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Monika Soni
Regional Medical Research Centre (ICMR) NER, Dibrugarh, Assam, India

Chandra K Bhattacharjee
Regional Medical Research Centre (ICMR) NER, Dibrugarh, Assam, India

Siraj Ahmed Khan
Regional Medical Research Centre (ICMR) NER, Dibrugarh, Assam, India

Prafulla Dutta
Regional Medical Research Centre (ICMR) NER, Dibrugarh, Assam, India

Correspondence
Prafulla Dutta
Regional Medical Research Centre (ICMR) NER, Dibrugarh, Assam, India

DNA barcoding as a complementary approach for species identification from dengue endemic regions of North East India

Monika Soni, Chandra K Bhattacharjee, Siraj Ahmed Khan and Prafulla Dutta

Abstract
Aedes aegypti and *Aedes albopictus* are rapidly expanding mosquito vectors responsible for transmitting Dengue, Chikungunya, Zika and Yellow fever. Regional climatic factors can trigger species drift consequently influencing epidemiological consequence. Though morphological identification is still widely adopted method of species identification, limited resources and absence of taxonomic expertise can adversely impact result outcome. The present study highlights resolution offered by DNA barcoding in species identification.
Results found *Aedes aegypti* as dominant and abundant vector species in urban areas. Sequence of *Aedes albopictus* shows 100% similarity whereas sequence of *Aedes aegypti* shows 99.77 - 100% similarity of COI gene when compared with BOLD database search. However, phylogeny revealed two clusters of each species based on similarity search, separating native population with rest of globe. Intra species divergences between two taxa, were found to be 0-2.6% and 0.5% respectively.

Keywords: COI, Dengue vectors, Morphological identification, DNA barcoding, Phylogenetics

1. Introduction
Dengue is a mosquito-borne viral infection, rapidly spreading worldwide and has become a major international public health concern [1]. According to the World Health Organization (WHO) 2.5 billion people are at risk globally where majority of population (70%) reside in Asia Pacific countries and 96 million (67–136 million) manifest clinically (with any severity of disease) [2]. India is showing an exponential increase of dengue incidence from all parts of country, including North-East region [3-7]. As per the National Vector Borne Disease Control Programme (NVBDCP) report of 2015, a total of 99913 dengue cases have been reported from almost all states and Union territories in India of which 220 (0.22%) patients expired [8].
Among vast diversity of vectors, two major mosquito species responsible for transmission of Yellow fever, Dengue, Chikungunya and Zika virus across the globe are known to be *Aedes aegypti* and *Aedes albopictus* [9, 10] and both the vectors are highly prevalent in India [11, 12]. Globalization, climatic shift and international trading have significantly expanded the geographic range and domestication of these mosquito species [13]. Ancestral form of *A. aegypti* still exists in forest of sub-Saharan Africa, known to feed on non human blood with habitats confined to tree holes [14]. Similar consequence could be anticipated for *A. albopictus* domestication, once known to be a forest mosquito. It is now viewed as a potential threat to public health, with several studies detecting arbovirus in this mosquito species [15, 16].
With increasing incidences of vector borne diseases and diversity of mosquito available expertise in mosquito identification becomes a critical step. It is prerequisite for effective entomological surveillance and vector incrimination program. Existing morphological keys are based on female characteristics only. Feature that distinguish these vectors viz. *A. aegypti* and *A. albopictus* lies on the thorax *A. aegypti* shows white silver marking in the form of lyre on the dorsal surface of thorax whereas *A. albopictus* marks a middle silver line down the thorax [17, 18] Freshly emerged adults shows clear external markings but eventually there may be cases when these external features could not be prominent or is lost subsequently. Further, fundamental changes developed within species inhabiting different climatic regions cannot be

identified with the traditional classification method. Sensitivity increase when sibling species are also co-circulating. All these circumstances pose difficulty in accurate species identification process.

DNA barcoding is an advance taxonomical identification approach which utilizes mitochondrial genome for species discrimination as it lack introns and do not show frequent recombination. Technique offers several advantages over traditional morphological identification method like clear discrimination between closely related species, identification of invasive species, recognition of cryptic species and prompt discrimination of mosquito species from a small piece of tissue from any developmental stage. The method utilized several genes among which mitochondrial cytochrome c oxidase subunit 1*(COI*) gene is common due to precise distinction at inter-species level [19]. Barcode of Life Data Systems (BOLD) is a growing reference library of DNA barcode i.e. (BOLD) contributing globally in acquisition, storage, analysis and publication of DNA barcode records [20]. Although, majority of the states in India are affected with dengue and other vector borne diseases, only few studies have reported molecular epidemiology and evolutionary analysis of dengue vectors forecasting scarcity of data for comparative analysi [21-23]. It is anticipated that geographic distribution and climatic factors can influence the vector potential of the local mosquito population. Assam and Arunachal Pradesh are endemic states of India for vector borne diseases [24, 25]. Further, before associating a species to a particular disease, its correct identification is extremely important when other sibling species are co-circulating, one of the essential requirement of successful vector control program. Therefore DNA based molecular approach was adapted for identification of dengue vectors circulating in these regions. Data generated will help to compare phylogenetic taxonomy at global scale. With increasing outbreaks of dengue and expansion to new areas, this detail study reinforces an understanding of diversity and population structure of dengue vector distributed across the region from two representative states of North East India.

2. Materials and methods

2.1. Sample collection sites and Sample collection:

Adults of mosquito species *A. albopictus* and *A. aegypti* were collected from the urban area of Sivsagar, Dibrugarh, Tinsukia, Tezpur and Biswanath Chariali of Assam and Pashigath of Arunachal Pradesh with previous history of dengue cases during June 2016 to September 2016. Areas with GPRS coordinates are shown in the map and marked as red color (Figure-1and Table1). Collection was carried out using BG-Sentinal Trap (Bio Gents GmbH, Germany) using commercial human bait during day time. Mosquitoes were brought to Regional Medical Research Centre (RMRC), Dibrugarh mosquito colony and identified by applying standard morphological keys and separated accordingly into two species respectively. All the biosafety measures were adopted as per institutional regulation and guideline for maintaining field collected mosquitoes. Adults were maintained on 10% glucose solution. Larval collection was carried out using dippers from different potential breeding habitats of *Aedes* mosquitoes like tire dumps, open battery boxes, and bamboo stumps. Thereafter, larvae were raised to adults for morphological identification. Separate mosquito colonies were raised according to collection site up to second filial generation of the each species. Females were given blood meal by artificial feeding using Hemotek membrane feeding systems (Hemotek Ltd, Bloomberg, UK) for egg collection and maintaining mosquitoes up to second generation.

2.2 Morphological Identification

Both the vectors collected from different locations were identified applying standard morphological keys [26, 27]. However, while adult collection using mosquito traps, sometimes the specimens are found damaged due to breakage of wings, removal of silver scales and for which the external morphological features were not prominent also highlighted in Figure 2.

2.3 Genomic DNA Extraction

Mosquitoes were sterilized in 95% ethanol, wings were removed and washed with sterile milliQ water before taken into downstream processing. Individual mosquito samples were grinded in liquid nitrogen using mortar and pistol and incubated with ATL buffer along with proteinase K (Qiagen, Inc., Hilden, Germany) solution for 2 hours at 56°C with pulse vortexing for 5-10 second. Total genomic DNA was extracted from each mosquitoes using QIAGEN DNeasy blood and tissue kit (Qiagen, Inc., Hilden, Germany) following manufacturer's instructions. The extracted DNA was stored at -20°C in aliquots until further used.

2.4 PCR amplification and sequencing

Polymerase Chain Reaction was carried out to amplify 735 bp region of mitochondrial COI gene using forward primers 5-GGATTTGGAAATTGATTAGTTCCTT and reverse primer 3 AAAAATTTTAATTCCAGTTGGAACAGC [28]. The total 50 µl PCR reaction consisted of 5 µl of extracted DNA, 25 µl of 2X Master mix containing Taq DNA Polymarase, dNTPs, $MgCl_2$ (Promega, USA), and 1.25 µl of each 10 µM/µl primer. Reactions were run on thermal cycler following conditions: Initial denaturation at 95 ^{0}C for 5 min; 35 cycles of 94 ^{0}C for 40sec, 45 ^{0}C for 1 min, 72^0 C for 1 minute; and a final extension at 72 ^{0}C for 10 min [28]. Amplicons were visualised on 1.5% agarose gel to check the integrity of the fragments and thereafter PCR products with expected band size (approximately 735base pair) (Figure3) were purified using the QIAquick Gel Extraction Kit (Qiagen, Inc., Hilden, Germany) according to manufacturer's instruction. Purified PCR products were sent for bidirectional sequencing at SciGenom Labs Pvt. Ltd., Kerela, India using same primers.

2.5 COI Sequence editing and species discrimination using DNA barcodes

Finch TV 1.4.0 (Geospiza, PerkinElmer) was used to check each chromatogram for nucleotide signal intensity and signal strength in order to obtain a more accurate reading. The ABI trace file generated after sequencing of COI mosquito sequences were trimmed and edited manually in BioEdit Sequence Alignment Editor v 7.0.9.0., and after editing a 550bp of. fasta formatted file was generated for each sequence [29]. The 550bp of COI nucleotide sequences of each mosquito species was compared with sequences previously deposited in National Centre for Biotechnology Information (NCBI) using nucleotide Basic Local Alignment Search Tool

(BLASTn). The sequences were also compared with reference sequences of barcode specific database namely barcode of life database (BOLD) (www.barcodingoflife.com) [29] for species identification using the "Identification Request" function. A similarity cut-off of 97% was used for species identification [19]. To compare the performance of DNA barcoding for species identification, 38 conspecific and congeneric sequences submitted by others investigators from different geographical areas were downloaded. These downloaded sequences along with the sequences generated in this study were used for neighbor joining (NJ) clustering analysis using MEGA 7 [30]. Primarily the sequences from this study specimen were chosen only to construct a neighbor-joining phylogenetic tree based on Kimura 2 parameter (K2P) genetic distance. The COI sequences of *Anopheles gambiae*, *Anopheles maculipennis* were used as an out-group for the construction of maximum-likelihood phylogenetic tree. The intra and interspecies nucleotide divergence were calculated using K2P in MEGA 7. The sequences generated from this study were processed and submitted to National Centre for Biotechnology Information (NCBI) with the accession numbers from KX227735- KX227743.

3. Results

All the collection sites showed the presence of dengue vectors widely distributed across the area (Figure 1). Collection sites which were surrounded with thick vegetation, waste tire dumps and human dwellings showed the mixed presence of both the vector species. However, few of the selected sites were predominant with single vector species. Genomic DNA extracted from whole mosquito sample yielded appreciable quality of (>10 kb) amplified PCR product as shown in Figure 3 with band size of approximately 735 bp.

Detailed taxonomic analysis revealed identification and prevalence of two species *A. aegypti* and *A. albopictus* despite of other sibling species reported from this region. The species *A. aegypti* comprised of 66.6% of the specimen still representing a dominant vector species.

The sequences obtained through standard DNA barcoding protocol were compared with public databases, viz. Gen Bank and BOLD. All the *Aedes albopictus* sequences showed 100% similarity whereas sequence of *Aedes aegypti* shown 99.77 - 100% similarity of COI gene when compared with geographically distinct similar species, based on BOLD database search (Table2).

Thirty eight sequences were retrieved from NCBI and BOLD databases from different geographical regions showing records of dengue, to determine the evolutionary distance model (Figure 4). Neighbor-Joining (NJ) and Maximum Likelihood (ML) phylogenetic tree was constructed in MEGA6.06 software with 1000 bootstrap replicates using Kimura-2-Parameter model (Figure5).

Based on the phylogenetic analysis, a global map is constructed depicting two clusters of dengue vectors for each species (Figure 4).Vector borne disease reported from these regions were also marked. Majority of the countries plotted are at high risk of Dengue, Chikungunya and yellow fever. However, in India still Dengue and Chikungunya are circulating but the risk of yellow fever could not be ignored. Also the available data on dengue vectors from India is comparatively less in correlation to increasing incidence of dengue outbreaks reported from the country.

Data analysis for sequence divergence found intraspecies divergence ranging from 0.0 to 2.0% and interspecies divergence ranged from 11.0 to 12.0%. Phylogenetic analysis reveals, both the mosquito species from northeast India forms a separate clad with Indian counterpart following few exceptions and is distinct from other parts of the globe (Figure 5). This depicts that the two mosquito species are confined to India.

3.1 Nucleotide base composition of the COI segment studied

Total nucleotide base composition of the CO1 fragment varied across the selected mosquito specimen with an expected AT bias. The sequences were highly AT rich, which ranged from 66.7 to 67.5% and GC content of 32.5to 33.3%. Difference of nucleotide base composition between two mosquito species is not observed.

3.2 Intra Species and Inter Specie homology

Intraspecific homogeneity at DNA level ranged from 98.4%-100% and 99.5% among *A. aegypti* and *A. albopictus*. Whereas both the group of mosquitoes had sequence homogeneity ranging from 88.2%-88.7% at nucleotide level.

4. Discussion

Mosquito fauna of Northeast India is known to be very diverse. The abundance of certain vegetations like banana, bamboo, pineapple etc., provides favorable breeding grounds for the growth of various *Aedes* species. Study carried out during 1999-2005 [31] has documented the presence of 191 species of mosquitoes out of which 43 species belong to *Aedes* alone from Northeast region of India. The state of Assam contributed the highest number of species (41 nos.) compared to the other six states. However, this study considered morphological classification as a gold standard method of species identification. However, the present study represents resolution and accuracy offered by phylogeny based molecular method over conventional approach of species identification. In an ongoing effort to build regional barcode library study has contributed samples from two most important vectors spreading dengue and other diseases from Assam and Arunachal Pradesh.

Several studies have utilized DNA Barcoding as a tool to distinguish morphologically similar *Aedes* species which are otherwise difficult to discriminate when the external features are washed away [28, 32-34].

Our findings showed close congruence of highest genetic homology based on mitochondrial COI gene i.e. 88.2%-88.7% between the two species of Aedes (*A. aegypti* and *A. albopictus*). The phylogenetic analysis reveals that both the mosquito species included in this study form a separate clad with other Indian counterpart with few exceptions and is distinct from other parts of the globe. Global map constructed (Figure 4) on the basis of available barcode sequences reveals the how the two vectors are distributed geographically. Map also depicts the earlier cases of vector borne disease reported from these regions. There are few places in the map where the vector is found but no disease reported. It is clear that developing country like India already facing economic burden of Dengue and Chikungunya is at high risk for yellow fever. This enforces the importance of large sequence repository required at global level to enhance our understanding of

distribution and diversity of mosquito fauna.

The composition of the target CO1 gene of the sequences were AT rich which is similar to earlier studies [35-37]. Intraspecies divergences noted between two taxa *A. aegypti* and *A. albopictus* were 0-2.6% and 0.5% respectively. Findings reported that the distance between the two dengue vectors was 0.5 and 1.3% (35) respectively lower than our estimated divergence between two mosquito species (12.3%-12.7%).

Recent studies show that vector competence is a phenomenon which may also vary at intraspecies level. One such study documents substantial variations in Mexico and United States *A. aegypti* population towards vector competence for dengue [38, 39]. Another study has documented that there are 11 cryptic species of *A. albopictus* subgroup among which *A. albopictus* is most widely distributed [40]. However no such data on species specific variation of vector competence is available from this region. It is found that still *A. aegypti* is the dominant vector in majority of the urban areas but the domestication of *A. albopictus* adaptation could not be ignored and there is scope for further studies in this aspect.

Table 1: Lit of *Aedes aegypti* & *Aedes albopictus* partial mitochondrial COI gene sequence submitted in gene bank along with geographic location.

Sl. No.	Mosquito Species	Place	Longitude	Gene Bank accession number
1	*A. aegypti*	Guwahati, Assam	91.4717234E&26.111092N	KX227735
2	*A. aegypti*	Tinsukia, Assam	95.357722E & 27.491074N	KX227743
3	*A. aegypti*	Dibrugarh, Assam	94.900615E&27.479737N	KX227737
4	*A. aegypti*	Pasighat, Arunachal Pradesh	95.332929E&28.059440N	KX227741
5	*A. aegypti*	Biswanath Chariali, Assam	92.928640E&26.788098	KX227739
6	*A. aegypti*	Sibsagar, Assam	94.648684E&26.999739N	KX227740
7	*A. albopictus*	Pasighat, Arunachal Pradesh	95.332929E&28.059440N	KX227738
8	*A. albopictus*	Sibsagar,Assam	94.648684E&26.999739N	KX227742
9	*A. albopictus*	Tinsukia, Assam	95.1953077E&27.215331N	KX227736

Table 2: Similarity of COI gene compared with geographically distinct similar species, based on Gene Bank and Barcode of life database (BOLD)

Studied species	Species matched by name		% Similarity	
	GenBank (BLASTn)	BOLD-IDS	GenBank (BLASTn)	BOLD-IDS
Aedes aegypti				
KX227735	*Aedes aegypti*	*Aedes aegypti*	100	100
KX227737	*Aedes aegypti*	*Aedes aegypti*	100	100
KX227739	*Aedes aegypti*	*Aedes aegypti*	100	99.82
KX227740	*Aedes aegypti*	*Aedes aegypti*	100	99.77
KX227741	*Aedes aegypti*	*Aedes aegypti*	100	100
KX227743	*Aedes aegypti*	*Aedes aegypti*	100	99.81
Aedes albopictus				
KX227736	*Aedes albopictus*	*Aedes albopictus*	100	100
KX227738	*Aedes albopictus*	*Aedes albopictus*	100	100
KX227742	*Aedes albopictus*	*Aedes albopictus*	100	100

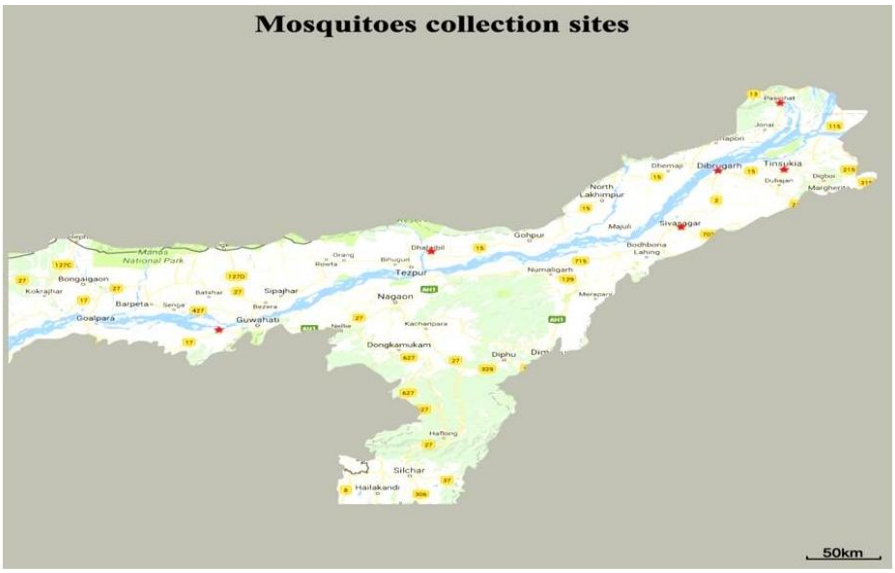

Fig 1: Represents the mosquito collection site from Assam and Arunachal Pradesh of India.

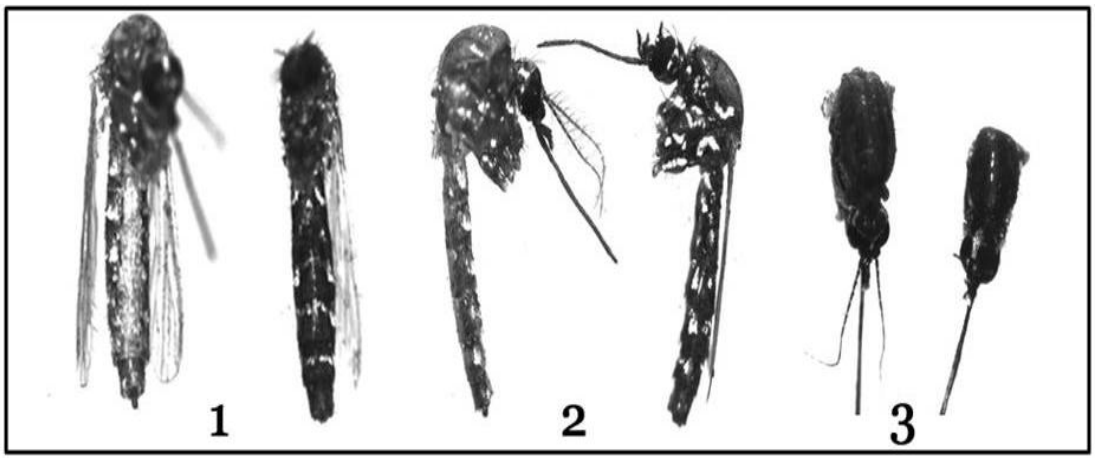

Fig 2: Represents the morphological comparison of *A. aegypti* and *A. albopictus* (1) ventral view (2) Lateral view (3) dorsal view

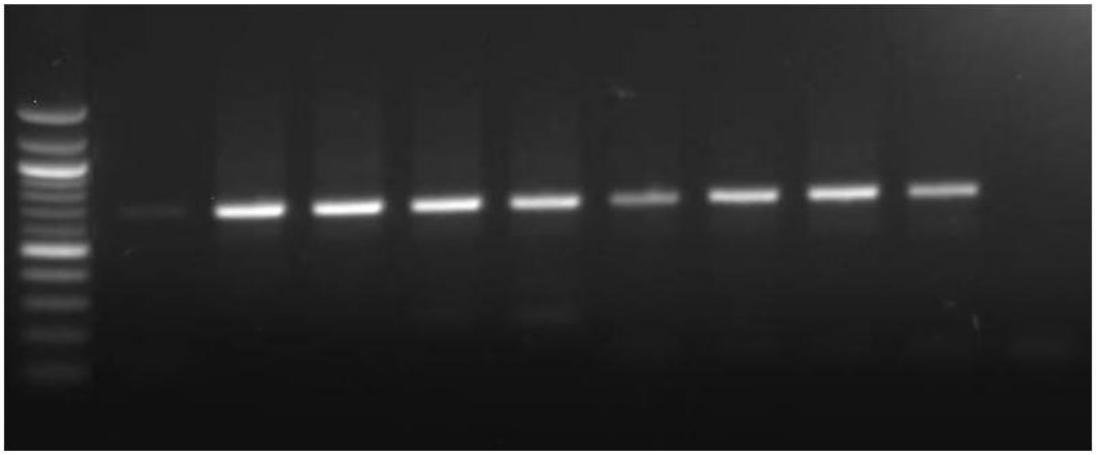

Fig 3: Represents the amplification of mitochondrial CO1 gene using the specific primers, each lane shows the amplified product approximately 735 base pair with marker.

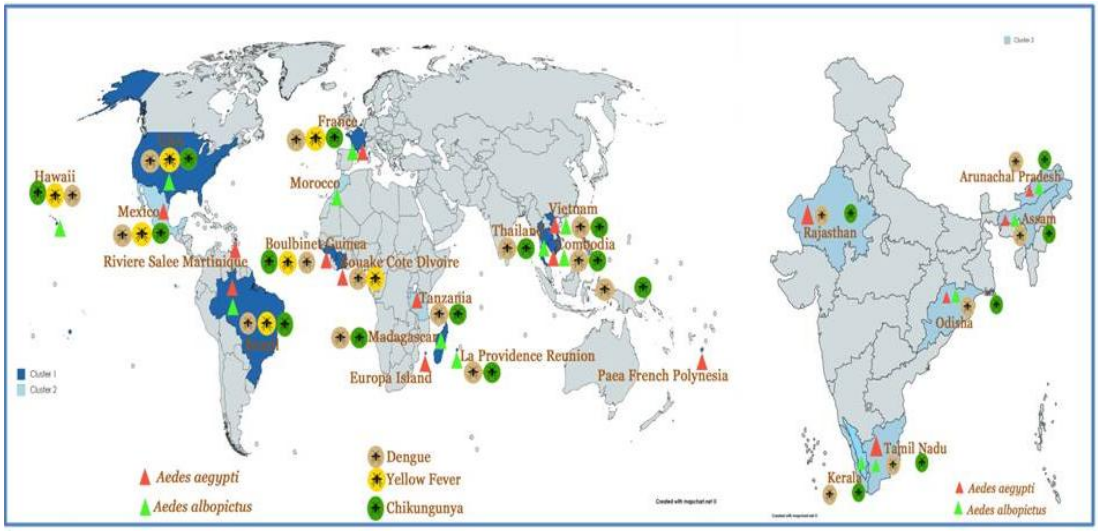

Fig 4: Represents Global and India map depicting similarity in dengue vectors from different geographic locations on the basis of barcode sequences. Aroboviral diseases reported from these regions are also highlighted.

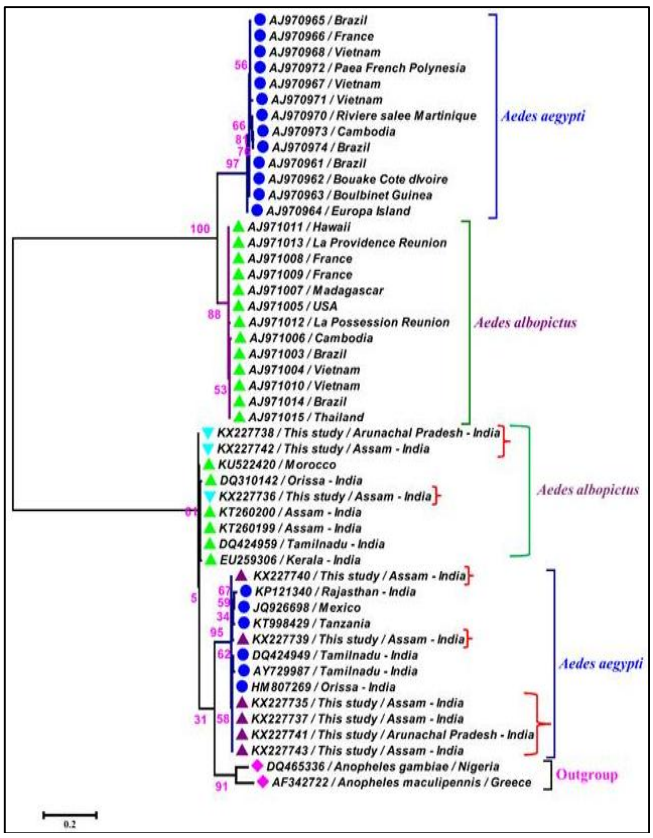

Fig 5: Explains about the phylogenetic tree of sequences from mosquito species from Assam and Arunachal Pradesh (highlighted by red curly bracket) in this study and sequences downloaded from databases using MEGA6 and K2P model.

5. Conclusion

The study highlighted the significance of molecular identification as a diagnostic tool in mosquito borne endemic settings when there are lack of resources and taxonomic expertise, eventually leading to misidentification of disease vectors. Study contributes as a preliminary step of initiating a regional barcode library of dengue vectors from one of the biodiversity hotspot of India. Knowledge of species or sibling species actively involved in spreading dengue outbreak in these regions is of extremely important for successful implementation of vector control strategy. For better representation of vector prevalence and species identification from this region it is important to expand regional DNA barcode database to be utilized in surveillance program. Hence, DNA barcoding could be a potential approach to sharpen our understanding of mosquito speciation process in vector endemic settings.

6. Author's contributions: MS is receiving the fund and designed the study, carried out field collection work and conducted laboratory experiments. PD supervised overall study and edited the manuscript. SAK was responsible for study design and overall coordination of the study. CKB assisted in laboratory works, analyzed the data and preparation of the manuscript. All authors read and approved the final version of the manuscript.

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