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Nucleotide Sequence and Secondary Structure Variations in ITS2-rDNA Region of the Members of *Anopheles culicifacies* (Diptera: Culicidae) Species Complex

K.H.S. Shanika, I.N. Harischandra, B.G.D.N.K. De Silva*

Center for Biotechnology
Department of Zoology, Faculty of Applied Sciences,
University of Sri Jayewardenepura

**Corresponding author*
Email : nissankakolitha@gmail.com

Abstract

Anopheles culicifacies, major vector of malaria in Sri Lanka is a five member species complex. Differences of the vector competence of siblings, is still poorly delineated. Therefore, the current study was carried out to observe any relationship of the variations in vector competence to ITS2 nucleotide sequences and secondary structure characteristics of the species complex. DNA was extracted from sibling species B and E, the ITS2 region was amplified and sequenced. Sequences for A, C and D siblings were retrieved from NCBI GenBank. The complex divided into two groups, AD and BCE based on primary and secondary structure of the ITS2 sequences. Secondary structures of all species had three helices where pyrimidine-pyrimidine mismatch in Helix II and a UUUGG motif at 5' of Helix III were displayed only for B, C and E. Among five types of loops, interior and exterior loops were more conserved than other loop types. Results showed the major vector sibling E and poor or non vector sibling B shares identical nucleotide sequence and secondary structure. Therefore, ITS2 secondary structure is independent of the vector competence of the sibling species.

Key words: *An. culicifacies*, Internal Transcribed Spacer 2, Secondary structures

Running title: ITS2 secondary structure analysis of *Anopheles culicifacies*

1. Introduction

Anopheles culicifacies is the major vector of malaria in Sri Lanka, consisting five sibling species provisionally designated as A, B, C, D and E (Green and Miles, 1980; Subbarao, *et al*., 1983; Vasantha, *et al*., 1991; Kar, *et al*., 1999). All the members of the complex have been reported from India whereas, only sibling B and E are found in Sri Lanka (Surendran, *et al*., 2000). Species A, C, D and E from the complex, are considered as most important and efficient vectors of transmitting *Plasmodium falciparum* and *Plasmodium vivax* while sibling B is considered to be poor or non-vector (Sinka, *et al*., 2011). Sibling species have been categorized into two groups by mitochondrial and ribosomal DNA variations as AD and BCE (Goswami, *et al*., 2005, Rhagavendra, *et al*., 2009, Singh, *et al*., 2004)

Sibling species B and E are morphologically identical in all life cycle stages and varies in vectorial capacity, host feeding preference, biting activity and insecticide resistance (Subbarao, *et al*., 1987, Joshi, *et al*., 1988, Raghavendra, *et al*., 1992). This puzzling behavioral pattern despite their morphological similarity, emphasize the prerequisite of accurate differentiation of the complex for vector control practices. Meaningless control measures without accurate differentiation of the particular sibling species may increase the threat of insecticide resistance development. Sri Lanka is currently in the malaria pre elimination stage struggling to prevent the disease re-emergence threat. As the neighboring India contains all the sibling species which have high prevalence of malaria cases to date, studying the entire complex is essential to Sri Lanka to identify the accurate vectorial capacity of different sibling species.

Internal Transcribed Spacer 2 (ITS2) within the tandemly repeated rDNA cistron of ribosomal DNA (rDNA) composed about 1000 repeated units in mosquito genomes (Kumar and Rai, 1993). Each unit consists of an external transcribed spacer (ETS), 18S gene, internal transcribed spacer I (ITS1), 5.8S gene, Internal transcribed spacer 2 (ITS2), 28S gene and non transcribed intergenic spacer (IGS) (Hillis and Dixon, 1991). The order (e.g., secondary and tertiary) RNA folding structures within the rRNA are necessary for pre-rRNA processing, maturation and subsequent ribosome synthesis and function. The utility of the internal transcribed spacers to differentiate closely related and cryptic species is well established (Collins and Paskewitz, 1996; Li and Wilkerson, 2005) and studies suggest that inferences from ITS2 sequences and secondary structures strongly correlate with taxonomic classification (Coleman, 2007). Further, secondary structures are particularly useful over primary sequences because they include information on

species morphology which are not seen in primary sequences (Caetano-Anollés, 2002). Therefore, the current study was designed to describe the complete ITS2 sequence and secondary structure characteristics of the members of *An. culicifacies* species complex compared to the universal eukaryotic ITS2 secondary structure and to the vector competence of different sibling species of the *An. culicifacies* complex.

2. Materials and Methods

2.1. Mosquito samples

Anopheles culicifacies female engorged mosquitoes were collected from mosquito prevalent areas of Monaragala (6° 54'N, 81° 10'E), Anuradhapura (8° 21'N, 80° 23'E), Kandy (7° 17'N, 80° 38'E), Kataragama (6° 40'N, 81° 32'E),Thanamalwila (6° 25'N, 81° 07'E) and Nikaweratiya (7° 43'N, 80° 07'E) during 2009-2012. Multiple collections were carried out in each sampling site. F_1 generation was raised in the laboratory to identify the sibling species status using mitotic karyotyping (Surendran, *et al*., 2000).

2.2. DNA extraction

Genomic DNA was extracted from whole dried individual mosquitoes following the method described in Ballinger and Crabtree, *et al*., (1992) with additional chloroform extraction step.

2.3. ITS2 amplification

Internal transcribed spacer 2 region was amplified (sample size: sibling B =12, E =23) using primers designed for the regions of 5.8S (ATC ACT CGG CTC ATC GAT CG) and 28S (ATG CTT AAA TTT AGG GGG TAG TC) (Dezfouli, *et al*., 2003) genes of ribosomal DNA cistron. PCR reaction consisted of 5 ng of the template DNA, 50 pmols of each primer, 200 μmoll-1 of each of the dNTP, 2.5 mmoll-1 $MgCl_2$, 5 μl of 10X reaction buffer and 1.25 U of Taq DNA polymerase (Promega). Samples were initially denatured for 5 minutes at 95 ºC followed by 25 cycles of denaturation at 94 ºC for 1 minute, annealing at 50 ºC for 1 minute and extension at 72 ºC for 1 minute. Final extension was 5 minutes at 72 ºC. Amplified products were purified and sequenced at Macrogen Inc, South Korea.

Sequences were assembled using DNA baser V. 3.5.4.2 software (http://www.dnabaser.com/index.html). Conserved flanking regions of 5.8s and 28s were identified using ITS2 database (http://its2.bioapps. 84biozentrum.uni-wuerzburg.de/) online and the complete ITS2 region was obtained. ITS2 region sequences of sibling species B and E were aligned in ClustalW alignment of Bioedit 7.2.0 software (Hall, 1999) and compared.

2.4. ITS2 Secondary structure Prediction

The ITS2 sequences, flanked by 25bp of 5.8S rRNA and 25bp of 28S rRNA were used to generate the secondary structure using RNA structure Version 5.6 (Mathews, *et al*., 2006). The resulted RNA structures were visualized by PseudoViewer 3 software (Byun and Han, 2009).

2.5 Analysis of nucleotide sequences and secondary structures

The predicted secondary structures were compared with the eukaryote-universal ITS2 secondary structure and identified the presence of characteristic features. Different types of loops present in RNA secondary structure that are interior, hairpin, exterior, multi and bulge were calculated manually.

ITS2 sequences of sibling species B and E sequenced in current study and genbank retrives sequences of A,C and D were analysis using DAMBE version 5.5.21software (Xia, 2013) to calculate the number of bases, nucleotide composition, GC content, substitution and indels (insertions and deletions).

3. Results

3.1. Nucleotide composition and length of ITS2 region

ITS2 region was 372 bp for all the sibling species B and E isolates extracted from Sri Lanka. Further, all sequences retrieved from GenBank for Sri Lankan and Indian B and E sequences were similarly in 372 bp length. As an exception, a single sequence of sibling B from Cambodia was 370bp. All sibling species A sequences considered in the current analysis were 370bp in length. Sibling species C and D sequences showed a wide length variation from 364bp-373bp and 311bp - 371bp respectively.

The base composition for sibling A and D was similar as 22.7% A, 28.9% C, 29.7% G, and 18.6% T while sibling B, C and E shared similar contents as 21.2% A, 29.5% C, 31.1% G, and 18.1% T. Furthermore, GC content of members of this complex was rich and ranged from 58.38% - 58.65% for sibling A and D while it was 60.59% - 61.54% for sibling B, C and E. a constant value of GC content belonged to the sibling species B and E, where A, C and D showed a variation in a range (Appendix I)

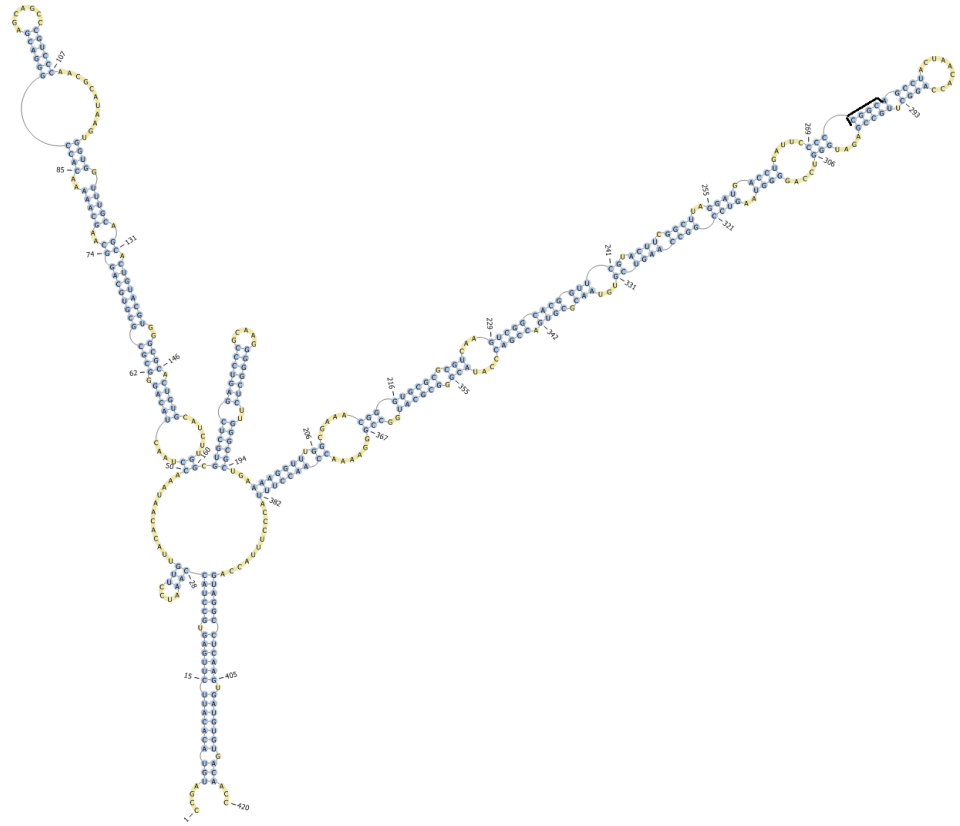
3.2. Nucleotide substitution patterns

The length differences of sequences have arisen due to insertion or deletion (indels) of bases and most of these indels are associated with short runs of a single base A or T and insertion of a 6 bp (TGGTGG) sequence. Sibling species A, B, C and E showed a similar number of insertions and deletions intra specifically while sibling D varied.

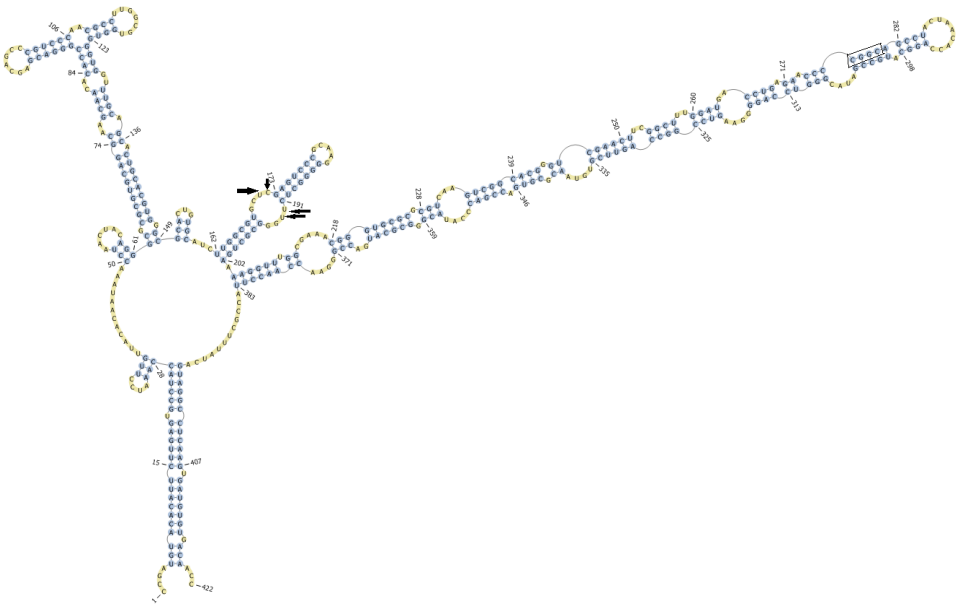
CG transversion (substitution) was not observed from any of the sibling species, however AC, AT and GT transversions and AG, CT transitions have appeared in different numbers. In the transition and transversion analysis of species, sibling species B showed intra sibling species variations in the number of AT transversions while all other sibling species had identical number among individuals. All other transitions and transversions were intra specifically identical while showing inter-specific variations. The number of substitution was higher than the number of indels in all siblings. Among substitutions, the number of transversions was higher than the number of transitions.

3.3. ITS2 secondary structures

The ITS2 secondary structures of the *An. culicifacies* B and E present in Sri Lanka were identical in shape and characteristics. Based on the secondary structure analysis, members of the complex of the *An. culicifacies* have shown two types of structures which, sibling A and D shares one type, and B, C and E species shares another common type (Figure 1).



(a)



(b)

Figure 1: Proposed secondary structures (a) B/C/E and (b) A/D groups. Arrows indicate the U-U mismatch in Helix II and red and orange circles represent the UUUGG and GCCG motifs in Helix III respectively.

The ITS2 secondary structure of *An. culicifacies* species complex mainly comprised of three main helices as helix I, helix II and helix III. The helix II was short and possessed a pyrimdine - pyrimidine mismatch (arrows in Figure 1) where as the helix III was longest and possessed CGGC motif at the 5' side to the apex of helix III.

In addition, five different types of loops as interior, hairpin, exterior, multi and bulge were observed in all five sibling species. Among them, bulge loops were highest in number. Among the loops of *An. culicifacies*, it showed that the number of all types of loops were varied except interior and exterior loops as they shared similar number of loops for all sibling species A, B, C, D and E (n=1 & 9 respectively). Based on the similarity of the number of loops of multi-branched, bulge and hairpin, sibling A and D shared similar number while others shares another (Figure 2). Number of multi-branched and hairpin loops were higher in BCE group while it was vise versa for bulge loops as AD group contained higher number of loops (Figure 2).

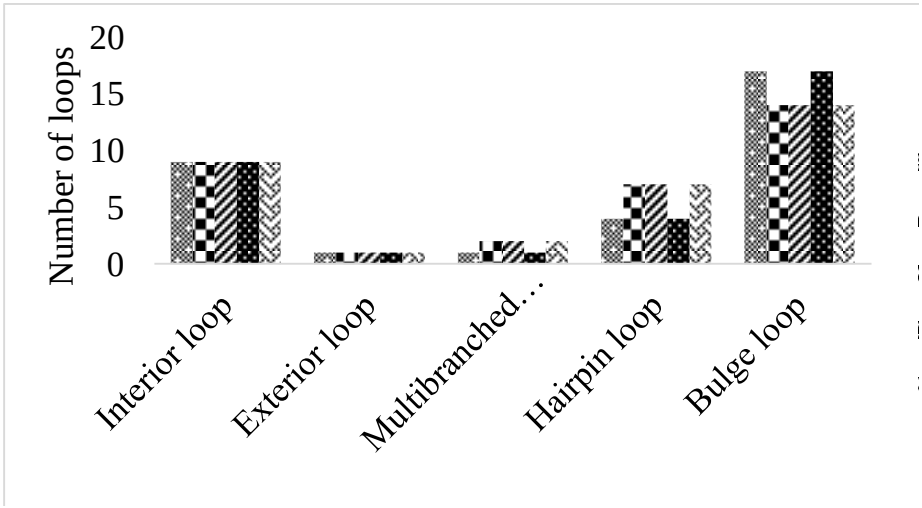


Figure 2: Comparison of different types of loops among sibling species of *An. culicifacies*

4. Discussion

The length of the ITS2 nucleotide sequence of *An. culicifacies* complex was within the range of the other Anophiline mosquitoes (300bp-600bp) (Wilkerson, *et al*., 2004) and the GC content and nucleotide composition were similar to other mosquito species, which fall in the range of 50-70% (Wesson, *et al*., 1992; Paskewitz, *et al*., 1993; Xu and Qu, 1997; Beebe, *et al*., 1999). Sibling species specific diagnostic characteristic of ITS2 nucleotide sequences have not been observed in the *An. culicifacies* sibling species complex.

In secondary structure analysis, helix III was the longest and helix II was the shortest among all the helices, which resemble the secondary structure predicted by Coleman (2007) for Eukaryotes. Characteristic Pyrimidine-pyrimidine mismatch in the helix II was not conserved for all the sibling species of *An. culicifacies* and only sibling species B, C and E possessed two mismatches as U-U and C-U (Figure 1). The helix III has un-branched in *An. culicifacies*. Further, helix I has not identical for all the sibling species, as A and D possessed un-branched helix. However, UGGU motif 5' to the apex of Helix III which has described by Schultz, *et al*., (2005) was not observed in any secondary structure. Instead, UUUGG was identified as a slight

modification of UGGU motif in all secondary structures of sibling species B, C and E and this motif was not found in sibling A and D. In addition all the structures of the five sibling species showed high purine content in helix III due to CGGC motif (Figure 1) which is important for RNA post transcriptional processing. AAA motif in between Helix II and Helix III was observed in all sibling species of *An. culicifacies*, but the order (Kotcheson, *et al*., 2010) was different among sibling species. Furthermore, a conserved sequence motif (GARTACATCC) that can be found throughout the family Culicidae (Coleman, 2007) was not present on the 5' of helix III of any sibling species.

The predicted secondary structures for all sibling have shown 3 domain models which differs with structures predicted for green algae, flowering plants, fruit flies (*Drosophila sp*.), parasitic flatworms, gastropods, and the mouse as they shown 4 domain models (Michot, *et al*., 1999; Schlötterer, *et al*., 1994; Mai and Coleman, 1997; Morgan and Blair, 1998; Joseph, *et al*., 1999, Coleman and Vacquier, 2002, Oliverio, *et al*., 2002; Gottschling and Plötner, 2004). Although, same number of domains (3 domains) had been recorded by Coleman (2007) for mosquitoes, there were some exceptions for *An.minimus, An. varuna* and *An.jayporiensis* (4 domains) whereas *An.subpictus* and *An.jamesii* with 5 and 6 domains respectively (Zomuanpuii, *et al*., 2013).

The most fundamental structural element in RNA is the stem, in which each nucleotide is base-paired with its complement. Apart from the stem, secondary structures of *An. culicifacies* sibling species consisted of loops, as hairpin, bulges, internal, exterior and multi-branch loops. These different types of loops are likely to be crucial to the evolution of modern organism and studies have underscored their importance in a variety of biological events (Moore, 1999; Leontis, *et al*., 2006). Bulges are regions of unpaired nucleotides situated along one strand of a duplex and are often found at protein binding sites (Wu and Uhlenbeck, 1987; Lilley, 1995). Hairpin loops are single stranded loops that bridge one end of a double stranded stem and serve as nucleation site for RNA folding or recognition sites for protein-RNA interactions. Internal loops represent interruptions in double stranded RNA caused by the presence of nucleotides on both strands that cannot participate in Watson-crick base pairing. These are important in protein binding sites and ribozyme cleavage sites (Coleman and Tsongalis, 2007). Loops with two or more enclosed base pairs and one closing base pair are called multi-loops or multi-branched loops whereas the open loop which is not closed by any base pair is called external or exterior loop. Among the

variations of loops, the number of loops was not varied within A, D or B, C, E groups. Therefore it can conclude that the functions determining of different types of loops are similar among two sibling groups AD and BCE.

The variations in the ITS2 secondary structures suggest that the differences and conserved nature observed in ITS2 secondary structures of sibling species are not "neutral" and are not simple accumulated random nucleotide changes, but bear a significant functional load as previously reported for three related mosquito genera (*Aedes*, *Psorophora*, and *Haemogogus*) (Wesson, *et al*., 1992). Intra spacer variable regions appear to co-evolve and ITS2 variation is constrained to some extent by its secondary structure.

Finally, the characteristics of secondary structures in *An. culicifacies* sibling species complex are not related to the vectorial capacity of siblings. The ITS2 region and respective secondary structures also congruent with the division of the species complex into two categories as shown in other ribosomal and mitochondrial molecular marker depicted identification methods.

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Appendix I

Table 2: Length of ITS2 region, nucleotide composition, substitution and indels of sibling species of *Anopheles culicifacies*

Species	Total bp	A	C	G	T	A+T (%)	G+C (%)	Substitution						Indel	
								Transition			Transversion				
								AG	CT	AC	AT	CG	GT	Insertion	Deletion
India-A1	370	84	107	110	69	41.35	58.65	3	5	5	6	0	3	4	8
India-A2	370	84	107	110	69	41.35	58.65	3	5	5	6	0	3	4	8
Iran-A1	370	85	107	109	69	41.62	58.38	3	5	5	6	0	3	4	8
Iran-A2	370	84	107	110	69	41.35	58.65	3	5	5	6	0	3	4	8
Iran-A3	370	84	107	110	69	41.35	58.65	3	5	5	6	0	3	4	8
Iran-A4	370	84	107	110	69	41.35	58.65	3	5	5	6	0	3	4	8
Cambodia-A	370	84	107	110	69	41.35	58.65	3	5	5	6	0	3	4	8
India-D1	371	85	107	110	69	41.51	58.49	3	5	5	5	0	3	5	7
India-D2	311	70	86	96	59	41.48	58.52	3	5	3	5	0	3	2	7
India-D3	370	84	107	110	69	41.35	58.65	3	5	5	5	0	3	4	8
India-D4	370	84	107	110	69	41.35	58.65	3	5	5	5	0	3	4	8
In-un1	370	84	108	109	69	41.35	58.65	3	5	5	5	1	3	4	8
SL-B*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-B*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-B*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-B	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
India-B1	372	80	110	116	66	39.25	60.75	2	5	5	5	0	3	6	6
India-B2	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
India-B3	372	79	110	116	67	39.25	60.75	2	5	5	4	0	3	6	6
India-B4	372	79	110	116	67	39.25	60.75	2	5	5	4	0	3	6	6
Cambodia-B1	370	78	109	116	67	39.19	60.81	2	6	4	4	0	3	6	8
Cambodia-B2	372	79	110	116	67	39.25	60.75	2	5	5	4	0	3	6	6
Cambodia-B3	372	79	110	116	67	39.25	60.75	2	5	5	4	0	3	6	6
Cambodia-B4	372	79	110	116	67	39.25	60.75	2	5	5	4	0	3	6	6
Cambodia-B5	372	79	110	116	67	39.25	60.75	2	5	5	4	0	3	6	6
India-C1	373	80	110	116	67	39.41	60.59	2	5	5	4	0	3	6	6
India-C2	372	79	110	116	67	39.25	60.75	2	5	5	4	0	3	6	6
India-C3	364	77	109	115	63	38.46	61.54	2	4	5	4	0	3	6	6
In-un2	373	79	111	116	67	39.14	60.86	2	5	6	5	0	3	6	6
Ch-Un1	372	79	110	116	67	39.25	60.75	4	5	5	5	0	3	6	6
Ch-Un2	373	79	110	116	68	39.41	60.59	5	6	5	5	0	4	6	5
India-E	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6
SL-E*	372	79	110	116	67	39.25	60.75	2	5	5	5	0	3	6	6