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Population genetic and phylogeographic relationships of the dengue vector *Aedes aegypti* inferred from three localities of Sri Lanka

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Abstract

Aedes aegypti is the major epidemic vector of dengue outbreaks in the world. Here, a 359 bp region of the mitochondrial NADH hydrogenase subunit 4 (ND4) gene was taken as the candidate locus for study purposes from 27 *Aedes aegypti* mosquito larvae collected from an inland mountain area (Badulla), and two distantly located coastal areas (Hambantota and Trincomalee) in Sri Lanka. Eleven parsimonious informative sites were observed, and 7 haplotypes were identified from the 27 samples. The coastal populations displayed higher genetic diversity compared to those from the inland mountain-contained region. The phylogenetic analyses of *Ae. aegypti* from Badulla, Hambantota and Trincomalee including published Sri Lankan and global samples (a total of 106 sequences) revealed two separate clades: a basal clade and a derived clade. Eighty percent of the samples found from Badulla, were of the basal haplotype (H1) clustered together with West African (Senegal) samples in close proximity to the outgroup. On the contrary, Hambantota and Trincomalee samples were found dispersed in both the basal and derived clades clustering closely with samples from Mexico, Brazil, Kenya, USA and Myanmar indicating their close genetic relationship to many New World, East African and Asian varieties. Taken together, our study proposes that colonization of *Ae aegypti* in Sri Lanka occurred through multiple founder events (ancient multiple invasions in coastal sites and a more recent West African invasion to the inland mountain-demarcated Badulla region) and continue to exist in mutational drift equilibrium following the settlement event.

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1. Introduction

Dengue is the most common and important arthropod-borne viral illness in humans and has affected nearly 3.9 billion people in about 129 countries (Brady *et al*., 2012; Bhatt *et al*., 2013). In Sri Lanka, an average of 61,000 dengue cases has been reported each year throughout the last decade with a sharp peak of 186,101 cases in 2017 (Epidemiology Unit, Ministry of Health, Sri Lanka). In the absence of a successful preventive vaccine, dengue control solely depends on controlling the *Aedes aegypti* is known as the primary vector of dengue fever, and is considered to have originated in Africa (Brown *et al*., 2013).

There are two recognized subspecies of *Aedes aegypti*, the ancestral form, *Aedes aegypti formosus* (*Aaf*) supposedly limited to Sub-Saharan Africa, and *Aedes aegypti aegypti* (*Aaa*), found globally in tropical and subtropical regions in association with humans (Moore *et al*., 2013). Outside Africa, the latter is commonly referred to as *Ae aegypti*, without its sub species name, assuming *Aaf* is restricted to Africa (Tabachnick and Powell, 1979). The two subspecies distinctly differ in morphological and behavioral characters: *Aaf* breeds in natural containers such as tree holes, feeds primarily on wild animals and carries darker scales (Moore *et al*.,

2013) while their first abdominal tergite is devoid of scales. *Aaa*, on the other hand, breeds in artificial containers, feeds on humans, and carries paler colored scales that are absent on the first abdominal tergite (Moore *et al*., 2013). However, the genetic affinities observed among the global collections of *Aedes aegypti* do not directly align with this subspecies definition and fall into two clades: an East African clade containing *Aaa* from East Africa, South America and the Caribbean and the New World and an Asian clade consisting of *Aaa* from Asia and Southeastern America and *Aaf* from East and West Africa (Moore *et al*., 2013). When considering the origin of *Aaa* outside Africa, Approximate Bayesian Computation supports a picture where the mosquitoes radiated initially from Africa to Americas, and from there to Asia due to trade between the New World and Old World (Gloria-Soria *et al*., 2016). Global studies conducted so far have suggested two phylogenetic patterns for *Aaa* distribution, either in two well supported clades (pattern I) (Gorrochotegui-Escalante *et al*., 2000; Bosio *et al*., 2005; Lima and Scarpassa, 2009) or a basal and derived group of which the derived group arises from the basal group (pattern II) (Bracco *et al*., 2007; Paduan and Ribolla, 2008).

Currently, a vast number of research studies have been conducted around the world on *Ae. aegypti aegypti* (referred hereafter as *Ae. aegypti*, going with the common usage) in line with its well-known vector role. In particular, the knowledge on the genetic heterogeneity and phylogeography of *Ae. aegypti* populations are required to understand the patterns of dengue transmission and will facilitate the designing of effective vector control strategies.

To date, a spectrum of genetic markers has been used in investigating the phylogenetic relationships and population genetics of *Ae. aegypti*. Among them, mitochondrial DNA (mtDNA) is widely used due to its numerous advantages (Paduan and Ribolla, 2008). Since mtDNA is inherited maternally in the absence of recombination, it serves as a better candidate for untangling phylogenetic relationships within and between species (Avise, 1994). In addition, mtDNA haplotype polymorphism allows detecting gene flow patterns and numerous other population genetics parameters. Consequently, many studies have utilized mtDNA to investigate the population genetics and phylogeography of *Ae. aegypti* around the world (Bosio *et al*., 2005; Herrera *et al*., 2006; Paduan and Ribolla, 2008; Twerdochlib *et al*., 2012; Núñez *et al*., 2016; Sousa *et al*., 2017; Dharmarathne *et al*., 2021). Among them, mitochondrial cytochrome oxidase subunit 1 (*COI*) gene, and mitochondrial NADH dehydrogenase subunit 4 (*ND4*) gene are the most widely used mtDNA markers. According to Moore *et al*. (2013), since the year 2000, fourteen publications and four unpublished datasets have used sequence data from the ND4 mitochondrial gene to compare *Ae. aegypti* collections, and in the contemporary the number of studies may have increased by a stronger incremental value. Nevertheless, in Sri Lanka, so far only one study has used the ND4 gene to investigate the phylogenetic relatedness of *Ae. aegypti* found in Sri Lanka (Fernando *et al*., 2020). However, mosquito vectors from intermediate zone have not been incorporated into this study. The intermediate zone of Sri Lanka has climatically and topographically different geographical pockets which are significantly isolated from other parts of the country. For example, Badulla district in the intermediate zone is unique due to its high elevation of 680 m (2,230 ft) above sea level, mountain ranges, deep narrow valleys, escarpments and dissected plateaus (Central Enviornmental Authority 1988). The Namunukula range of mountains that consist of nine mountain peaks with the highest point of 2,016 m (6,614 ft), overshadows the area. In addition, the very short dry seasons with intermediate rainfalls and relatively cool temperatures in the region are likely to create a unique habitat for mosquitoes, which is not generally present in other areas of the country (Central Enviornmental Authority, 1988). The high elevation, the presence of Namunukula mountain range and other topographical features might be hindering the dispersal range of mosquitos and may be acting as a barrier to gene flow with the surrounding country. Thus, it would be important to analyze the *Ae. aegypti* phylogeny in Sri Lanka incorporating populations from such unique geographical pockets to understand the true dimensions of genetic diversity and phylogenetic relationships of *Ae. aegypti* mosquitoes present in the country. Further, given the mosquito flight ranges and possible passive migration through human mediated means, use of juvenile mosquito stages from different breeding sources provides a better opportunity to include unrelated individuals and to avoid incidental sampling of siblings in mosquito population studies. This is especially important in capturing the existing variability where sample numbers analyzed are low. It further facilitates a higher level of accuracy in the identification of the larval species compared to the use of adults. As such, the present study intends to improve the knowledge on phylogenetic relationships of Sri Lankan *Ae. aegypti* using the ND4 gene from larval samples from sources that were not investigated earlier, namely Badulla (intermediate mountainous zone) and Hambantota (dry zone).

2. Material and Methods

2.1 Ethical Clearance

Ethical clearance for this study, including the sampling of mosquitoes, was granted by Ethical Review Committee of Institute of Biology, Sri Lanka (ERC IOBSL 122 04 15).

2.2 Sample collection, identification and DNA extraction

Mosquito larvae were collected from three localities in Sri Lanka representing three districts and two climatic zones; Provincial General Hospital premises (6.9906°N, 81.0530°E), Badulla district, Intermediate zone; Railway station (8.5784°N, 81.2410°E), Trincomalee district, Dry zone; Fishery harbor (5.9873°N, 80.7330°E), Hambantota district, Dry zone (Fig. 1). In each locality, larvae were sampled from a variety of natural breeding habitats (discarded plastic containers, used tires, discarded bottles and tins, clay pots etc.) found within a radius of 500 m. Only one mosquito larva was randomly selected from any given breeding habitat/ container to avoid incidental sampling of siblings. *Ae. aegypti* larvae were morphologically identified using standard taxonomical keys (Amerasinghe, 1995) and preserved in absolute alcohol until further processing. DNA was extracted from a total of 27 mosquito larvae from Badulla (n = 10), Trincomalee (n = 10), and Hambantota districts (n = 7) using Promega Wizard DNA extraction kit (Promega, USA). The sample collection was carried out from March 2018 to September 2018.

2.3 PCR amplification and sequencing

Extracted DNA samples were subjected to PCR amplification to generate 359 bp fragments of ND4 gene using primers (ND4+: 5-TGATTGCCTAAGGCTCATGT-3; ND4-: 5-TTCGGCTTCCTAGTCGTTCAT-3) designed by Gorrochotegui-Escalante *et al*. (2000). Each 25 µL PCR reaction mixture contained 50 ng of template DNA, 1.5 mM $MgCl_2$ (Promega, USA), 0.2 mM dNTPs (Promega, USA), 0.4 µM of each primer (IDT, USA), 5 µL of 5X PCR amplification buffer and 0.3125 units of *Taq* polymerase (Thermo Fisher Scientific, USA). The PCR program involved an initial denaturation at 94ºC for 4 min, followed by 35 cycles at 94ºC for 1min, 57ºC for 1min, 72ºC for 1min, and a final extension at 72ºC for 10min. PCR amplicons were visualized on 2% agarose gels for qualitative analysis and were sent to Macrogen, Korea for bidirectional Sanger sequencing. Quality of all chromatograms was manually checked before further analysis. The consensus sequences were generated by aligning the forward and the reverse sequences in Bioedit 7.2 (Hall, 1999), and were verified using the online NCBI BLAST search (http://blast.ncbi.nlm.nih.gov) against previously deposited *Ae. aegypti* ND4 sequences.

2.4 Phylogenetic analysis

A total of 106 sequences were used for phylogenetic analysis: 27 ND4 sequences obtained from the present study together with 73 sequences obtained from previously published studies, and six *Aedes* species used as outgroups (Table 1). The sequences were aligned with ClustalW multiple alignment tool in Bioedit 7.2 (Hall, 1999). PhyML 3.0 (Guindon and Gascuel, 2003) was used to resolve the phylogeny of the samples based on maximum likelihood algorithm. Bootstrap support which denote the proportion of replicate trees with a particular clade as in the original phylogeny using the original sequence alignment, was calculated with 1,000 replications. Accordingly, three phylogenetic trees were built; the first one analyzed all samples of the current study, the second analyzed all Sri Lankan sequences available at NCBI databases along with present samples and the third investigated the relationship of all Sri Lankan samples with several selected global samples (Table 1).

2.5 Statistical analysis

Sequences aligned with each other and with reference *Ae. aegypti* ND4 sequence obtained from NCBI database were analyzed using DnaSP 5.1 (Librado and Rozas, 2009) to infer on the number of polymorphic sites, number of different haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π) and average number of nucleotide differences among individuals (k). Arlequin 3.5 (Excoffier *et al*., 2007) was used to estimate the variance in haplotype frequencies within and among populations using Analysis of Molecular Variance (AMOVA), the pairwise population F_{ST} estimates and the tests of neutrality-Tajima's D test (Tajima, 1989) and Fu and Li's F test (Fu, 1997). The statistical significance of AMOVA, F_{ST} estimates and Tajima's D test was evaluated with an in-built permutation test in Arlequin using 1,000 permutations. The level of gene flow (Nm) was estimated using population differentiation based on the formula $N\text{m}=F_{ST}/(1-F_{ST})$. The relationships between the haplotypes were deduced using Median Joining algorithm implemented in PopART (Leigh, 2015).

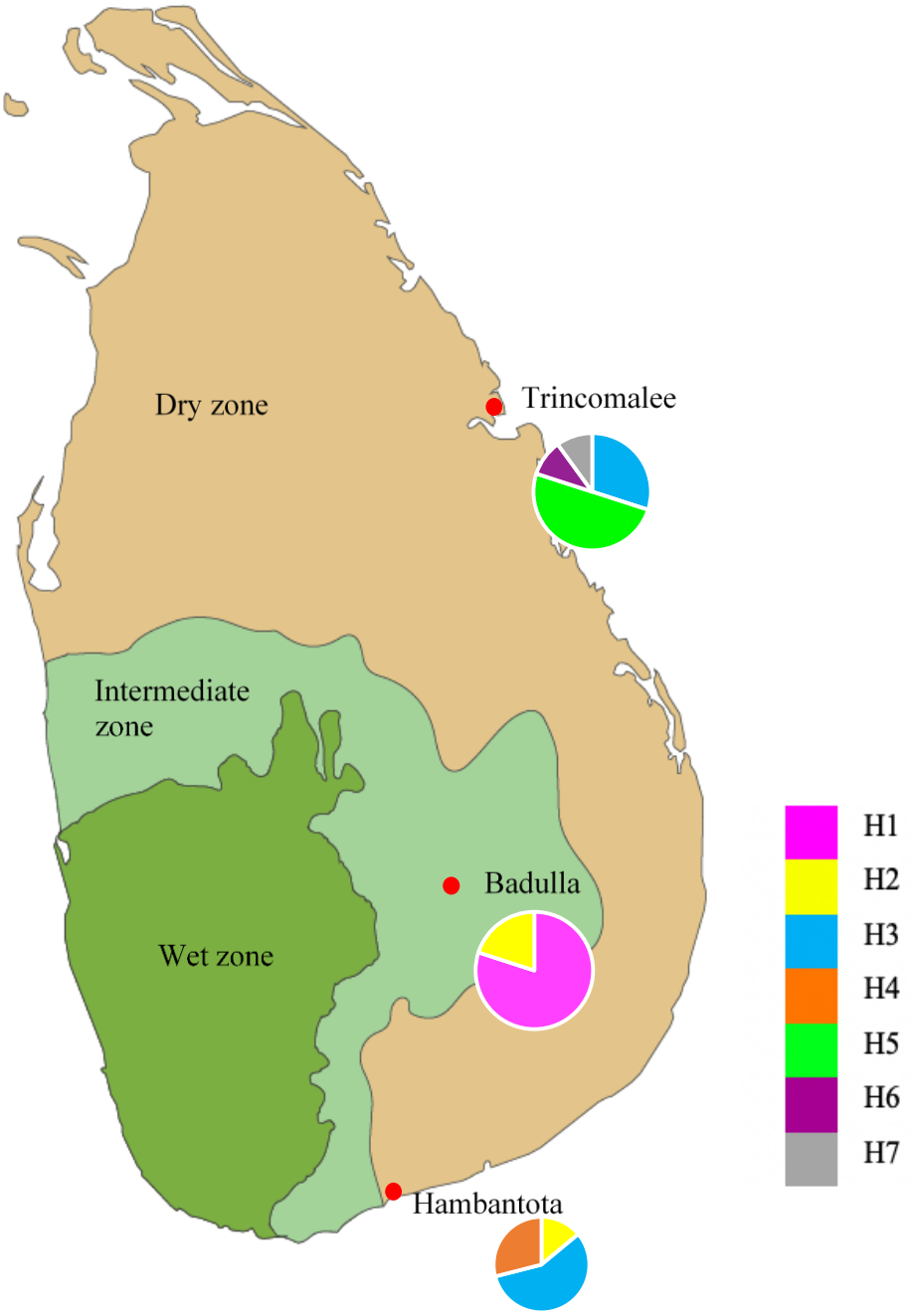

Fig. 1. *Aedes aegypti* sampling locations in Sri Lanka. The pie charts show the frequencies of each haplotype in the three populations. Circle size is proportionate to the number of individual mosquitoes in each population

3. Results

3.1 Genetic variation

The three mosquito populations analyzed in the study revealed an overall moderate genetic diversity compared to studies conducted elsewhere. Among the total 27 ND4 fragments analyzed, there were 11 parsimony informative polymorphic sites (Table 2); 10 with single transitions (C↔T at 16, 22, 73, 133, 142, 190, 235 and 313 bp positions and G↔A at 155 and 268 bp positions) and one with a single

transversion (A↔T at 271 bp position). The average nucleotide differences observed among the individual mosquitoes (k) was 4.803 with a nucleotide diversity (π) of 0.0134. The haplotype diversity observed was substantially high (Hd= 0.821) with a total of 7 haplotypes for the 27 samples collected (Table 3).

However, notwithstanding this overall picture, the genetic diversity measures calculated for Badulla population differed from those calculated for Hambantota and Trincomalee populations. In particular, the nucleotide diversity (π) and average number of nucleotide differences (k) observed in Badulla population were 0.0020 and 0.711, respectively, which were 6 to 8 folds less compared to those of Hambantota (π=0.0167; k=6.000) and Trincomalee (π=0.0136; k=4.489) populations. This was accompanied by a similar five-fold reduction in number of polymorphic sites (Badulla:2; Hambantota:11; Trincomalee:9). Likewise, both the haplotype diversity (Hd) and haplotype number (H) showed a two-fold reduction in Badulla population (Hd=0.356; H=2) compared to Hambantota (Hd=0.667; H=3) and Trincomalee (Hd=0.711; H=4) populations. Further, out of the 10 samples from Badulla, eight had the same haplotype (H1), indicating a genetic homogeneity among the Badulla population. This points at the possibility of a recent founder event or a genetic bottleneck in the Badulla population compared to the other two sites.

Among the three sites analyzed in the present study, the mosquitoes occupying Hambantota area displayed the highest genetic diversity with respect to all k, π and Hd values (k = 6, π = 0.0167, Hd = 0.667). However, the number of haplotypes observed in Trincomalee area was larger by one haplotype compared to that of Hambantota. Likewise, the number of unique haplotypes found in Trincomalee samples was higher (3; H5, H6 and H7) compared to that of Hambantota (2; H2 and H4). This may have been caused by the disparity in sample size between the two areas, as Trincomalee carried three additional samples compared to Hambantota.

It is also interesting to note that none of the seven haplotypes observed in the current study were shared among all three populations, though haplotypes 2 and 3 are shared between two populations; H2 for Badulla and Hambantota and H3 for Hambantota and Trincomalee (Table 2 and Fig. 5). Haplotypes 6 and 7 were singletons and detected only in Trincomalee population. In addition, five unique haplotypes were also identified; haplotype 1 from Badulla, haplotype 4 from Hambantota, and haplotypes 5, 6 and 7 from Trincomalee *Ae. aegypti* populations, indicating a relatively high genetic diversity in the present sample. Further, when these haplotypes were compared with the sequences reported earlier by other authors, H6 and H7 were found only in the present study, while H2 had been reported only in Sri Lanka previously. Except these three haplotypes (H2, H6 and H7), which are found to be unique to Sri Lanka, other four haplotypes (H1, H3, H4 and H5) have been reported previously from various countries around the world.

According to the results obtained with AMOVA, substantially high (31.9%) amount of variation among the studied samples was attributed to the genetic differences among population (F_{ST}= 0.319; p<0.05) indicating a population substructure among the study sites. When pairwise comparisons were performed for the three populations, mosquito samples collected from Badulla showed a substantial population differentiation with Hambantota (F_{ST}= 0.4702) and Trincomalee (F_{ST}= 0.4539) samples (p<0.05). However, there was no population structure detected among the samples collected from Hambantota and Trincomalee. Parallel to this, a high level of gene flow was observed between Trincomalee and Hambantota sites while less than one migrant per generation had been exchanged between Badulla - Hambantota and Badulla -Trincomalee (Table 4).

The tests of Neutrality conducted with Tajima's D (ranged from 0.019 to 2.35) and Fu's F (ranged from 1.523 to 4.058) indicated positive values for all three populations. However, none of the values were statistically significant (p > 0.05).

3.2 Phylogenetic analysis

According to the ND4 sequence analysis performed in the present study, the *Ae. aegypti* mosquitoes of Sri Lanka forms two distinct clades (bootstrap support = 100%) (Fig. 2). The mosquitoes sampled from Hambantota and Trincomalee were grouped together in both clades whereas the specimens from Badulla were restricted to only one clade. Accordingly, clade 1 (Bootstrap support = 95%) consisted of three haplotypes (H3, H6 and H7) collected from Hambantota and Trincomalee, while the clade 2 (bootstrap support = 75%) enclosed four haplotypes (H1, H2, H4 and H5) sampled from Badulla, Hambantota and Trincomalee. A few individual mosquitoes from Badulla indicated a shared haplotype with Hambantota population but not with Trincomalee population (Fig. 2).

When all the samples of the current study were analyzed together with the 40 selected Sri Lankan samples that were analyzed by Fernando *et al.* (2020), two distinct clades were revealed (bootstrap support = 100%): one clade consisting of samples from Trincomalee (N=11; 5 from current study).

Table 1. Sampling locations and the NCBI accession numbers of mtDNA ND4 sequences used for the phylogenetic analysis.

Location/ Country of Collection	Number of Sequences	Accession Numbers	Reference
Badulla, Sri Lanka, Asia	10	MW239031, MW239032, MW239033, MW239034, MW239035, MW239036, MW239037, MW239038, MW239039, MW239040	Current study
Hambantota, Sri Lanka, Asia	07	MW239041, MW239042, MW239043, MW239044, MW239045, MW239046, MW239047	Current study
Trincomalee, Sri Lanka, Asia	10	MW239048, MW239049, MW239050, MW239051, MW239052, MW239053, MW239054, MW239055, MW239056, MW239057	Current study
Galle, Sri Lanka, Asia	01	KY496642	Fernando *et al*., 2020
Colombo, Sri Lanka, Asia	01	KY496643	Fernando *et al*., 2020
Trincomalee, Sri Lanka, Asia	08	KY496644, KY496645, KY496646, KY496647, KY496648, KY496649, KY496650, KY496651	Fernando *et al*., 2020
Kandy, Sri Lanka, Asia	17	KY476368, KY476369, KY476370, KY476371, KY476372, KY476373, KY476374, KY476375, KY476376, KY476377, KY476378, KY476379, KY476380, KY476381, KY476382, KY476383, KY476384	Fernando *et al*., 2020
Puttalam, Sri Lanka, Asia	13	KY476385, KY476386, KY476387, KY476388, KY476389, KY476390, KY476391, KY476392, KY476393, KY476394, KY476395, KY476396, KY476397	Fernando *et al*., 2020
Senegal, West Africa Kenya, East Africa	22	JX427511, JX427515, JX427516, JX427517, JX427518, JX427519, JX427520, JX427521, JX427524, JX427525, EU446267, EU446268, EU446271, EU446273, EU446275, EU446276, EU446278, JX427526 (*Aedes metallicus*: Outgroup), JX427529 (*Aedes vittatus*: outgroup), JX427527 (*Aedes luteocephalus*: outgroup), JX427530 (*Aedes unilineatus*: outgroup), JX427528 (*Aedes longipalpis*: outgroup)	Moore *et al*., 2013
Brazil, South America Senegal, West Africa USA, North America	02	EU650417, EU650411	Lima and Scarpassa, 2009

Brazil, South America Kenya, East Africa	01	AY906841	Paduan and Ribolla, 2008
Mexico, North America USA, North America Brazil, South America Venezuela, South America Peru, South America Senegal, West Africa Kenya, East Africa Myanmar, Asia Thailand, Asia Tahiti, Asia Cambodia, Asia Singapore, Asia	06	AF203356, AF203348, AF203346, AF203344, AF334863, AF334860	Gorrochotegui-Escalante *et al*., 2000
Cameroon, West Africa	01	EF562504	Paupy *et al*., 2008
Senegal, West Africa USA, North America Guinea, West Africa Uganda, East Africa Singapore, Asia Brazil, South America Cameroon, West Africa	04	DQ176836, DQ176848, DQ176845, DQ176837	Bracco *et al*., 2007
Senegal, West Africa Venezuela, South America Thailand, Asia	01	DQ440274	Ribeiro *et al*., 2007
Brazil, South America Mexico, North America Myanmar, Asia	01	EF153747	GenBank
Brazil, South America	01	EF153761 (*Ae. albopictus:* outgroup)	Costa *et al*., 2006

Table 2. Variable sites of the seven haplotypes found for 27 *Ae. aegypti* mosquitoes

	Polymorphic site											Number of haplotypes observed (N)			
Haplotype	016	022	073	133	142	155	190	235	268	271	313	N_{Total}	$N_{Badulla}$	$N_{Hambantota}$	$N_{Trincomalee}$
H1	T	T	T	C	T	G	T	T	A	A	C	8	8		
H2	T	T	T	C	T	A	T	T	A	T	C	3	2	1	
H3	C	C	C	T	C	G	C	C	G	A	T	7		4	3
H4	T	T	T	C	T	G	T	T	A	T	C	2		2	
H5	T	T	T	T	T	G	T	T	A	T	C	5			5
H6	C	C	T	T	T	G	T	T	G	A	T	1			1
H7	C	C	C	T	C	G	C	C	G	T	T	1			1

Table 3. Sequence polymorphisms and genetic variability reported for *Ae. aegypti* populations in the current and previous studies from Sri Lanka and Brazil, Venezuela, Mexico and Thailand

Collection	N	Length in bp	Sequence overlapping	P	H	π	Hd	k	Reference
Sri Lanka	27	359		11	7	0.0134	0.821	4.803	Present study
Badulla	10	359		2	2	0.0020	0.356	0.711	Present study
Hambantota	7	359		11	3	0.0167	0.667	6.000	Present study
Trincomalee	10	359		9	4	0.0136	0.711	4.489	Present study
Sri Lanka[a]	40	349	Y	17	24	0.0198	-	6.9231	(Fernando *et al*. 2020)
Parana, Brazil	156	311	Y	12	8	0.01556	0.702	4.82	(Twerdochlib *et al*. 2012)
Brazil[b]	125	374	Y	15	24	0.01740	0.787	6.542	(Paduan and Ribolla 2008)
Venezuela[c]	1144	359	Y	18	7	0.01877	-	6.073	(Herrera *et al*. 2006)
Mexico[d]	574	387	Y	15	7	0.01434	-	5.549	(Gorrochotegui-Escalante *et al*. 2000)
Brazilion Amazon	123	380	Y	16	13	0.0115	0.666	4.399	(Lima and Scarpassa 2009)
Thailand[e]	1346	359	Y	17	7	0.00793	-	2.847	(Bosio *et al*. 2005)

N: number of *Ae. aegypti* mosquitoes collected, P: number of polymorphic sites, H: number of haplotypes, π: nucleotide diversity, Hd: haplotype diversity, k: average number of nucleotide differences. Y: the ND4 region analyzed overlaps with the ND4 region analyzed in the current study. [a-e] denote different regions where sampling was done in each country. [a]Kandy (n=17), Trincomalee (n=8), Puttalam (n=13), Colombo (1) and Galle (1) in Sri Lanka. [b]Alagoas, Ceará, Mato Grosso Sul, Paraná, Rondônia and São Paulo. [c]West Coast, Lake, West Region, Inland Region, Central Region, North Region, East Region and South Region. [d]Nuevo Leon, Tamaulipas and Veracruz. [e]Chiang Mai, Mae Sot, Kamphaeng Phet, Bangkok, Phang-nga, Hat Yai and Sadao.

Table 4. Population differentiation and gene flow among the *Ae. aegypti* populations of the three different locations

	Badulla	Hambantota	Trincomalee
Badulla	0	0.89	0.83
Hambantota	**0.4702** (120)	0	Infinity
Trincomalee	**0.4539** (173)	-0.0133 (294)	0

Upper diagonal shows the geneflow values estimated using $N_m=F_{ST}/(1-F_{ST})$. Lower diagonal shows the population pairwise F_{ST} values. Within parentheses is the geographical distance in km. Bold denotes significance at α =0.05.

Hambantota (N=4; all from current study), Kandy (N=8) and Galle (N= 1) (bootstrap support = 94%); second clade consisting of samples from Badulla (N=10; all from current study), Trincomalee (N=7; 5 from current study), Hambantota (N=3; all from current study) and Kandy (N =9), Puttalam (N=13), Colombo (N=1) (bootstrap support = 77%) (Fig. 3).

When the phylogenetic tree was further extended to include ND4 haplotypes reported from six other countries of the world, Sri Lankan *Ae. aegypti* samples resolved in to two clades. In clade 1 (bootstrap support=100%), haplotypes from Trincomalee (H3, H6 and H7) and Hambantota (H3) were clustered with 16 more global haplotypes (EU650411, EU650417, AF203356, AY906841, DQ440274, AF203344, AF334863, AF334860, DQ176836, EU446267, EU446268, EU446271, EU446273, EU446275, EU446276 and EF562504), which have been collected from Cameroon, Kenya, Brazil, Mexico, USA and Senegal that were previously reported to be on the derived clade in a global phylogenetic analysis (Moore *et al*., 2013).

The clade 2 (Bootstrap support=75%) consisted of haplotypes from Trincomalee (H5), Hambantota (H2 and H4) and Badulla (H2) and seven other global haplotypes resolved in to the basal clade in the same previous phylogenetic analysis (Moore *et al*., 2013). These seven specimens were coming from countries such as USA, Brazil, Mexico, Myanmar, Kenya and Senegal (AF203348, DQ176845, DQ176848, AF203346, EF153747, EU446278 and DQ176837) and indicated a higher genetic affinity to H2, H4 and H5 haplotypes of the present study. Haplotype 1 (H1) of Badulla was found separately associated with clade 2 along with samples from Senegal closer to the outgroup (JX427511, JX427515, JX427516, JX427517, JX427518, JX427519, JX427519, JX427521 and JX427525) (Fig. 4), indicating H1 to be the basal haplotype observed in the present study.

3.3 Haplotype network analysis

The haplotype network indicated a similar pattern to the phylogenetic trees discussed above. Haplotypes 1, 2, 4 and 5 were clustered closely to each other at one end of the network. Haplotypes 1, 2 and 5 were two mutations apart from each other while they all were only one mutation apart from haplotype 4, indicating a subtle sequence divergence among them. On the other hand, these haplotypes were comparatively distant from haplotypes 3, 6 and 7 revealing at least 5 mutational steps. The common star-like pattern was not observed in the haplotype network generated here (Fig. 5). Haplotype distribution among the three mosquito populations indicated that the haplotype 1 which is the basal haplotype in the present study was exclusive to Badulla mosquito samples only. Further as indicated in the Fig. 1, the populations located along the coastal line of the country were harboring comparatively more haplotype diversity than the inland Badulla population.

4. Discussion

The present study was carried out to understand the population genetics and phylogenetic relationships among the *Ae. aegypti* mosquitoes in three dengue endemic areas in Sri Lanka using a 359 bp region of mtDNA, the ND4 sequence. As observed by Moore *et al*. (2013), *Ae. aegypti* mosquitoes around the world have originated from one of two ancestral clades; a basal clade associated with West Africa and a clade derived from the basal clade, which consist of mosquitoes from East Africa. In the present study too, the phylogenetic analysis of *Ae. aegypti* that includes samples from Sri Lanka and six other countries were resolved in to two clades, but without a clear demarcation between the basal and derived clades.

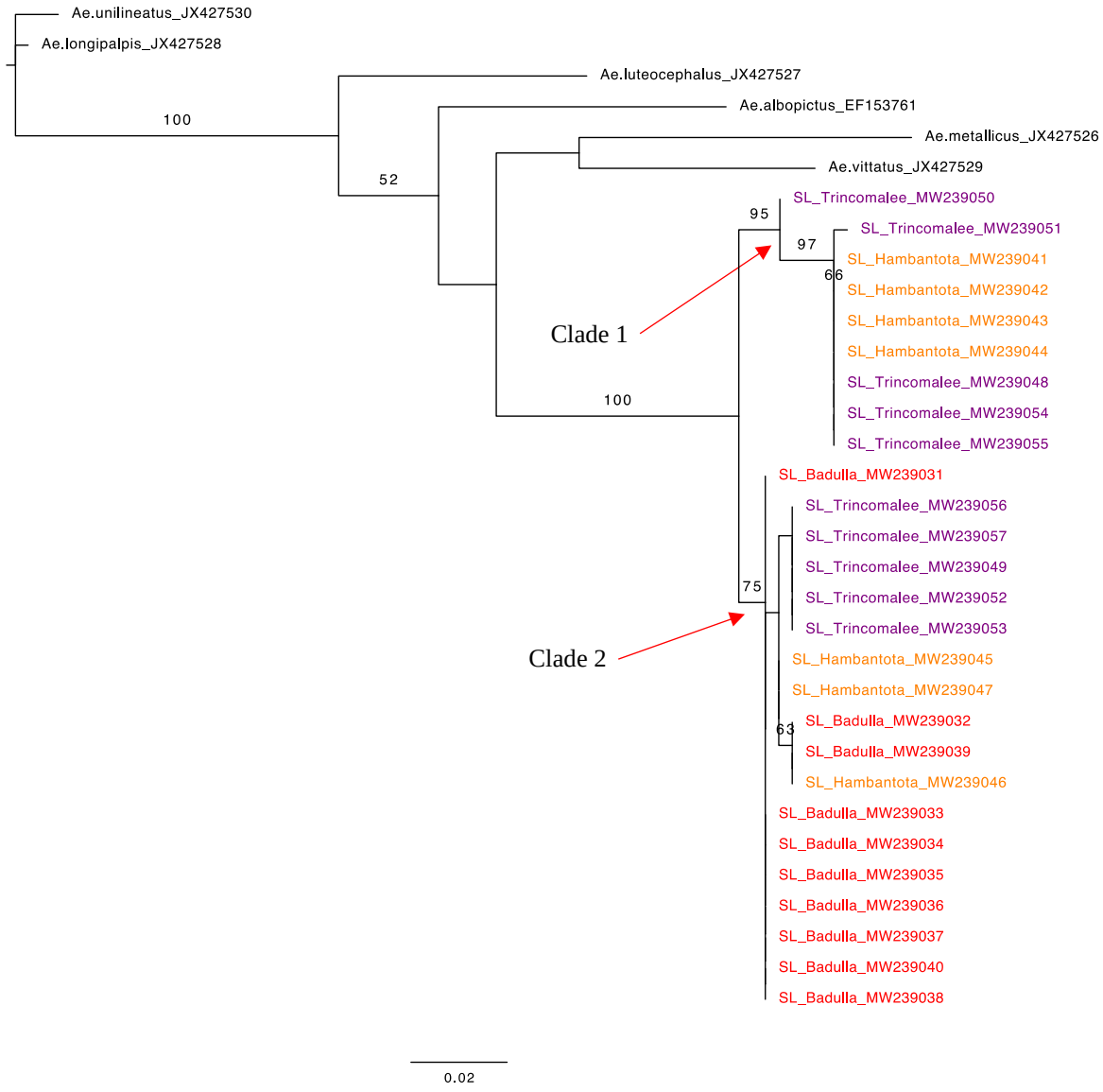

Fig. 2. Maximum likelihood tree showing phylogenetic relationships among the 27 ND4 haplotypes in *Ae. aegypti* collected from Badulla (red), Hambantota (orange) and Trincomalee (purple) populations in Sri Lanka generated with PhyML. Bootstrap support (>50%) is shown above each branch.

Fig. 3. Maximum likelihood tree showing phylogenetic relationships among the 67 ND4 haplotypes in *Ae. aegypti* collected from Badulla, Hambantota, Trincomalee, Galle, Colombo, Kandy and Puttalam in Sri Lanka (current study and Fernando *et al*., 2020) generated with PhyML. Bootstrap support (>50%) is shown above each branch.

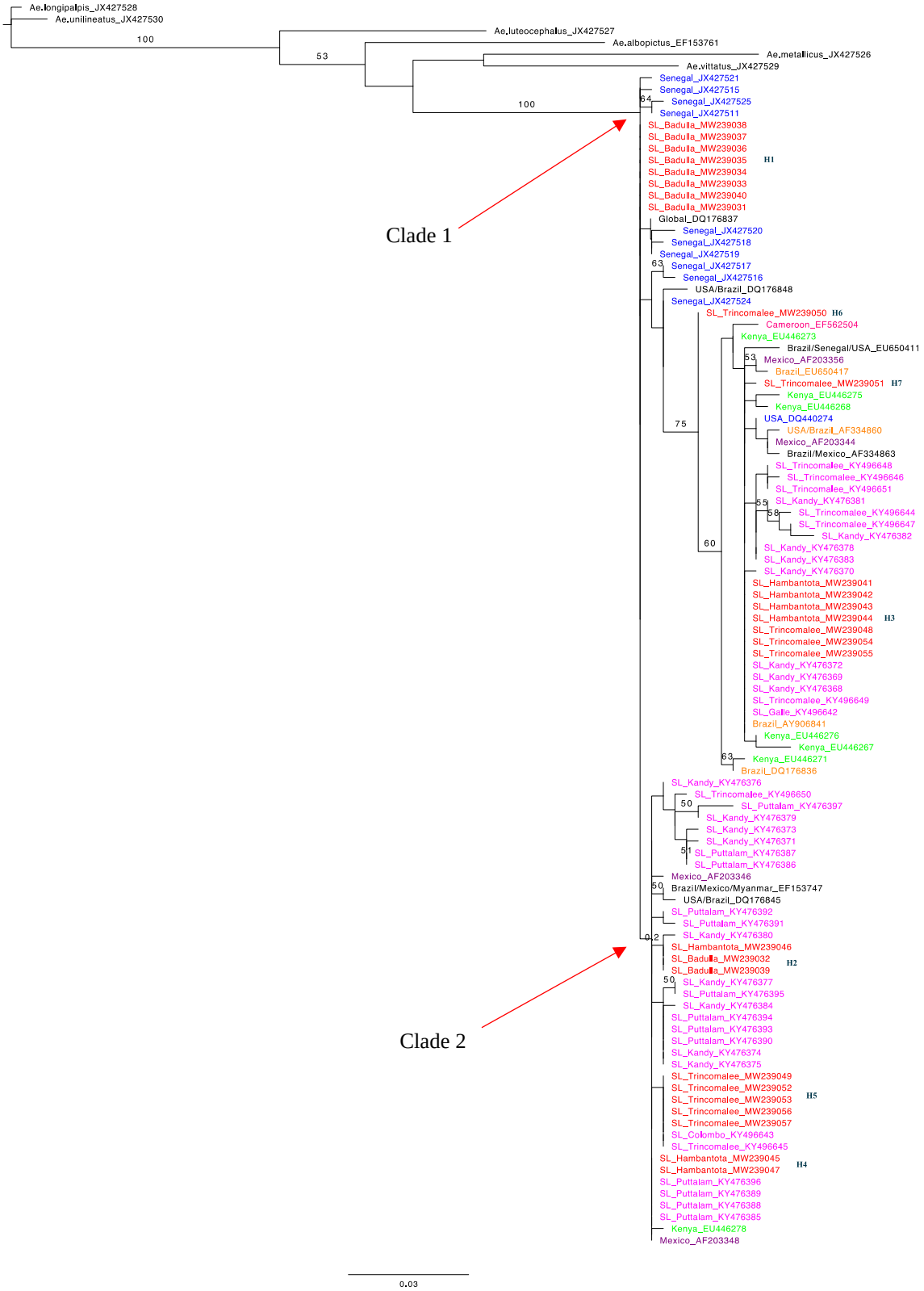

Fig. 4. Maximum likelihood tree showing phylogenetic relationships among the 106 ND4 haplotypes in *Ae. aegypti* collected from Sri Lanka, Senegal, Kenya, Brazil, Mexico, USA and Myanmar. Sri Lankan samples collected from the present study and the previous study (Fernando *et al*., 2020) are labeled in red and pink, respectively. The sequence named as Global represents Guinea, Uganda, Singapore, Cameroon, Brazil, Myanmar and Senegal. H1-H7 refer to the haplotypes generated in the present study. Bootstrap support (>50%) is shown above each branch.

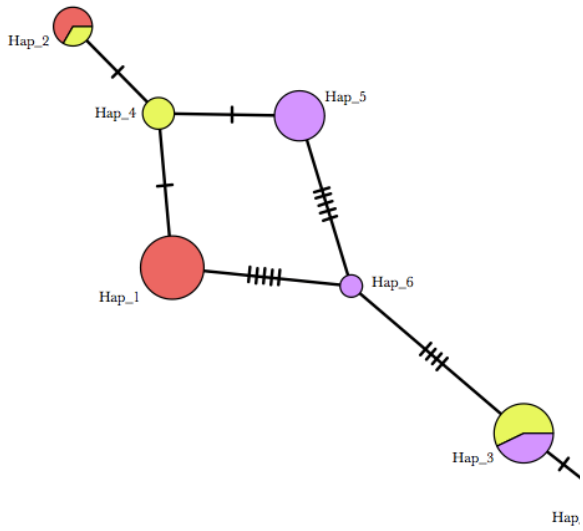

Fig. 5. Median-joining haplotype network showing relationships among the seven haplotypes. Circle size is proportionate to the number of individual mosquitoes found for each haplotype. Hatch marks represent the number of mutational steps.

Nevertheless, one clade consisted of 17 global *Ae. aegypti* sequences previously assigned to the basal clade, and the second clade consisted of 16 sequences previously assigned to the derived clade. Based on this observation, the two clades obtained in the current analysis can be considered to be synergistic with the phylogenetic tree reported earlier for global ND4 haplotypes. Accordingly, the two clades identified in the present analysis are also designated as basal and derived clades. Interestingly, haplotypes from Trincomalee and Hambantota occupied both clades, while the two haplotypes observed for Badulla were restricted to the basal clade. Further, among the two haplotypes detected in Badulla, haplotype 2 was grouped with other Sri Lankan samples in the basal clade, while haplotype 1, which is unique to Badulla was resolved together with samples from Senegal in to a separate group located closer to the outgroup. This observation suggests the existence of a basal group of *Ae. aegypti* mosquitoes in Badulla, whose ancestry could be directly linked to West African Senegal populations, where *Ae. aegypti* originated from. Interestingly, this haplotype 1 found in Badulla is also shared globally (Global DQ176837) with many countries including Uganda, Singapore, Brazil and Myanmar in addition to other West African countries like Guinea and Cameroon. Considering the fact that *Ae. aegypti* had originated in West Africa, and was introduced later to New World and Asia, the presence of Senegal haplotypes in many parts of the world including Badulla in Sri Lanka could be attributed to multiple direct invasions of *Ae. aegypti* occurred from West Africa towards many parts of the globe including both New World and Asia (i.e. single focal expansion). When Badulla is considered, this invasion should be quite recent, as the genetic diversity of the Badulla population (π=0.002) is approximately six fold lower than the genetic diversity reported for Senegal (π=0.0119) (Fernando *et al*., 2020). According to Tabachnick and Powell (1979), after a founder event, a minimum of 10^5 generations is needed prior to restoring the heterozygosity to the level of original population. This indicates that the Badulla population should be much younger to the Senegal population in an evolutionary time scale.

On the other hand, the haplotypes of coastal Hambantota and Trincomalee populations found in both the basal and the derived clades clustered together with the haplotypes reported from other countries in the world such as Brazil, Mexico, Kenya, USA, Cameroon and Senegal. This genetic analogy of local haplotypes to multiple other haplotypes reported globally from several countries may indicate possible multiple invasions of *Ae. aegypti* to coastal Sri Lanka. It is believed that *Ae. aegypti* was introduced to Asia, during the late 19th century, with the development of the shipping industry, specifically through the cargo ships carrying used tires (Service, 1980; Tracy, 1990). Sri Lanka, being a well-known trading hub at that time with two natural ports at both Hambantota (Ray, 2003) and Trincomalee (Siriweera, 2002) might have been an easy target for possible multiple invasions of *Ae. aegypti* from various countries that have made use of the two ports during their sea voyages. This further suggests that Hambantota and Trincomalee populations had been open historically to accumulate new genetic material from different geographical areas, diluting the presentation of ancestral haplotypes within the respective populations.

When genetic diversity indices (π, Hd, k and number of haplotypes and polymorphic sites) of Hambantota and Trincomalee populations were compared with those available from other countries, they were approximately comparable and much higher than what had been observed for Badulla. This high genetic diversity in Hambantota and Trincomalee populations implies that these populations are likely to have a much larger effective population size and /or population settings that had been well-established compared to the Badulla population. These inferences are supported by the climatic, geographical and economic characteristics of both

areas. Bordering the coastline of Sri Lanka, both Hambantota and Trincomalee, are located in low altitudes below 10 meters, with average annual temperatures around 27ºC and alternate dry and wet seasons providing optimal conditions for mosquito breeding and survival. In Hambantota, mosquito samples were collected from a busy fishing harbor, while in Trincomalee, the collections were made from the Railway Station. Both collection sites are of close proximity to two of the three major harbors in Sri Lanka (Hambantota International Port and Trincomalee Port), which are actively involved in passenger and cargo transportation at both local and international levels. In addition, the second largest airport in Sri Lanka is located in Hambantota, while the domestic China Bay passenger terminal belonging to China Bay Airport in Trincomalee is only 6 km apart from the Trincomalee sampling site. Thus, both collection sites are regularly visited by numerous vehicles, facilitating human mediated mosquito migrations on a regular basis. Numerous studies around the world have previously shown that marine transportation assists mosquito migration across the sea, increasing the level of gene flow among populations (Fonzi *et al*., 2015; Guagliardo *et al*., 2019). In addition, a study from Japan reported the presence of *Ae. aegypti* at Narita International Airport and inside aircrafts (Sukehiro *et al*., 2013), which provides evidence for human mediated aerial transportation of mosquitoes. As such, the geographical locality of the Hambantota and Trincomalee populations has been a major contributing factor for the possible multiple invasions and the high degree of genetic distribution between countries as inferred by the phylogenetic analysis. Moreover, Ngugi *et al*. (2017) have shown that *Ae. aegypti* in coastal regions produce significantly more immature populations than those in non-coastal regions. Since genetic diversity depends to a large extent on the actual population size, this high genetic diversity may be an indication of the relatively large effective population sizes in Hambantota and Trincomalee areas.

The observed genetic diversity for Trincomalee and Hambantota areas is significantly less than what had been reported previously for Sri Lankan *Ae. aegypti* population using ND4 sequences (Table 3). For example, Fernando *et al*. (2020) have reported an average nucleotide difference of 6.9 for the overall population of Sri Lankan *Ae. aegypti*, which is 1.4 times higher than the overall value we have observed in the present study. Likewise, the value of nucleotide diversity (π) reported is significantly higher than the present study. Further, within the 40 samples used for ND4 phylogenetic analysis, 24 haplotypes were reported (60 haplotypes for 100 samples) compared to the total of seven haplotypes that we have observed for 27 samples (25 haplotypes for 100 samples). It might be possible that an increase in sampling size would initially increase the number of haplotypes detected until all common haplotypes have being sampled. However, detecting 60 haplotypes per 100 individuals is a surprisingly high observation and had not been reported before from any other global *Ae. aegypti* population typed for ND4. The highest number of haplotypes reported so far for *Ae. aegypt*i ND4 sequence, 24, was previously reported for a Brazilian sample consisting of 125 mosquitoes (19 haplotypes per 100 individuals). Notwithstanding this disparity, when the genetic diversity observed in the current study was compared to the previous studies conducted elsewhere in the world, the results observed for the two coastal areas in the country (Hambantota and Trincomalee) are approximately comparable to those observed in Brazil, Mexico, Panama, and Venezuela , although Thailand has exhibited a lower level of genetic diversity compared to all other populations (Table 3).

Unlike Hambantota and Trincomalee, Badulla is located in an inland mountainous area with no direct access to aerial or coastal transport connectivity from other countries. The minimum distance to Badulla from Sri Lankan coast line is about 134 km, while the closest airport is situated 131 km away from Badulla. Accordingly, any non-native mosquito invasion should first cross the coastal belt of Sri Lanka, before being transferred to Badulla through a land continuum. However, our results show that the Badulla mosquito population is relatively isolated from the other two Sri Lankan populations studied as indicated by the high population differentiation (F_{ST}>0.4) and low gene flow (Nm<1) values obtained for Badulla-Hambantota and Badulla- Trincomalee populations. The same was also reflected in the substantial genetic structure (F_{ST} = 0.319) observed in the AMOVA analysis. The restricted gene flow between Badulla samples with the other two locations might be due to its high altitude of 680 m, which restricts the flight range of mosquitoes as has been observed previously in other countries (OCED, 2018). Thus, there is a little chance for Badulla population to be supplemented with new variants that invade Hambantota or Trincomalee. This isolated nature of Badulla population is partly reflected by its relatively low genetic diversity as discussed earlier. On the other hand, as observed by Keyghobadi *et al*. (2006), the genetic diversity of mosquito populations tends to reduce with increasing altitudes. In addition, several studies conducted in India, Kosovo, Galapagos Islands and Hawaii have illustrated that the mosquito abundance is relatively low in areas of mid to high altitudes

compared to those of low altitudes (Kalra *et al*., 1997; Ahumada *et al*., 2004; Asigau *et al*., 2017; Muja-Bajraktari *et al*., 2019). This in turn is associated with low effective population sizes and leads towards low genetic diversity. Further, the low temperatures which generally accompany higher altitudes are also capable of affecting the mosquito life cycle and behavioral patterns. For example, a study conducted in Albania on *Ae. albopictus* has revealed that low temperatures reduce mosquito development rates resulting in large bodied mosquitoes with increased wing sizes specially among females (Prudhomme *et al*., 2019) slowing their movement at high altitudinal areas. Aytekin *et al*. (2009) also described that at low temperatures, *Anopheles superpictus* show longer egg incubation periods and low larval survival rates. Another study done in East St. Louis, Missouri, USA showed that the overall production of adult mosquitos is lower in cooler climates compared to more warmer counterparts (Alto and Juliano, 2001). Thus, it is possible that the more central location and high altitudes in Badulla may have affected mosquito development and behavior, with a negative impact on both population size and gene flow causing the mosquitoes to retain the more basal haplotypes (basal haplotype H1, and H2 which is only two mutation steps away from H1) with less genetic diversity, differentiating from the other two mosquito populations analyzed in the current study.

On the other hand, *Ae. aegypti* samples collected from Hambantota and Trincomalee are quite panmictic with a maximum level of gene flow between the two localities, despite the 294 km distance that separates them. The geographical locations of the two populations, as well as the various transportation routes that link the two sites as discussed above, might be actively contributing to the long-distance mosquito migrations leading to high geneflow between the two localities. The recent developments in infrastructure and increased urbanization in both Hambantota and Trincomalee might have augmented this geneflow, leading to the observed genetic admixture among the two populations.

These genetic substructures observed among the three populations were also reflected in our phylogenetic analysis. In the phylogenetic tree illustrated from all 27 *Ae. aegypti*, only haplotype 1 sampled from Badulla made a separate clade while haplotype 2 associated with only one sample from Hambantota, highlighting their genetic homogeneity and restricted gene flow with other populations. In contrast, mosquitoes from Hambantota were clustered with those from Trincomalee in both clades denoting the genetic proximity between the two populations. The same basic structure was retained in the phylogenetic tree, when the previously published sequences from Kandy, Puttalam, Galle, Colombo and Trincomalee were incorporated in to the phylogenetic tree, supporting the relatively isolated nature of Badulla *Ae. aegypti* mosquitoes and panmictic nature of Hambantota and Trincomalee populations. Further, the majority of Hambantota and Trincomalee mosquitoes were clustered together with haplotypes previously generated from Kandy, Puttalam, Trincomalee, Galle and Colombo populations without any identifiable pattern that approximate their geographic distribution.

Although it is plausible to suspect a recent population expansion in both Hambantota and Trincomalee areas based on the relatively large diversity indices and high mosquito migrations recorded, neither revealed population expansion according to Tajima's D and Fu's F tests. The two tests of neutrality are known to reveal the demographic history of populations in the recent past and positive values obtained for them denote reductions of population size such as genetic bottleneck and/or balancing selections, whereas negative values indicate population expansions and/or positive selections (Tajima, 1989; Pichler, 2002). Although both the Tajima's D and Fu's F values were positive for all three populations in the current study, none of them was statistically significant for any population, which exclude that the populations under study have undergone size reductions associated with bottlenecks or balancing selections. This further suggests that the low genetic diversity detected in Badulla area is likely to have caused by a recent founder event rather than a genetic bottleneck, which may have caused by topographical factors or mosquito control campaigns. Accordingly, it is fair to infer that the mosquito populations analyzed here have neutrally evolved under mutation drift equilibrium during the recent past.

5. Conclusion

Our analysis on mitochondrial ND4 sequences obtained from three localities in Sri Lanka is suggestive of multiple invasions that have occurred during *Ae. aegypti* colonization within the country. The initial colonization events are likely to have occurred in the coastal areas, which has several climatic and topographical features desirable for mosquito population expansion. The central mountain area is likely to have undergone a more recent direct invasion from the ancestral West African variant. The population structure which was observed between the coastal and central mountain areas may have been colonized/established at least

in part by the high altitudes in the latter that hinders the mosquito flight range. The same must have contributed towards the relatively low genetic diversity observed in the central mountainous area. The panmictic nature of the two populations occupying the two distantly located coastal sites, Hambantota and Trincomalee is suggestive of long-distant passive mosquito migrations occurring within the country. Although, these findings need to be confirmed with more extensive set of samples, considering the limited number of samples upon which these inferences are based on, the present study points at the need for an international collective attempt to contain the dengue vector from those affected countries like Sri Lanka.

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7. Conflict of Interest

Authors declare that they have no conflict of interest.

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