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Secondary structure and restriction based analysis of the ribosomal DNA internal transcribed spacer regions (ITS 1 and 2) of allopatric populations of *Anopheles stephensi* (Diptera: Culicidae)

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Abstract
Accurate identification of anopheline species is essential for vector incrimination and implementation of appropriate control strategies. Correct vector identification is very important to design strategies for managing vector borne diseases. Moreover, many closely related species of mosquitoes with differing ecological and host preferences are nearly indistinguishable morphologically. These factors mean that the identification of mosquitoes to a species or even a genus is often difficult. As a consequence, DNA-based approaches to mosquito identification, genetic diversity, and molecular phylogeny have gained increasing adoption. However, species and population determination is complicated by cryptic morphology and intra-individual variation. Herein, we propose a simple and reliable PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) based method for the differentiation of allopatric populations of *Anopheles stephensi* by using internal transcribed spacers (ITS 1 and 2).

Keywords: *Anopheles stephensi* populations, Allopatric, Secondary Structure Analysis, ITS 1, ITS 2.

1. Introduction
Eukaryotic ribosomal DNA (rDNA) has several properties that make it useful for studying genetic variability and divergence within and between species: tandemly repeated genes, secondary structure of transcribed regions, differential rates of evolution between spacers and coding regions, and concerted evolution [1, 9, 13, 30, 33]. rDNA is composed of tandemly repeated transcriptional units separated from each other by intergenic spacers. Each transcriptional unit, in turn, includes spacers and regions coding for the ribosomal subunits. Like most other insect groups, in mosquitoes too, studies have been carried out using sequences of internal transcribed spacers (ITS) of rDNA [5, 9, 14, 15, 22, 30]. Analysis of these hypervariable regions are known to provide information about the inter and intraspecific genetic relatedness of mosquitoes of interest.
Recently, DNA based methods of identification have been used as tools for unequivocal species determination. Among them, molecular analysis of the ITS regions of rDNA has proved of great value in species differentiation [19]. In *Anopheles*, ITS 1 variation is readily detected among individuals of a single species [3, 11] and this variation has been used to identify populations or strains within a species [3, 17, 21, 32]. In contrast, ITS 2 variation is low or not detectable within a species. Considering that accurate identification of Anopheline species is essential for vector incrimination, the purpose of this study was to develop a rapid and accurate high throughput assay based on Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of ITS 1 and 2 sequences for reliable identification of the allopatric populations of *Anopheles stephensi*.

2. Materials and Methods
Mosquitoes
Gravid females of *An. stephensi* (population A) were collected from resting and breeding sites in the cattle sheds of village Beladhayani near Nangal (105 km North-West of Chandigarh) during the period of September, 2006 to October, 2008.

These field collected gravid females were allowed to lay eggs on a wet filter paper placed in the test tubes. The eggs received in this way were used to raise a colony of larvae in the mosquito rearing laboratory. The larval stocks were fed on protein rich diet consisting of a mixture of finely powdered yeast and dog biscuits mixed in the ratio of 6:4 [29, 8]. The freshly hatched female specimens were stored in dry eppendorf tubes until processed for the extraction of DNA while the adults of population B were procured from an urban area in south India (Malaria Research Centre, Goa). The ITS 2 sequences of 5 different populations of *An. stephensi* were taken from GenBank.

Morphological identification
Morphological identification was carried out under a microscope using morphological key by [31].

DNA Extraction
DNA was extracted from the legs of individual female mosquitoes by following the protocol of [2] and the one previously standardized in this laboratory [7, 16].

Amplification of rDNA ITS 1 and ITS 2 region
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: ITS 1- FP-5' -CCTTTGTACACACCGCCCGT-3', RP-5'-GTTCATGTGTCCTGCAGTTCAC-3'; ITS 2- FP- 5'-TGTGAACTGCAGGACACAT-3', RP- 5'-TATGCTTAAATTCAGGGGGT-3' [27, 22]. The amplification reactions were performed as per the scheme of [33] and [6]. 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 the accession numbers were obtained.

Restriction analysis of PCR products
Amplified DNA from both ITS 1 and 2 sequences of populations A and B were digested in separate reactions by using three restriction enzymes viz. *Hae*III, *Pst*I and *Hpa*II. Digested DNA was analyzed on 2% agarose gels in 1x TAE buffer.

Secondary structure prediction
Secondary structure based analysis of ITS 1 and ITS 2 sequence of *An. stephensi* was done with the application of RNAdraw Web Server program included in Vienna RNA package [20]. Structures inferred by RNAdraw were examined for common stems, loops, and bulges.

Identity and Similarity Analysis
The percentage of identity and similarity of ITS 1 and 2 sequences of all the populations were also analysed.

Phylogenetic Analysis
A phylogenetic tree (phylogram) was also prepared by using Gene Bee Service programme [34].

3. Results
In figure 1a,b, lane M carries the bands of standard DNA ladder while lanes A and B have the amplified products from ITS 1 and 2 of populations A and B of *Anopheles stephensi*. The length of ITS 1 sequence was found to be 685 bp in population A and 642 bp in population B while the ITS 2 sequence was found to be 589 bp and 600 bp in population A and B, respectively. The length difference of ITS 1-rDNA was 43 bp and of ITS 2 was 17 bp between the two populations. The length of the ITS 2 sequences of population C-E taken from GenBank ranged from 458-652 bp. The DNA of these bands was sequenced and accession numbers were obtained: ITS 1- population A (EU847222), population B (EU847221); ITS 2- population A (EU847233), population B (HQ703001). The accession numbers of sequences obtained from PubMed for ITS 2 region were as follows: population C (EF192275) (Nadasahib), EF192275 D (AY157316) (Chabbar), population E (AY157678) (Khash), population F (EU346653) (Hewizeh) and population G (EU346652) (Kourna). The ITS 1 and 2 sequences of all the populations were then aligned with Clustal W multiple sequence alignment algorithm (www.ebi.ac.uk/clustalw/). These sequences from all the populations were found to be G: C rich. Therefore, to distinguish between allopatric populations on the basis of ITS 1 and 2, amplicon sizes were not sufficient and we developed a PCR-RFLP assay for the same.

PCR-RFLP of ITS1 Sequence
The ITS 1 amplicons were digested with three restriction enzymes viz. *Hae*III, *Hpa*II and *Pst*I yielding distinct banding pattern with *Hae*III and *Pst*I unique to both the populations (Fig. 1c, d, Table 1), while no site of *Hpa*II enzyme was found in either population.

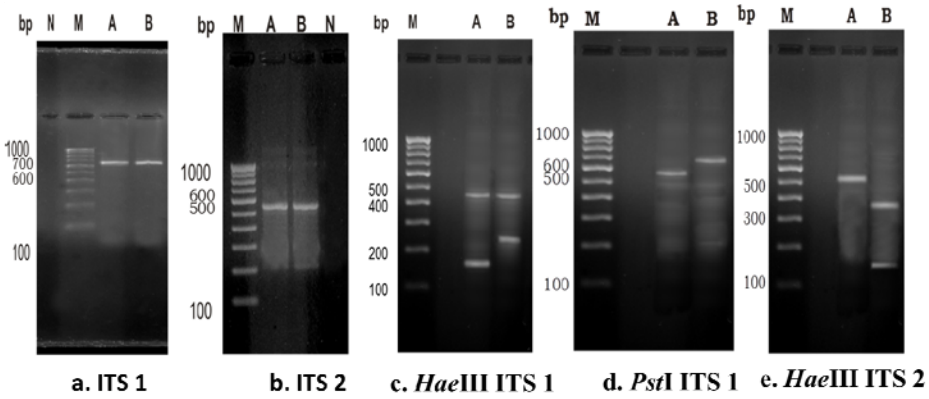

Fig 1: a, b: PCR amplified ITS1 & 2 sequence) of populations A and B of *An. stephensi*. Lane N- negative control, Lane M- Gene ruler, Lane A- DNA band from pop. A, Lane B- DNA band from pop. B.
c,d,e- HaeIII and PstI restriction endonuclease digests of the amplified products from the ITS1 and 2 sequence of pop.A and B of *An. stephensi*. Lane M- Gene ruler, Lane A- digestion products (DNA band) of pop.A, Lane B- digestion products from (DNA band) of pop.B.

Table 1: PCR product size after the digestion of ITS1 and 2 sequence of all the populations of *An. stephensi* by using restriction enzymes *Hae*III, *Pst*I and *Hpa*II.

Sequence	Population	PCR product size in bp	RFLP-PCR product size in bp		
			HaeIII	PstI	HpaII
ITS1	A	642	440, 172	68, 572	No restriction site
	B	685	440, 245	No restriction site	No restriction site
ITS2	A	590	508, 82	No restriction site	No restriction site
	B	532	380, 152	No restriction site	No restriction site
	C	458	372,86	No restriction site	343,115
	D	652	No restriction site	No restriction site	441,211
	E	652	No restriction site	No restriction site	442,210
	F	601	No restriction site	No restriction site	387,214
	G	648	No restriction site	No restriction site	431,217

PCR-RFLP of ITS 2 Sequence

The ITS 2 amplicons of population A and B were also digested with *Hae*III, *Hpa*II and *Pst*I. In addition, bioinformatic analyses of other 5 populations were also predicted by NEB cutter. With *Hae*III restriction enzyme the three populations A, B and C showed distinct RFLP patterns while the populations D, E, F, G show distinct patterns with *Hpa*II enzyme (Fig. 1e, Table 1). No cutting site was observed with *Pst*I enzyme. Therefore, *Hae*III and *Hpa*II enzymes were found to be suitable for distinguishing allopatric populations of *An. stephensi*.

All the sequences of ITS1 and 2 regions were also aligned with Clustal W multiple sequence alignment algorithm (www.ebi.ac.uk/clustalw/). These sequences from all the populations were found to be G: C rich. In Fig. 2 the loci marked with asterisks (*) indicate those regions where nucleotide sequences are identical. Dashes (-) were used to indicate the insertions and/ or deletions (indels). In addition to the regions marked as * and -, some regions marked with arrows provide examples of the differences in the complementary bases in the sequences arising due to substitutions in the form of transitions and transversions (ts/tv). From these results the correlation of indel bias with intron length was also analysed (Table 2). The number of deletions in a sequence is expected to be more in those cases where intron length is short, therefore in the present case the indel bias show positive correlation with intron length in both ITS 1 and 2 sequences of all the populations.

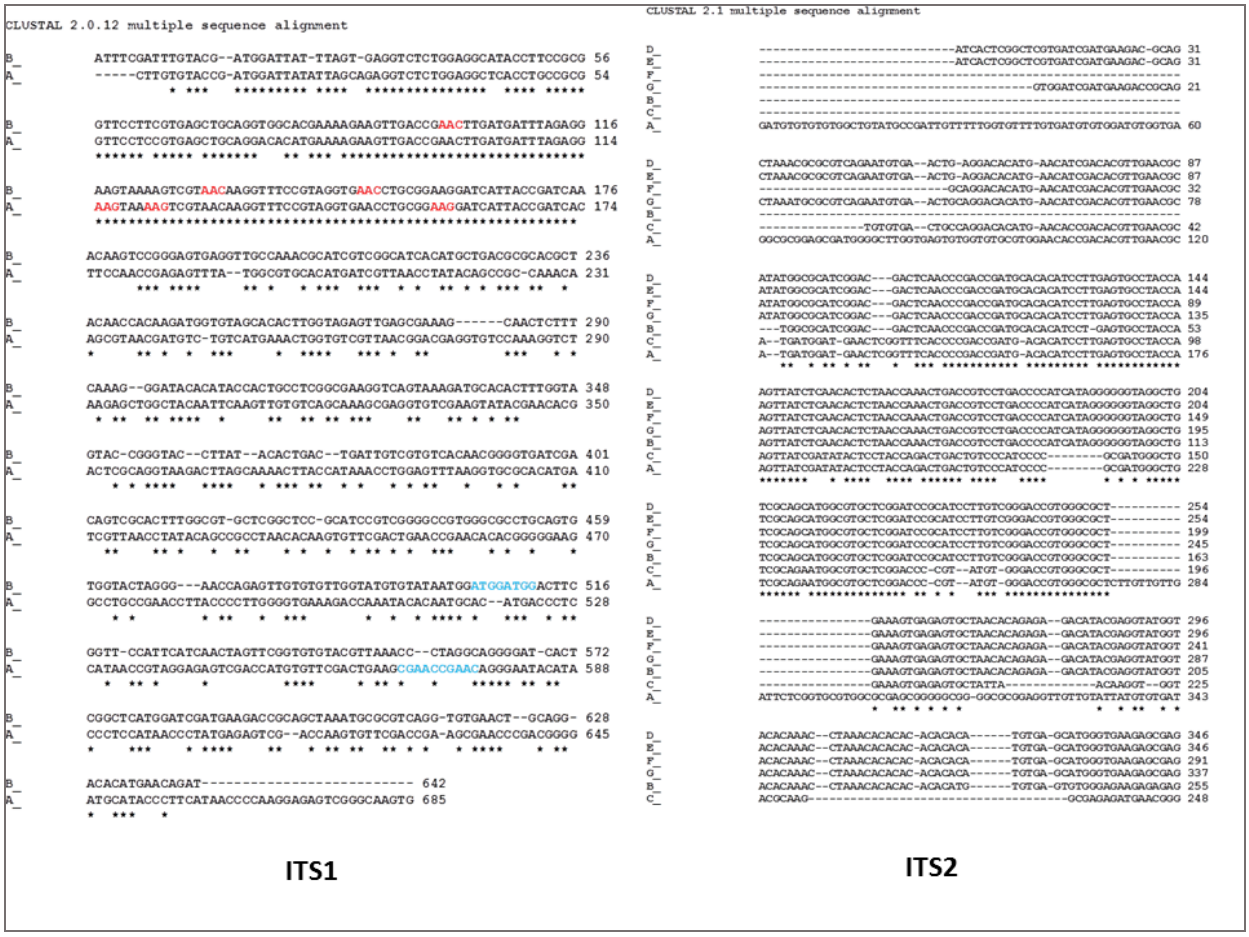

Fig 2: Multiple sequence alignment of ITS1 (pop.A, B) and 2 sequence (pop.A, B, C, D, E, F and G) of *An. stephensi* *- identical bases, -- insertions/ deletions (indels).

Table 2: Correlation of indel bias with intron length of ITS1 and 2 sequence of all the populations of *An. stephensi*.

Sequence	Population	No. of deletions	No. of insertions	Correlation
ITS1	A	15	60	+
	B	60	15	+
ITS2	A	143	206	+
	B	206	142	+
	C	278	20	+
	D	84	287	+
	E	84	287	+
	F	137	222	+
	G	90	238	+

Secondary structure Analysis

Once the sequence characteristics of ITS 1 and 2 were analyzed, the secondary structures of rDNA ITS 1 and 2 were predicted by using RNAdraw programme. The structural energy, number of stems, minimum, maximum heat formation, different types of loops such as hairpin, bulge, interior, multibranch and tetra loop and common loops were studied.

ITS 1 secondary structure analysis

The secondary structures of ITS 1 sequence of all the four populations were generated and none of the type of loop was found to be common. There was difference in the structural energy between the populations and only one motif was found to be common (Fig. 3, Table 3).

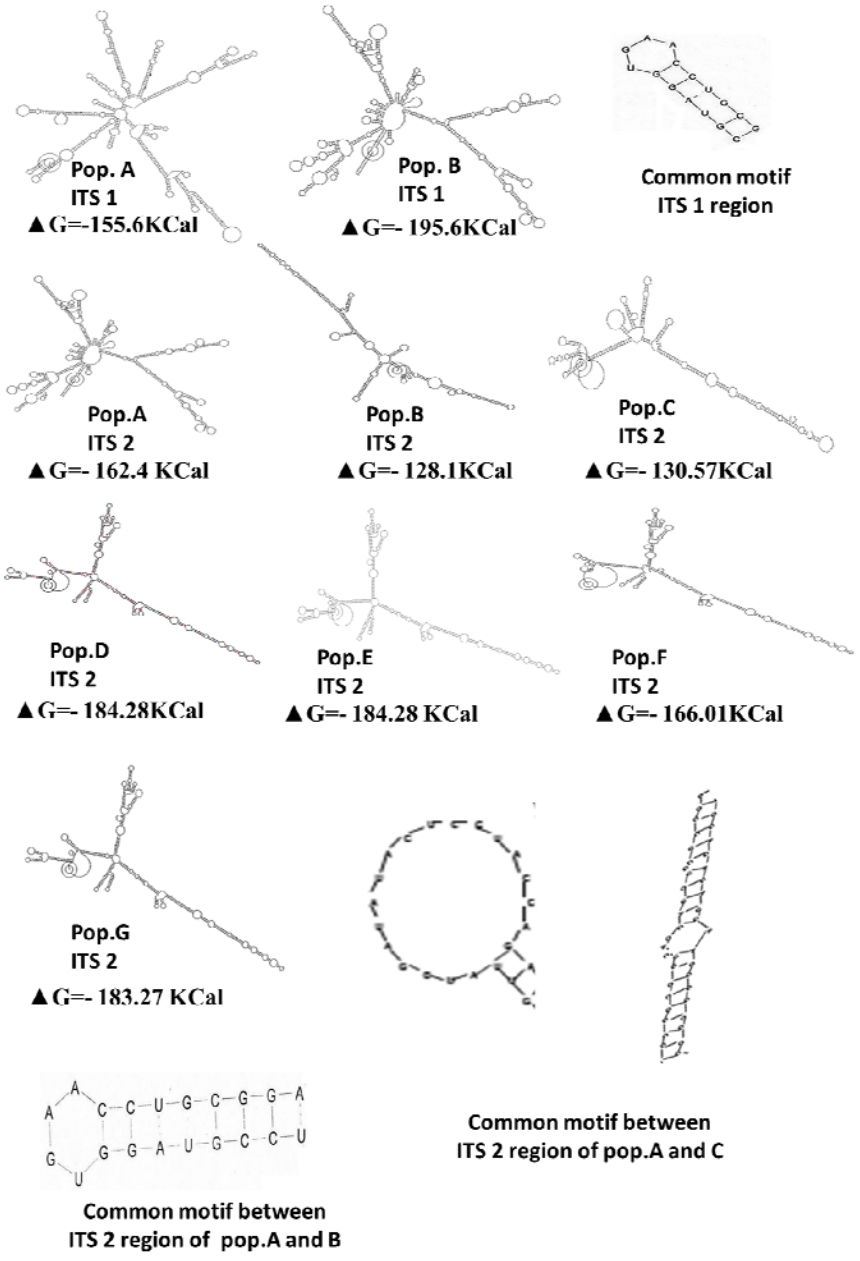

Fig 3: Secondary structure of ITS1 and 2 sequence of all the populations of *An. stephensi* along with their common loops.

Table 3: Different type of loops in ITS1 and ITS2 sequence of all the populations of *An. stephensi* based on rDNA 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	16	9	5	0	3
	B	9	17	1	7	5
ITS2	A	7*	12*	12	2*	1*
	B	10	8	9	1	4
	C	7*	12*	11	2*	1*
	D	8*	1*	14*	5*	5*
	E	8*	1*	14*	5*	5*
	F	7	3	13	5*	5*
	G	8*	1*	14*	5*	5*

*- common loop

ITS 2 secondary structure analysis

Secondary structures were drawn of all the seven allopatric populations and it was found that population A shared maximum number of common type of loops with population C, population D with E and population F with G (Table 3, Fig. 3). The structural energy was also in accordance with the type of common loops (Table 4). The population D, E, F and G structure shared 99% similarity while their structures were found to be different from the populations forming separate clade i.e. populations A,B and C. The populations A and C which forms a clade had 2 motifs in common while populations A and B shared only one motif.

Table 4: Length, 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 on rDNA secondary structure of all the populations of *An. stephensi*.

Sequence	Population	G-C	A-U	G-U	No. of stems	Energy (kcal)	Min. heat formation (kcal)	Max. heat formation (kcal)
ITS1	A	100	62	16	41	-155.6	5.885	30.860
	B	105	78	32	45	-195.6	3.089	79.880
ITS2	A	94	50	58	40	-162.4	7.904	40.332
	B	81	43	13	30	-128.1	2.490	26.482
	C	79	38	22	30	-130.57	3.065	48.1501
	D	121	68	22	48	-184.28	3.548	68.7169
	E	121	68	22	48	-184.28	3.548	68.7169
	F	108	61	19	42	-166.01	3.052	66.5475
	G	119	69	20	48	-183.27	3.047	69.463

In addition, ITS 1 sequences have been found to display high levels of sequence variation even among individuals [11]. As such, it will be necessary to determine the extent of ITS 1 sequence variation. The level of variation of ITS 1 sequences enabled the identification of populations of this species. We were not able to detect more ITS 1 sequence variation due to lack of ITS 1 sequence available on GenBank.

Phylogenetic Analysis

A phylogram was generated with the help of Gene Bee Service programme [34] (Fig. 4). Populations F and G from Hewizeh and Kourna district of Iraq, and D and E from Chabbar and Khash of Iran formed the first and second clades while populations A and C from Nangal and Nadasahib formed the third clade, and B from Goa joins the third clade in accordance with their allopatric status.

Identity and similarity analysis

To study percentage of homology between the ITS 1 and ITS 2 sequence of different populations the identity and similarity was calculated by on line calculator.

ITS1 sequence

The population A showed 52.6% identity and 57.9% similarity with population B indicating wide difference in the nucleotide set up of both the populations which might generated the difference in restriction site of a particular enzyme.

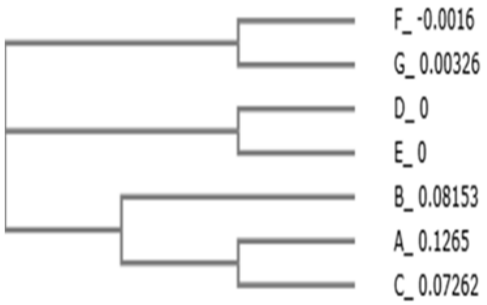

Fig 4: The phylogram of ITS2 region of seven populations of *An. stephensi*.

ITS2 sequence

The population D showed 100% identity and similarity with population E, population F and G showed 93.5% whereas population A and C showed 63.7% identity and 64.9% similarity, population A and B had 44% identity and 47% similarity, population B and C had 61.7% identity and 64.2% similarity (Table 5).

Table 5: Percentage of Identity and similarity of ITS2 sequence between pop. A, B, C, D, E, F and G of *An. stephensi.*

IDENTITY RESULTS							
D	100%						
E	100%	100%					
F	91.19%	91.19%	100%				
G	96.61%	96.61%	93.49%	100%			
B	73.71%	73.71%	80.62%	74.52%	100%		
C	59.21%	59.21%	64.36%	60.02%	61.65%	100%	
A	46.47%	46.47%	43.08%	45.12%	44.03%	63.68%	100%
	D	**E**	**F**	**G**	**B**	**C**	**A**

SIMILARITY RESULTS							
D	100%						
E	100%	100%					
F	91.19%	91.19%	100%				
G	96.61%	96.61%	93.49%	100%			
B	75.47%	75.47%	82.38%	76.28%	100%		
C	61.78%	61.78%	66.8%	62.6%	64.22%	100%	
A	49.32%	49.32%	45.52%	47.69%	47.01%	64.9%	100%
	D	**E**	**F**	**G**	**B**	**C**	**A**

4. Discussion

In recent years, rapid progress has been made in the development of molecular approaches to the identifications of mosquitoes. 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 transcribed spacers are thought to contain conserved structures important in forming the mature ribosomal amplicon [34, 19]. Consequently, the identifying and quantifying levels of intragenomic and intraspecific variations among ITS sequences are of paramount importance [18]. Such difficulties in morphological identification of sibling species and allopatric populations prompted this study.

Molecular techniques that can differentiate closely related species on the basis of their ITS 2 are well documented in *Anopheles,* and the low level of intra-specific sequence variation has proven useful as the basis for species-specific PCR assays, including the African *An. (Cellia) gambiae* Giles complex [26] and the Holarctic *An. (Anopheles) maculipennis* Meigen group [10, 24]. ITS 2 sequences have also been used in taxonomic studies of closely related species in the subgenus *Nyssoryhnchus,* including *An. nuneztovari* Gabaldón [12] and *An. oswaldoi* [28], for species characterization [19], and in phylogenetic reconstruction [25].

A few years ago a PCR based assay for a quick and reliable identification of six Palearctic sibling species of the *An. maculipennis* complex was presented by making use of the differences in the nucleotide sequence of ITS 2. A similar study was also carried out on *Anopheles (Nyssorhynchus) benarrochi, An. (Nyssorhynchus) oswaldoi* and *An. (Nyssorhynchus) rangeli* which are the common anthrophilic mosquitoes in the southern Colombian State of Putumayo. Although *An. rangeli* is easy to identify, the morphological variants *An. benarrochi* and *An. oswaldoi* found in the same region were not always easy to separate. Therefore, a rapid PCR based molecular method could easily distinguish these two species in southern Colombia. In this, the sequence data of ITS 2 was analysed for studying the extent of variations in the progenies of *An. benarrochi* and *An. oswaldoi*. As per their results, the ITS 2 sequences were 540 bp in length in *An. benarrochi* and 531 bp in *An. oswaldoi*. These sequences showed no intra-specific variation and ungapped inter-specific sequence divergence was only 6.4%. When RFLP based results were obtained by using specific restriction enzyme species diagnostic banding patterns were recovered following digestion of the ITS 2 amplicons with an enzyme *Hae*III. These bands revealed a composition of 365, 137 and 38 bp in *An. benarrochi* and 493 and 38 bp in *An. oswaldoi*. [23] amplified ITS 2 region of two morphologically closely related species of *An. annularis* group i.e. *Anopheles (Cellia) philippinensis and Anopheles (Cellia) nivipes* in which the sequence data revealed that no interspecific differences were found and both these species shared as much as 85.2% sequence similarity.

Our data clearly showed that PCR-RFLP of ITS 1 and 2 amplicons yielded unique patterns for each of the seven species and, thus, allowed unambiguous identification. Therefore, the ITS 1 and 2 PCR-RFLP assay designed in this study rapidly and reliably identified all the populations of mosquito specimens.

Fairley et al [11] investigated the level of intragenomic heterogeneity of the ITS array (ITS 1, 5.8S, and ITS 2) of *An. aquasalis* mosquitoes and found greater levels of sequence and length variation in the ITS 1 than in the ITS 2. Bhargavi *et al* [4] studied the ITS 2 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 -149.38 kCal and -148.23 kCal followed by *Cx. tarsalis* -129.2 kCal, *Cx. vishnui* -115.3 kCal, *Cx. pseudovishnui* -105.66 kCal and *Cx. tritaenorhynchus* -82.57 kCal. 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.

5. Conclusion

In conclusion, PCR-RFLP and sequence analysis of ITS 1and 2 reported in this study can be used as a diagnostic tool for discrimination of allopatric populations of this species with overlapping morphology. The correct species assignment is of great relevance because multiple species with geographic ranges have been implicated in malaria transmission. It seems the rDNA spacer provides a useful marker to study population structure and in turn the machinery of concerted evolution. The knowledge of species that are of regional importance will contribute to the design and implementation of effective vector

control measures for malaria prevention. The ITS 1 and 2 PCR-RFLP assays have a potential to be useful for rapid and accurate identification of large numbers of mosquitoes, and may assist in carrying out extensive epidemiologic and vector incrimination studies.

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