAG GACACAT-3', RP-5'-TATGCTTAAATTCAGGGGGT-3' (Sharpe et al.2000; Porter and Collins, 1991). The amplification reactions were performed as per the scheme of Williams et al. (1990) and Chaudhry and Kohli (2007). The PCR products and standard DNA ladder were electrophorased in 2% agarose gel.

Sequencing: The amplified products were sequenced and aligned with Clustal W multiple sequence alignment algorithm (www.ebi.ac.uk/clustalw/). The sequences were submitted to GenBank and accession numbers obtained.

PCR-RFLP analysis: Both ITS1 and 2 sequence products of population A and B were digested by using three restriction enzymes viz. *Hae*III, *Pst*I and *Hpa*II.

Interspersed and tandem repeats analysis: Both ITS1 and ITS2 sequences of population A and B were subjected to Spectral Repeat Finder (SRF) and Tandem Repeat Occurrence Locator (TROLL) programmes (Sharma *et al*. 2004; Benson, 1999) for identifying presence of interspersed and tandem repeats, respectively.

Secondary structure prediction: Secondary structure based analysis of ITS1 and ITS2 sequence of *Ar. subalbatus* was done with the application of RNAfold Web Server program included in Vienna RNA package (Hofacker, 2003). Structures inferred by RNAfold were examined for common stems, loops, and bulges.

RESULTS AND DISCUSSIONS

A single parameter of species identification is not sufficient, necessitating adoption of more than one parameter for species discrimination. The advances made in the field of molecular biology have made it possible to solve some of these problems. The PCR related approaches use amplification of random or selected regions of the genome by using sequence specific primers. These modified protocols of basic PCR technique have proved to be of immense practical utility in DNA diagnostics of mosquitoes (Munstermann, 1995; Cornel *et al*., 1996). These techniques (RAPD, RFLP) have the ability to detect differences between DNA of closely related organisms, studying population biology and genetic mapping. In addition to Random Amplified Polymorphic DNA-PCR (RAPD-PCR) (Wilkerson *et al.,* 1993; Ayres *et al.,* 2002), Restriction Fragment Length Polymorphism-PCR (RFLP-PCR) (Goswami *et al.,* 2005) and Arbitrary Fragment Length Polymorphism-PCR (AFLP-PCR) (Pieter *et al.,* 1995) have also been developed as the PCR technique variables to characterize DNA from different sources such as ribosomal DNA (Fritz *et al*., 1994; Djadid *et al.,* 2006), mitochondrial DNA (Foley *et al.,* 1998; Shouche and Patole, 2000), repetitive sequence DNA, telomeric DNA, internally transcribed spacer (ITS) DNA (Fritz *et al.,* 1994; Prakash *et al.,* 2006). But the most widely applied PCR assays target rDNA. It is organized as a tandemly repeated array of conserved genes punctuated by fast evolving non coding ITS1 and 2. Therefore, it is logical to use these spacers for the study of population genetics and present study is the first attempt to separate two populations of *Ar. subalbatus,* one each from north and south India.

ITS1 and ITS2 sequence divergence: Accordingly, the length of ITS1 sequence in population A and B varied from 674-757bp while ITS2 sequence in population A and B varied from 589-606bp (Figure1, Table 1). The sequences were submitted to GenBank with following accession numbers: ITS1 sequence, EU847227 and EU847226; ITS2 sequence, EU847231 and EU847230 for populations A and B, respectively.

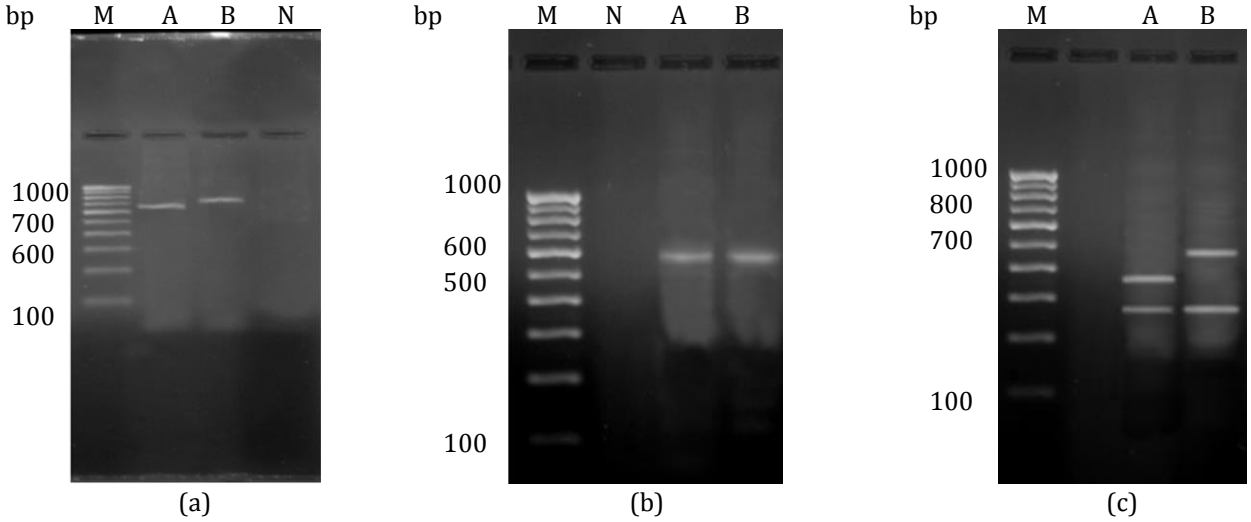

Figure 1. (a) PCR amplified ITS1 sequence of pop.A and B of *Ar. subalbatus*. Lane M- Gene ruler, Lane A-DNA band from pop.A, Lane B- DNA Band from pop.B, Lane N- negative control; (b) PCR amplified ITS1 sequence of pop.A and B of *Ar. subalbatus*. Lane A- DNA band from pop.A, Lane B- DNA Band from pop.B; (c) HaeIII restriction endonuclease digests of the amplified products from the ITS1 sequence of pop.A and B of *Ar. subalbatus*. Lane M- Gene ruler, Lane A- HaeIII digestion products of pop.A, Lane B- HaeIII digestion products of pop.B.

Therefore, in population level studies due to their hypervariable nature these spacers have been quite useful in drawing sequence based genetic kinship among species. For example, Dezfouli et al. (2002) amplified ITS2 region of *Anopheles fluviatilis* of South and South-East provinces of Iran in which all PCR generated segments were found to be 374bp in length. Therefore, no intaspecific variation was observed in the length of ITS2 spacer. Similarly, in our earlier investigations by Kaura et al. (2010), ITS2 sequence of seven different populations of *An. Subpictus* was found to be conserved in as many as six populations in which it varied in length from 491-687bp. Apart from the base pair length variation of ITS1 and 2 spacers, the G:C and A:T content and correlation of indel bias with intron length was also analyzed. Here, ITS1 and ITS2 sequences in both populations were also found to be G:C rich (Table 1).

Table 1. Average bp length and GC, AT content of ITS1 and 2 sequence in population A and B of *Ar. subalbatus*.

Sequence	Population	Total bp length	G:C%	A:T%
ITS1	A	674	52	47.8
	B	757	50.1	49.7
ITS2	A	589	54	46
	B	606	55	45

Dezfouli *et al*. (2002) studied the G:C content of ITS2 sequences of different populations of *An. fluviatilis* in which it was found to be 50% in all the populations. When ITS2 sequence of seven different populations A, B, C, D, E, F and G of *An. subpictus* were studied by Kaura *et al*., (2010), the sequence of all the populations was found to be GC rich. In addition to this, the number of insertions and deletions were also calculated. In ITS1 sequence, population A had 97 deletions and 9 insertions while population B had 9 deletions and 97 insertions. Similarly, ITS2 sequence revealed that population A had 33 deletions and 15 insertions while population B had 15 deletions and 33 insertions (Figure 2). Therefore, both ITS1 and 2 sequences of population A and B of this species showed positive correlation with intron length.

The variation in the intron length is mainly due to the presence of highly repetitive sequences, which results in higher base pair length of introns that make them useful for the study of inter- and intra-genomic variations at the level of populations and species. As a consequence of this, it was desirable to study different types of repeats in both the spacer sequences in the populations of this species. Studies carried out on this aspect of sequence characteristics have shown that repetitive sequences are presumed to be important in a number of regulatory functions and are principle causes of genomic instability (Zhang and Hewitt, 1997, 2003). The repetitive sequences with high level of polymorphism also influence the functional DNA (Tautz *et al*., 1986; Kashi *et al*., 1997). Banerjee et al. (2007) studied the interspersed and tandem repeats in ITS2 of 18 mosquito species from diverse geographical locations which included nine species each from genera *Aedes* and *Anopheles.* According to them dimer frequency in the interspersed repeats of all the species showed considerable variation as the dimer CA was found to be common in four species of genus *Anopheles* while the dimers with base sequence TC, GC, TG and GC of four species of genus *Aedes* had the same copy number 16.

Repeats analysis: In ITS1 sequence as revealed by SRF, pentamer and polymer were missing from population A. In comparison, population B had pentamer GAGGT and three types of polymers with base sequence CCGAAG, CATGACCC and CCGAACACA (Table 2). Dimers were not found in population B and none of the interspersed repeats was found to be common in both populations. In ITS2 sequence, the results obtained by application of SRF revealed that pentamer and polymer were missing from population, while in comparison, population B had pentamer AGAAA and two types of polymers CCCCTCTCT and CCCCTCCCCT (Table 2). Trimer and tetramer were not found in population B. In order to find tandem repeats, ITS1 and ITS2 sequences of both populations of *Ar. subalbatus* were also subjected to TROLL and none of the repeats was found to be common in both populations (Table 3).

Table 2. Spectral repeat finder based analysis of ITS1 and 2 sequence analysis.

Sequence	Population	Dimer		Trimer		Tetramer		Pentamer		Polymer	
		Sequence	Copy No.	Sequence	Copy No.	Sequence	Copy No.	Sequence	Copy No.	Sequence	Copy No.
ITS1	A	TG	35	AAC	24	CGTG	43	-	-	-	-
	B	-	-	ACC	30	GAAG	26	GAGGT	18	CCGAAG	14
										CATGACCC	14
										CCGAACACA	6
ITS2	A	CG	31	-	-	TGAC	10	ACCAT	7	ACGGGT	5
								CCTAC	7	ACCTTGAGT	5
								GTGCT	7	GTATCGCAC	5
	B	-	-	GTG	21	CGTG	44	-	-	CTCGGCGTG	6

CLUSTAL 2.0.12 multiple sequence alignment

```
A_       AACGATTTGTACCACCGGGC---GTCTATTGTGGTCTCTGCCCAGGCGATTGTTCGGCGG 57
B_       ---CTTGTGTACCGATGGATTATATTAGCAGAGGTCTCTG--GAGGCTCACCTGCCGCGG 55
              * ******   **      *     * ********   ****     * * ****

A_       TCCCTTCGTGTATCTGCTTGACTCATGAAAA----TTGACCGAACTTGATGATTATATAG 113
B_       TTCCTCCGTG-AGCTGCAGGACACATGAAAAGAAGTTGACCGAACTTGATGATT-TAGAG 113
         * *** **** * ****  *** ********    ****************** ** **

A_       GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGGTAC 173
B_       GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGATCA 173
         ************************************************** * *

A_       CCCCCCACCGCCAAACAATGATG-----ACGAGAGTTGGAGCG---AGCGAGCGAGCGAG 225
B_       CTTCCAACCGAGAGTTTATGGCGTGCACATGATCGTTAACCTATACAGCCGCCAAACAAG 233
         *  ** ****  *    ***  *     * **  ***         ***   * * * **

A_       TGTAGTGTTGT-TGTC----------TGTGGCGAGTACACGGCGAGCCCCCA---CCACC 271
B_       CGTAACGATGTCTGTCATGAAACTGGTGTCGTTAACGGACGAGGTGTCCAAAGGTCTAAG 293
         ***  * *** ****          *** *  *    ***  * * **  *   * *

A_       AGTGGGCTCC-------GTCCGGCCACCGCAGAGAGG-----AACAACACAAACACAACA 319
B_       AGCTGGCTACAATTCAAGTTGTGTCAGCAAAGCGAGGTGTCGAAGTATACGAACACGACT 353
         **  **** *       **   * ** *  ** ****     **  * ** ***** **

A_       ACCA---AAAACCTGGTGCG--TTACCCTGTTCTGTGCTGTGTGAACTA-GCCTTCCGTT 373
B_       CGCAGGTAAGACTTAGCAAAACTTACCATAAACC-TGGAGTTTAAGGTGCGCACATGATC 412
         **   ** ** * *      ***** *   *  **  ** * *  *  **      *

A_       GCTG-CTGCTGCTGCTGCTGCTGCTGCGCTCCCCCCCCCCATGTCGTCAAGGGTTGT 432
B_       GTTAACCTATACAGCCGCC-TAACACAAGTGTTCGACTGAACCGAACACACGGGGGAAGG 471
         * *  *   * * ** **     *    * * **  *    **       *  ***  *

A_       CATTACCGATCACTTCCAACCGAGAGTTTATGGCGTGCACATGAT---CGTTAACCTATA 489
B_       C-CTGCCGAACCTTACCCCTTGGGGTGAAAGACCAAATACACAATGCACATGACCCTCCA 530
         *  * **** *  * **    * *     *   *    ***  **   * * * ***  *

A_       CAGCCGCCAAACAAGCG-TAACGTCATTACC--GATCACTTCCAACCGAGAGTTTATGGC 546
B_       TAACCGTAGGAGAGTCGACCATGTGTTCGACTGAAGCGAACCGAACAGGGAATACATACC 590
         * ***    * *  **   * **  *   *   * *    * *** * ** *  **  *

A_       GTGCACA------TGATCGTTAACC---TATACAGCCGCCAAACAAGCGTAACGATGTCT 597
B_       CTCCATAACCCTATGAGAGTCGACCAAGTGTTCGACCG--AAGCGAACCCGACGGGGATG 648
         * ** *      ***  **  ***   * * *  ***  ** * * *   ***  *

A_       GTCATGAAACTGGTGTCGTTAACGGATGTCTGTCATGAAACTGGTGTCGTTAACGGACGA 657
B_       --CATACCCTTCATAACCCCAAGGAGAGTCGGGCAAGTGTCATGGACCACATACGGGGAA 706
         ***     *  *  *   ** *   *** * ** *   *  *   *    ****   *

A_       GGTGTCCAAAGGTCTAA---------------------------------- 674
B_       GGCATACCGTACCTTACCCCAAGGAGAGTTGGACCATGGTCCTACGTCCCA 757
         **  * *       **
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(i)

CLUSTAL 2.0.12 multiple sequence alignment

```
A_      ------------GACGCCGTGACGCATATGCACACGTACTACAGTACGATGTACACATTT 48
B_      CCCCCTCCCCCCCCCCCCCTCCCCTCCCCTCCCCCCCCCCCCCCCCCCCCTCCCCCCCGCTTGTCC 60
        * ** *  *       * * *   *  *  * *      *   *

A_      TTGGTGCCTATAT------TTATCCATTCAACTGTGCACGCCCCGCGTGTACGCGTAAGT 102
B_      TCGTCGCCTCTGTAGCAGCTGCACCCCTCGTTCGTACTCTTCCCCTCTCTCCAGATATGT 120
        * *  **** * *      *   **  **    ** * *  ***   * * *   ** **

A_      GACGTT--TTCCTAAC-CGCCACCACCACCCCCGGTTT------GGTAGAAAAACAAAAA 153
B_      GATGGCGGCACACAGCACGCGAAAGTCTCGCATGGCCACTCGGGGGTGAGAGGTCGGAGT 180
        ** *      *  * * *** *    * * *  **         ***   *   *  *

A_      AACGGCGCCAGTTGTTCGGGGGGCCGCCGCCGGATGATGACAACTTCCATTATAAACAAC 213
B_      GCGGCCGCTCGGCGCTCGCGGG-CGGTCTCCGAATGTAAGAGAGGACGGAGGTGACGGTT 239
        * ***  *  * *** *** * * * *** ***      *   *     * *

A_      AACACCACCATCATCATGATCTGGGTGTGGGGGGGGGTGGGTGGTGTTTGTTTTTTTCCA 273
B_      GTGTGTCCTGTGCACACTCTTAGGATGCAGAGAGAAGAAAGTCGTGAGGAGAGAGATCCG 299
        *  *   **   *  ** **  * * *  *   ** ***          ***

A_      TCACCCCAAATAATAGATCCTCCTTCGTAG----GCCGCAAAAAAAGTGTGACTACCCCC 329
B_      G-AAGAAGAGTAATAGGGCATGCGCAGGGGTGGCGCCGCACGCAAAGAGATAAAAAACAA 358
        *     * ******  * * *   *  *    ******   **** *  *  *  *

A_      CCCCTTTAAAAATAAAAAAAATGGTGTTGGTTGG-GTTTGTGGGGGTGGTGGGGGTTGTG 388
B_      AGCAAAACAGAAGAGGAGAAAGGGAAAAAATGAAAGAAAGAGAGGGAAGAGCGTGCAACC 418
        *     * ** *  * *** **      *    *   * * ***  * * * *

A_      GTGGGGTTGTTTGTTGGTTTTGTTTGGTGGTTGTGTTGGGGGGGGGGGCGGGGGGAGTGG 448
B_      AGGGAAAGAAAGGGAAGCACAATGGAAAAGCGGGTTAGCCTCGATTCACAACATTAGAGA 478
        **        *   *     *      *  *  * *    *     *      ** *

A_      TT--GGCGTGTTCGTTGTGTGGTGCGGATGGTGGATGTGAGTTGTGTGCGGTGTGTTCGT 506
B_      TTTAGGACAATTAGCTTACTCATCCGTAGATATCTTATTATTTAC---CAAGGTTAGCGT 535
        **  **    ** * *   *  * ** *       * * * **     *   **   ***

A_      CTGGTGGTTGTCGTGTGTCTCGAGTGTCTGTCGCTGCTCGCTTCGTAGGAGGTGTGGCTA 566
B_      TGCGAGCTTATTGATTATA-CAGATTTCTGTCATAAATCAGACATCAAGCACCACAGATA 594
        * * ** * *  * *  *   * ******     **       * *       * **

A_      TGTGTGCGGCTGATGTGGGTGTG 589
B_      TACATACATATG---------- 606
        *   * *   **
```

(ii)

Figure 2. Multiple sequence alignment of ITS1 (i) and 2 (ii) sequence of pop.A and B of *Ar. subalbatus* (*- identical bases, --insertions/deletions (indels), type of repeat (red- interspersed, blue- tandem repeat).

Table 3. Tandem repeats in ITS1 and 2 sequence of population A and B of *Ar. subalbatus.*

Sequence	Population	Sequence
ITS1 Sequence	A	(GAGC)*4, (AGTGT)2, (AACACA)2, (CTGTG)2, (TGC)10, (C)10
	B	(CGAAC)2
ITS2 Sequence	A	(TAAAAA)*2 , (G)11
	B	(C)13, (CCCCT)2

*- copy number

Restriction sites divergence: Restriction digest of ITS1 sequence of population A and B by *Hae*III produced DNA bands of distinct sizes, while none was produced with *Pst*I and *Hpa*II. No band was produced from ITS2 sequence of population A and B by using either of the three restriction enzymes (Table 4). RFLP-PCR

technique was applied for the first time by Reno et al. (2000) to differentiate two sibling species *Aedes triseriatus* and *Aedes hendersoni* by mplifying ITS1 and 2 sequences. Of them, *Hae*III was found to be an ideal endonuclease for obtaining differences at intragenomic level from ITS1 sequence.

Table 4. PCR product size after the digestion of ITS1 and 2sequence of population A and B of *Ar. subalbatus* by using restriction enzymes *Hae*III, *Pst*I and *Hpa*II.

Population	PCR product size in bp	RFLP-PCR product size in bp		
		HaeIII	PstI	HpaII
A	674	238, 388	No restriction site	No restriction site
B	757	471, 286	No restriction site	No restriction site

ITS1 and 2 Secondary structure analysis: Once the sequence characteristics of ITS1 and 2 were analyzed by using various parameters, further information regarding species relatedness and intraspecies variations could then be drawn by studying the ITS1 and 2 based RNA secondary structure prediction from their functional folding patterns (Wesson et al., 1992; Severini et al., 1996; Joseph et al., 1999; Dassanayake et al., 2008). Accordingly, the RNA secondary structure of populations of Ar. subalbatus showed considerable variation in the results obtained by the application of RNAdraw programme (Figure 3). Different types of loops such as hairpin, bulge, interior, multibranch and tetra loop were studied. The number of all type of loops varied except multibranch loop which was found to be same in both populations (Table 5). The common motifs in secondary structure of both populations were also studied and only two motifs were found common in populations of both ITS1 and 2 sequences (Figure 3).

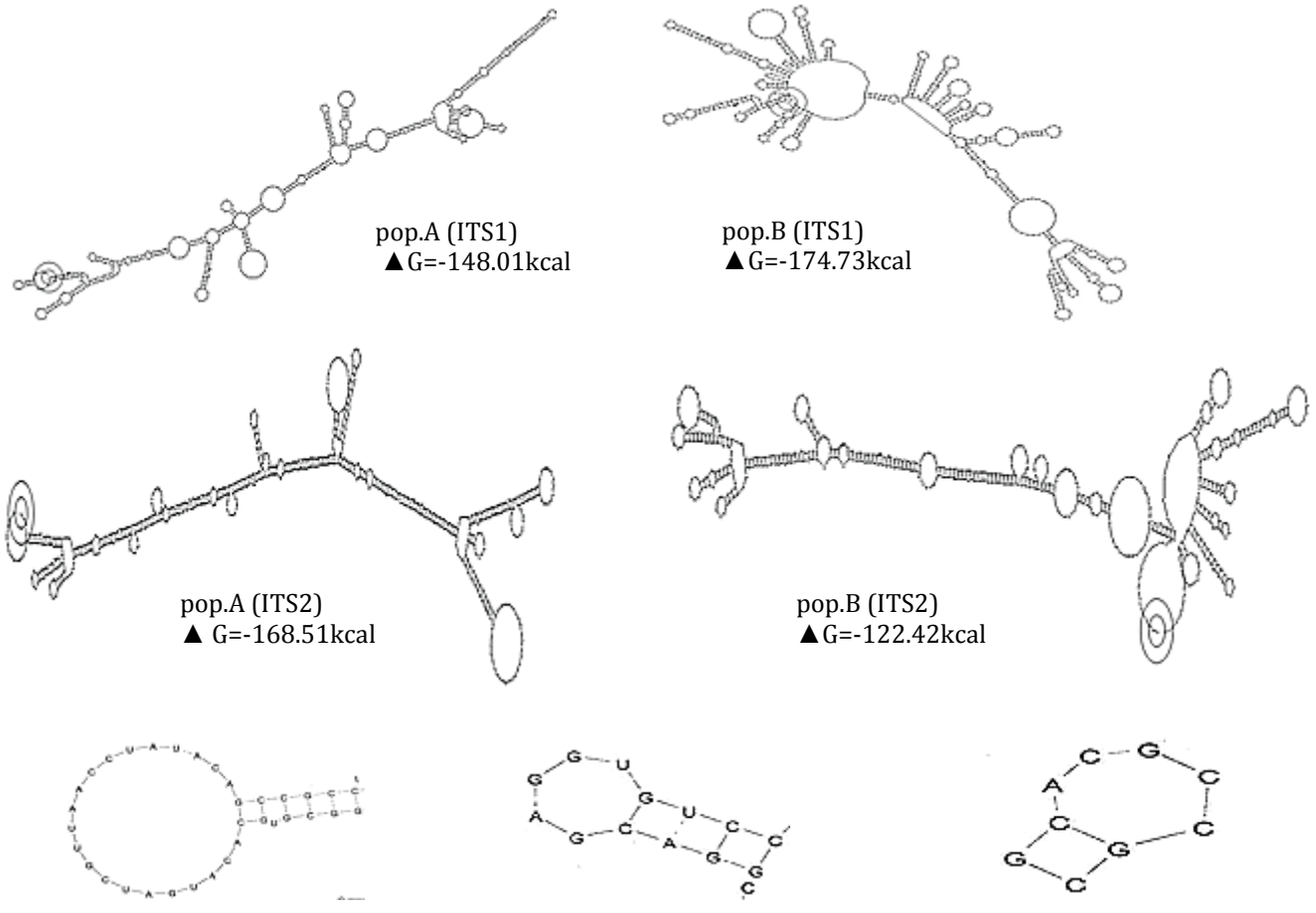

Figure 3. Secondary structure of ITS1 and ITS2 SEQUENCE of pop.A and pop.B of *Ar. subalbatus* along with their common loops.

Table 5. Different type of loops in ITS1 and ITS2 sequence of population A and B of rDNA based secondary structure.

Sequence	Population	No. of hairpin loop	No. of bulge loop	No. of internal loop	No. of multibranch loop	No. of tetra loop
ITS1	A	9	13	14	3	5*
	B	13	15	13	4	5*
ITS2	A	6	19	7	2*	2
	B	10	15	12	2*	1

*- common loop

Similarly, the parameters for the study of RNA secondary structure prediction such as structural energy, maximum and minimum heat formation, G+C and G-C, A-U and G-U content were also analyzed (Table 6). It was found that differences were observed in G-C and A-U combinations, number of stems, structural energy within both the populations from both ITS1 and 2 sequence. Therefore, there is lesser rRNA structural homologies between allopatric populations. Bhargavi *et al*. (2005) studied the ITS2 sequence based RNA secondary structure of different species of *Culex* belonging to different geographical locations. In this study, *Cx. pipens and Cx. quinquefasciatus* had highest negative energy of -149.38kCal and -148.23kCal followed by *Cx. tarsalis* -129.2kCal, *Cx. vishnui* -115.3kCal, *Cx. pseudovishnui* -105.66kCal and *Cx. tritaenorhynchus* -82.57kCal. As for the common motifs two motifs with UGUCG and CUUCGGUG were found to be highly conserved in all the seven species covered in their study.

Table 6. Length, G+C content (%), G-C, A-U, G-U base pair number, number of stems, energy (kcal) and minimum and maximum heat formation (kcal) for the secondary structure of ITS1 and 2 sequence based rRNA of population A and B of *Ar. subalbatus*.

Sequence	Population	G+C	G-C	A-U	G-U	No. of stems	Energy	Min. heat formation (kcal)	Max. heat formation (kcal)
ITS1	A	51%	103	73	25	47	-148.01	5.716	46.391
	B	49%	122	84	19	54	-174.73	6.315	34.619
ITS2	A	52%	99	52	50	39	-168.51	6.258	61.697
	B	53%	88	58	25	40	-122.42	2.592	39.462

CONCLUSION

Therefore, from the present comparative data it is evident that study of ITS1 and 2 spacers, repeats and their secondary structure is suitable for differentiating allopatric populations of *Ar. subalabtus*. It may also be suggested that both populations are adapted to different set of environmental conditions. Further studies on more populations and their nucleotides, repeats and secondary structures can help in understanding intraspecific variations in this important non-anopheline vector of human disease.

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