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# Mitochondrial-*COII* sequence polymorphism reflects spatial genetic clustering of *Anopheles culicifacies* sibling species E in Sri Lanka

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## ABSTRACT

*Background & objectives:* Malaria infects around 216 million people annually with estimated 445,000 deaths globally. *Anopheles culicifacies* is the vector of malaria in Sri Lanka, a complex of five morphologically identical sibling species of which precise identification using DNA–based methods is still under experimentation. This study was carried out in Sri Lanka to observe the utility of BCE-PCR assay based on mitochondrial Cytochrome Oxidase II (*COII*) developed in India, in sibling species B and E identification in Sri Lanka, to characterize nucleotide and corresponding amino acid sequences of *COII* region in major vector sibling species E in Sri Lanka and to analyze the spatial distribution pattern of sibling species E in Sri Lanka using microsatellite markers.

*Methods:* BCE-PCR was carried out for the samples to identify their sibling status. Sequencing of *COII* region was then carried out to investigate the genetic diversity of Sri Lankan sibling species E, sequences were aligned and compared; microsatellite genotyping was carried out and the spatial clustering pattern was analyzed.

*Results:* Identification of sibling species B and E using BCE-PCR was confusing due to the heterogeneity in the *COII* region of sibling species in Sri Lanka. Non-synonymous substitutions were detected in *COII* gene amongst sibling species E. Spatial distributed two clusters were detected in the studied population.

*Interpretation & conclusion:* Existence of genetic variants among sibling species is suggested in Sri Lanka. Further, the pattern of sibling species identification in BCE-PCR was reflected in the spatial clustering of sibling E in Sri Lanka.

**Key words** *Anopheles culicifacies* sibling E; *COII*; microsatellites; spatial clustering

## INTRODUCTION

Malaria is one of the most affecting mosquito-borne disease causing 216 million cases worldwide and estimated 445,000 deaths annually[1]. *An. culicifacies* is the major vector of malaria in the Indian subcontinent and Sri Lanka, and comprises of a complex of five morphologically identical sibling species as A, B[2], C[3], D[4] and E[5]. Sibling species B and E have been reported in Sri Lanka[6]; the sibling B is considered as a poor or non-vector while E is the major vector in Sri Lanka[7].

Cytogenetic examination based on the polymorphism of Y-chromosome in males of the late III or IV instar larvae is the standard method of practice in the identification of sibling species[5, 8] where the technique is often difficult to be carried out without expertise knowledge and is also time-consuming. In such backdrop, the development of DNA/PCR based method that reliably differentiates sibling species in *An. culicifacies* complex is the most sought for. However, such methods based on number of DNA markers to identify all five-sibling species in the complex have been unsuccessful[9–12]. Two-step multiplex PCR assay (D3-PCR/ITS2-*Rsa*l assay followed by either AD-PCR or the BCE -PCR assay) based on sequence differences within the Cytochrome Oxidase II (*COII*) region has been developed to distinguish all five-sibling species found in India[13].

This method to distinguish all five sibling species in India[13] has been used in later studies successfully; in first detection of sibling species E in Odissa[14], in Madhya Pradesh[15] and recently in Tyagi *et al*[16]. However, the study has carried out the validation of the assay[13] using mosquitoes collected from various parts of India and has concluded that this method cannot be used universally to distinguish all sibling species in the complex[17]. This study has collected samples from Ramanathapuram in India, which is closely situated to Sri Lanka and has shown various banding patterns for the BCE-PCR assay[17]. The BCE-PCR assay has not been used to distinguish the sibling species found in Sri Lanka.

Currently, indigenous malaria cases are not reported from Sri Lanka and they have obtained the World Health Organization (WHO) certification for a malaria free country. However, imported malaria cases from Indian, Afri-

can, Pakistan regions are increasingly reported[18]. Sibling species E in Sri Lanka is generally wide spread in the dry zone, and has longer life span and is generally resistant to insecticides that are frequently applied as major vector control practice in dry zone compared to sibling species B[7]. Since the vector is highly established in the environment, any dispersal of malaria parasite, *Plasmodium sp.* population in the country will immediately and successfully spread the disease among people. Therefore, genetic, behavioral and biochemical changes and variations of vector mosquitoes is important to predict the future possible out-breaks and disease spreading patterns, and to control the vector mosquitoes. Studies to characterize the population genetics and biochemical variations are poor for the *An. culicifacies* sibling species. Sibling species E population in Sri Lanka has been recently identified as sympatrically distributed three gene pools according to the microsatellite elicited non-spatial Bayesian cluster analysis[19].

Therefore, this study was undertaken to examine the utility of BCE multiplex PCR developed by Goswami *et al*[13] and then to characterize nucleotide and corresponding amino acid sequences of *COII* region in major vector sibling species E in Sri Lanka. Further, the relationship between the microsatellite elicited spatial distribution of sibling species E and polymorphism of *COII* region reported amongst the sibling species E was determined.

## MATERIAL & METHODS

*Sample collection and DNA extraction*

*An. culicifacies* female mosquitoes were collected from six different localities, Anuradhapura (8° 32'N, 80° 41'E), Kandy (7° 28N, 80° 63'E), Nikaweratiya (7° 74'N, 80° 11'E) Thanamalwila (6° 43'N, 81° 13'E), Monaragala (6° 89'N, 81° 34'E) and Katharagama (6° 28'N, 81° 26'E) of Sri Lanka during May 2010 to December 2012 (Fig. 1). The locations were selected to represent all the mosquito prevalent geographic areas in dry and intermediate climatic zones of Sri Lanka. The mosquitoes were collected using cattle bait hut/trap collection method with the assistance of the entomological teams attached to the Anti-Malaria Campaign in Sri Lanka. Egg laid mosquitoes were cytogenetically identified[8] using IV instar larvae in $F_1$ progeny. Genomic DNA was extracted from mosquitoes using a phenol: chloroform extraction method[20]. The homogenized mosquitoes in SDS 10%, Lysis buffer and Proteinase K 5mg/mL were incubated at 50°C for 10 h. Then the purification of DNA in the solution was carried out using phenol: chloroform: isoamyl alcohol 25:24:01 solution followed by chloroform: isoamyl alcohol 24:01

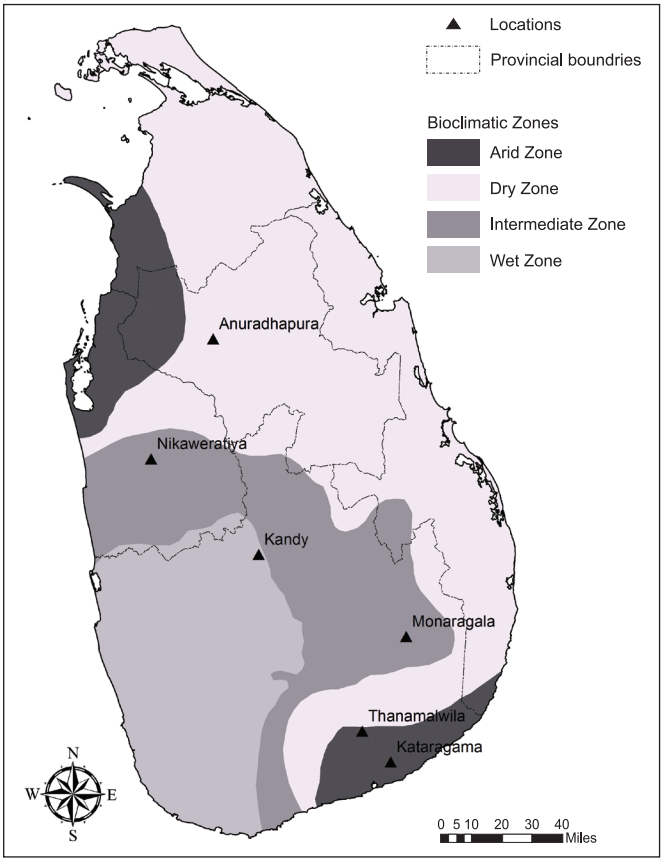


*Fig. 1:* A map showing the sampling sites.

solution. DNA was precipitated using 100% ethanol and re-suspended in 100µL of TE pH 8.0.

*Sibling species status using BCE multiplex PCR*

Wild caught mosquito DNA (n=210; 32, 31, 39, 38, 37 and 35 from Anuradhapura, Kandy, Nikaweratiya, Monaragala, Thanamalwila and Kataragama respectively) and 53 cytogenetically identified samples (Sibling B - n=6 and Sibling E - n=47) were used for the sibling species identification using BCE-PCR[13]. One forward and three reverse primers (BCEF and BCR, CR & ER) were used in PCR. A 25µl PCR mixture contained 1.5mM $MgCl_2$, 1x GoTaq® reaction buffer, 200µM dNTPs, template DNA (30ng), 25pmol of BCEF and ER, 12pmol of BCR, 30pmol of CR primers and 1.25U GoTaq® *Taq* DNA polymerase (Promega). The cyclic conditions were adopted from Goswami *et al*[13]. Non-amplified samples of this PCR were amplified for mitochondrial *COII* and nuclear D2 regions to verify the good quality of DNA. Post PCR products were run in 2% agarose gels to verify the banding pattern.

*Amplification and sequencing of COII region of mitochondrial DNA*

Cytochrome Oxidase II (*COII*) region was amplified using universal primers, C2J-3138 and C2N-3686[21] for 20

samples including all obtained banding profiles of BCE PCR. In a 25 µL of total reaction mixture, approximately 20ng of the template DNA, 50 pmols of each primer, 200 µM of each of the dNTP, 2.5 mM $MgCl_2$, 5 µL of 10X reaction buffer and 1.25U of *Taq* DNA polymerase (Promega GoTaq® flexi, USA) were included. The cyclic conditions were; initial denaturation at 94ºC for 4 min, followed by 40 cycles, each of denaturation at 95ºC for 40 sec, annealing at 50 ºC for 1 min, and extension at 68 ºC for 1 min. Final extension was at 72ºC for 10 min (Eppendorf; Germany). The products were electrophoresed at 80v for 45 min (Labnet, USA) and visualized by Alpha Imager mini gel documentation system (Protein simple, USA). The column purified (Qiagen purification kit) PCR products [sibling species B (n=6); sibling species E (n=8); non amplified samples for BCE-PCR (5); single band at 178 bp sample (1)] were sequenced in both directions from the Macrogen Inc, South Korea.

Nucleotide sequences were translated into amino acid sequences without stop codons and aligned using ClustalW multiple alignment. Sequences were deposited in the NCBI GenBank (sibling species B; KP197041-KP197044, KJ466962 and sibling species E; KP197037 -KJ475440, KJ093623, and KJ093624).

*Microsatellite genotyping*

Cytogenetically identified sibling species E (n=193; 29, 36, 42, 33, 32 and 31 for Anuradhapura, Kandy, Nikaweratiya, Monaragala, Thanamalwila and Kataragama respectively) were amplified using 8 microsatellite markers (ACA59, AcAIIB5, AcAVB93, AcAVB93A, AcAVIB213, AcAVIIIB40, AcA36, AcA75)[22] and genotyped[19]. Data were analyzed for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) and also simulated under a scenario of isolation-by-distance at mutation-migration-drift equilibrium[19].

*Spatial genetic cluster analysis*

The spatially explicit clustering method implemented in the program TESS 2.3[23] that builds a spatial individual neighborhood network using the Voronoi tessellation was used to generate the possible clusters in the population. In spatial genetic cluster analysis, individuals are assigned probabilistically to genetic clusters based on their multilocus genotypes to maximize HWE and minimize LD. As this method does not assume predefined populations, it is useful for identifying spatial discontinuities between samples. The method assumes that spatially proximate individuals are more likely to be genetically related than those that are not and therefore, use the spatial location of individuals as a prior[23].

The TESS analysis was run for 100,000 burn-ins followed by 10,000 run-in sweeps for *K* (2–10) using without admixture model[23]. Analysis was carried out providing geographic coordination of sampled locations and geographic distances between locations. The number of clusters (*K*max) was determined by plotting mean Deviance Information Criterion (DIC), a statistical measure of the model prediction capabilities value per each *K* value against the *K*max[24]. Three dummy points from where samples were not collected were selected to modify the TESS network topology as Colombo (6° 91'N, 79° 86'E), Batticaloa (7° 70'N, 8° 68'E) and Killinochchi 9° 37'N, 80° 41'E).

## RESULTS

*Determination of sibling species status of An. culicifacies using mitochondrial COII based on BCE-PCR assay*

A portion of 41.91% of total screened mosquitoes (n=210) was identified as sibling species B and E which were identified by a single band of 248 bp in sibling B and two bands at 248 bp and 178 bp in sibling E as described in Goswami *et al*[13] (Fig. 2). Among these, 24 samples (11.43%) were identified as sibling species B and 64 samples (30.48%) were identified as sibling species E. There were nine samples (4.28%) that did not fall into any of these groups and produced a single band of 178bp while 113 samples (53.81%) did not give any bands in BCE-PCR assay. However, these non-amplified samples gave expected bands in other PCR assays using *COII* and D2 regions of *An. culicifacies* genome indicating the suitability of DNA in PCR amplification.

In the analysis of cytogenetically identified samples, six samples of sibling species B and eight samples of sibling species E were confirmed that they belonged to their respective sibling species by BCE-PCR assay, however, the rest of 39 cytogenetically identified samples of sibling species E did not produce any band in BCE-PCR assay

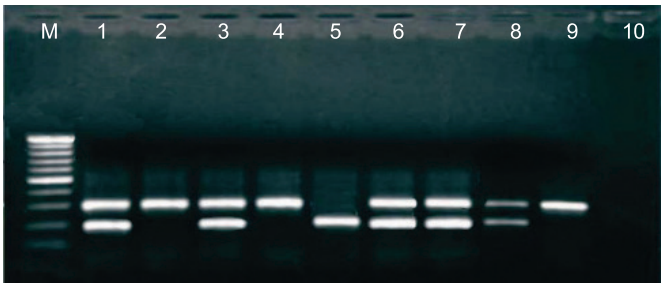


*Fig. 2:* Agarose gel image showing amplified BCE-PCR fragments. Lane 1, 3, 6, 7, 8 -sibling species E, Lane 2, 4, 9 -sibling species B, Lane 5-sample with 178bp single band, Lane 10 -unamplified sample and Lane M - 100bp DNA ladder (100-1000bp).

indicating that these are genetically heterogeneous group of sibling species E.

It was found that the mosquitoes collected from different localities showed different percentages of identified sibling species B, E and non-amplified samples when this assay was carried out. The samples collected from Monaragala, Thanamalwila and Kataragama locations showed higher percentages of both sibling species B and E while Anuradhapura, Kandy and Nikaweratiya localities showed higher percentage of non-amplified samples (Fig. 3). Further, samples collected from Monaragala and Thanamalwila and Kataragama showed higher percentage of sibling species E than other three locations (Fig. 3).

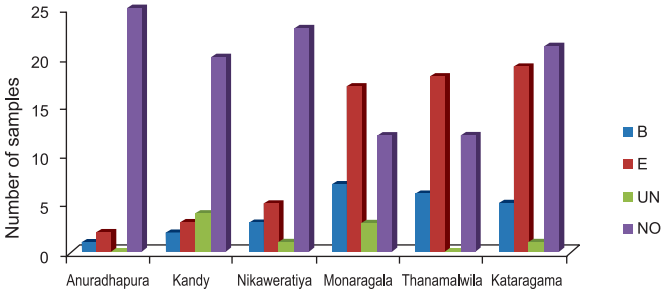


*Fig. 3:* Spatial distribution of sibling species in sampled localities according to BCE-PCR. B-identified as sibling species B, E-identified as sibling E, UN-only single band amplified at 178 bp, NO-non amplified samples.

*Nucleotide variations and amino acid sequence analysis of mitochondrial COII region of sibling species B and E*

Cytochrome Oxidase II sequence (530bp) of sibling species B and E showed intra-species nucleotide variations. There were 20 nucleotide positions varying among sibling species E sequences while it was five positions amongst sibling species B. The sequence heterogeneity was also observed in the region to which the reverse primers of BCE-PCR, ER and BCR bind to yield 178 bp and 248 bp bands respectively (reverse primer CR amplifies only for sibling species C). Fig. 4 shows the 3' ends of BCEF, BCR and ER primers and the substitutions at 255$^{th}$ position (3' end of ER) and the 334$^{th}$ position (3' end of BCR) of sibling species. The amino acid substitutions were apparent in mitochondrial region characterized in this study and such substitutions were observed in the amplified *COII* coding region (175 amino acids) of studied species B and E samples respectively.

Nine positions showed amino acid substitutions among cytogenetically identified sibling species E sequences (Table 1); 18th position Phenylalanine (F) – Tyrosine (Y), 19th Threonine (T) – Asparagine (N), 38$^{th}$ position Alanine (A) to Glycine (G), 64$^{th}$ position Threonine (T) to Proline (P), 105th Aspartic acid (D) – Tyrosine (Y), 109th Arginine (R) – Proline (P), 110th Isoleucine (I) – Tyrosine (Y) – Leucine (L), 119th Arginine (R) – Leucine (L) and 157$^{th}$ position Serine (S) to Proline (P). One position (110$^{th}$ position) was reported having three amino acid

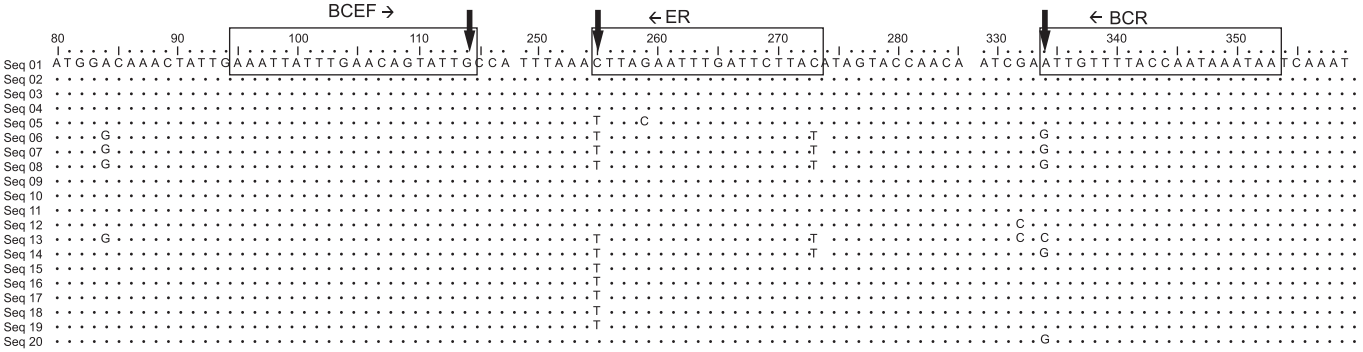


*Fig. 4:* Nucleotide substitutions at BCE-PCR primer binding sites
Primer sequences are shown in squares. Nucleotides at 3' ends are shown by down headed arrows. The 5'-3' direction of primers is shown by the arrows along with the primer names. Seq 1,2,3, 4, 9,10,11,12 – sibling species E, Seq 5,15,16,17,18,19 – sibling species B, Seq 6,7,8,13,14 – non amplified samples, Seq 20 - single band at 178 bp.

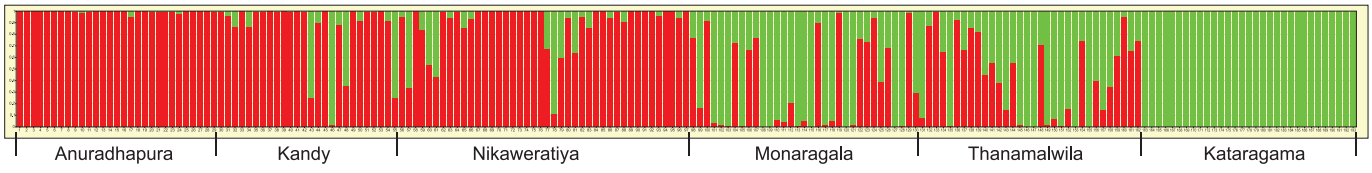


*Fig. 5:* Graphical representation of TESS for *K*max, *K*=2 without admixture model.
Anuradhapura, Kandy and Nikaweratiya sampling sites show a higher proportion of samples under the red colored cluster while Monaragala, Thanamalwila and Kataragama sites share the highest proportion of green colored cluster.

Table 1. Amino acid substitutions of sibling species B and E sequences of India and Sri Lanka Ind E01 (GenBank accession no. AJ534646) and Ind B01 (AJ518810) sequences by Goswami et al., 2016 were used to compare the amino acid substitutions of sequences isolated from Sri Lanka. Seq 1,2,3, 4, 9,10,11,12 – sibling species E, Seq 6,7,8,13,14 – non amplified samples, Seq 20 - single band at 178 bp, Seq 5,15,16,17,18,19 – sibling species B

| Sequence name | Amino acid position | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 11 | 18 | 19 | 38 | 58 | 64 | 85 | 99 | 105 | 109 | 110 | 119 | 143 | 157 | 165 | 167 |
| Ind-E01 | G | F | T | A | E | T | E | T | D | R | I | R | T | S | C | E |
| Seq 01 | | | | | | | | | | | | | | P | | |
| Seq 02 | | | | | | | | | | | | | | P | | |
| Seq 03 | | | | | | | | | | | | | | P | | |
| Seq 04 | | | | | | | | | | | | | | P | | |
| Seq 06 | | Y | N | | | | | | | | V | | | P | | |
| Seq 07 | | Y | N | | | | | | | | V | | | P | | |
| Seq 08 | | Y | N | | | | | | | | V | | | P | | |
| Seq 09 | | | | | | | | | | | | | | P | | |
| Seq 10 | | | | | | | | | | | | | | P | | |
| Seq 11 | | | | | | | | | | | | | | P | | |
| Seq 12 | | | | | | | | | Y | P | | L | | P | | |
| Seq 13 | | | | | | | | | | P | L | | | P | | |
| Seq 14 | | | | | | | | | | | V | | | P | | |
| Seq 20 | | | | G | | P | | | | | | | | P | | |
| Ind-B01 | G | | | A | E | T | E | T | | | V | | S | P | C | E |
| Seq 05 | | | | | | | Q | | | | | | | P | | |
| Seq 15 | | | | | | | | | | | | | T | P | | |
| Seq 16 | S | | | | | | | | | | | | T | P | | |
| Seq 17 | | | | | | | | | | | | | T | P | | |
| Seq 18 | | | | | | | | | | | | | T | P | | |
| Seq 19 | | | | G | | | | | | | | | T | P | | |

variations within the studied population while all other positions possessed two variations.

Sequences of sibling species B showed four amino acid substitutions among the sequences at 11th position Glycine (G) – Cysteine (C), 38th position Alanine (A) – Glycine (G), 85$^{th}$ position Glutamic acid (E) to Glutamine (Q) and 143$^{rd}$ position Serine (S) to Threonine (T)

*Spatial genetic cluster analysis*

Spatial geographic coordinates and geographic distances between populations were taken into consideration in TESS genetic analysis. Plots of Deviance Information Criterion (DIC) values against the *K*max values showed the plateau starting at *K*max = 2 indicating that the number of clusters is *K*=2. Fig. 5 represents the color bar diagrams for *K*max without admixture model. Anuradhapura and Kataragama localities which located most distantly (239 km apart) show completely different color bars. Kandy and Nikaweratiya sampling populations shares Anuradhapura color pattern while Monaragala and Thanamalwila locations were similar to the Kataragama location. Anuradhapura and Nikaweratiya locations situated in upper (towards northern) part of the country while other three locations are in southern part of the country. This clustering of localities shows congruency with the BCR-PCR banding profiles of each locality.

## DISCUSSION

The precise identification of sibling species in *An. culicifacies* complex is crucial in malaria surveillance and control programs. BCE-PCR assay developed previously to identify the sibling species in India, failed to identify sibling species present in Sri Lanka correctly and only 41.91% of samples (11.43% as sibling species B and 30.48% as sibling species E) showed a congruency to the BCE-PCR banding profiles of sibling species B and E reported from India[12]. However, using the same assay, it was reported that cytogenetically identified 75 sibling species B and 28 sibling species E of India were correctly identi-

fied except one isolate of sibling species B[13] indicating that sibling species B and E present in Sri Lanka are not identical to the same sibling species in India. Although number of studies have shown successful identification of sibling species using this PCR assay[14–16], the findings of the BCE PCR validation assay[17] has shown some of the samples could not be identified using this PCR assay.

Further, loci of *COII* region to which ER and BCR primers in the BCE-PCR annealed were observed to have nucleotide substitutions in some isolates thus this being the case of not amplifying the corresponding regions of these isolates in BCE-PCR. According to the ClustalW multiple alignment of the current study, substitutions appeared commonly in these primer binding sites while occasional substitutions were found in other nucleotide positions (Fig. 3). Therefore, it is apparent that the loci of BCE-PCR primers annealed (3' ends) were the nucleotide positions where high rate of substitutions observed in all five-sibling species in the complex, further confirming the unsuitability of the method to distinguish sibling species in Sri Lanka. This variation could be due to the fast evolving nature of mitochondrial gene regions and the single base differences used in BCE-PCR primer designing[13, 17] might be unsuccessful when substitutions occur at those base positions. It has been reported that alterations and interchanging morphological characteristics of *An. subpictus* sibling species complex in Sri Lanka[25] show differences of the same sibling species found in India and Sri Lanka.

Sibling species B is the poor or non-vector in Sri Lanka and highly susceptible to commonly used insecticides, and their fecundity and longevity are less than the major vector sibling species E[7]. As the abundance of sibling B is very low in all populations surveyed and is a poor vector, only the sibling species E, the major vector species was of concern in further analysis. The percentages of the different sibling species identified by BCE-PCR were varied in six localities studied in the current study. Three locations in the northern part of the country which is separated by the central mountain range shared similar pattern in BCE-PCR identification while was found a different pattern for the locations in southern part. This central mountain range has been identified as a possible barrier to the gene flow among *An. culicifacies* sibling species E population in Sri Lanka[19].

BCE-PCR profiles and the heterogeneity in nucleotide sequences and corresponding amino acid sequences of *COII* show that the sibling species E in Sri Lanka is genetically diverse giving an indication of the high intra-species heterogeneity among individuals. The sibling species E has a longevity and has successfully adapted to survive against the insecticides used in Sri Lanka to date, compared to its counterpart sibling species B, leaving them more opportunity to exchange their genetic materials through mating[7]; this should be the reason to have such genetic diversity of species E observed in this study. Further, reported genetic diversity amongst Sri Lankan sibling species E as compared to the Indian counterpart might be due to the evolutionary processes that independently acted on mosquito populations in these two countries following separation of Indian subcontinent and Sri Lanka 15 million years ago[26].

Spatial analysis of genetic clustering shows two spatially distributed clusters in the studied sibling species E population. As observed in BCE-PCR banding profiles, Anuradhapura, Kandy and Nikaweratiya localities were in a single cluster while Monaragala, Thanamalwila and Kataragama in another cluster. This clustering pattern strengthens the finding reported in previous study that the barrier formed by central hills of the country restricts gene flow between these two regions[19]. Similarly, *An. arabiensis* in Africa has shown extreme population differentiation even 25km apart with limited gene flow due to elevated topography barriers[27]. Generally, marine separation of land masses, presence of physical/geographic barriers, isolation by distance and island populations have distinct species diversity and these factors act as evolutionary bridges to enhance the independent evolution of species[28–32]. By looking at the results of current and previous studies, it shows, sibling species E is different in Sri Lanka and India as well as different localities within Sri Lanka, where one can consider as intra-species genetic heterogeneity.

These results might indicate spreading newly evolving alleles among species in different geographic areas such as insecticide resistance to achieve advantages against environmental changes or parasite susceptibility in the population. It is essential to understand the changes of major vector species and their impact on disease transmission potential to prevent any future possibilities of re-emergence of malaria in Sri Lanka. Therefore, it is essential to understand the genomic variations of major vector species and their impact on malaria transmission. These baseline data are required to prevent any future possibilities of re-emergence of malaria in Sri Lanka.

## CONCLUSION

The BCE PCR assay to distinguish all five sibling species of *An. culicifacies* in India cannot be used to differentiate sibling species present in Sri Lanka due to the nucleotide and amino acid variations within the sibling species E population in Sri Lanka. The variations in the

mitochondrial *COII* region has a relationship to the special distribution pattern of the sibling species E.

*Conflict of interest:* None

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