

Morpho-molecular aspects of two deathly mosquitoes from Manipur, India

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ABSTRACT: The morphometrics along with the molecular approach of two species *Anopheles* species and *Aedes aegypti* (L), was taken up. Live mosquitoes and their larvae were gathered from various locations throughout Manipur, including Thoubal, Imphal East, and Imphal West. Using the appropriate keys and comparing them with the data at hand, the morphometric identification was completed. After extracting DNA from adults and sequencing it using Cytb primers, a phylogenetic tree was created using identical sequences from the NCBI Genbank. *Anopheles* in present studies reports two species, *Anopheles longirostris* and *An. jamesii* but the molecular work is based only on *An. longirostris*. Though their niche is overlapping, both *Anopheles* are quite distinct from one another. While *An. longirostris* is a mosquito species that is a significant malaria vector than *An. jamesii*. *Aedes aegypti* was more prevalent in valley districts. © 2026 Association for Advancement of Entomology

KEY WORDS: Vectors, *Anopheles longirostris*, *An. jamesii*, *Aedes aegypti*, phylogenetic tree

INTRODUCTION

Certain mosquitoes are responsible for dreaded human diseases like malaria, dengue, yellow fevers, Japanese encephalitis etc. as they can spread pathogens like harmful viruses, nematodes, and protozoa (Soghigian *et al.*, 2020). With the synergic effect of urbanization and climate change, the gravity of the disease burden due to mosquito as vector multiplied many folds. The life cycles of mosquitoes are accelerated by higher temperatures, which results in faster reproduction and larger populations. The mosquito vectors exhibit quite different resting, biting, vectorial capacity and habitat to show different epidemiology of respective species (Sriwichai *et al.*, 2016; Chaiphongpachara and Laojun, 2020). This necessitates for true

identification of the vector species for formulating species specific vector controls after accurate vector information for effective control (Payakkapol *et al.*, 2016; Sumruayphoet *et al.*, 2020). But absence of certain morphological key features due to hasty handling of the specimens in the process of capturing them in the fields, leading to misidentification (Chaiphongpachara *et al.*, 2022). Furthermore, there are several *Anopheles* mosquito species form complex groups, comprising morphologically indistinguishable sibling species (Muller *et al.*, 2013). The knowledge in molecular biology has undergone significant progress, leading to the development of techniques useful for species identification (Walton *et al.*, 1999). Therefore, the use of modern molecular biology techniques for the species-based identification of mosquito vectors is

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an extremely alternative to standard morphological methods (Samung *et al.*, 2016). The morphological approach is still an important approach for species identification. Molecular approaches that efficiently differentiate conspecific mosquitoes such as the barcode region (Hebert *et al.*, 2003) improve identification accuracy considerably, but are time consuming, expensive in terms of post-polymerase chain reaction processing and depend heavily on DNA sequencing (Ajamma *et al.*, 2016). The species of mosquito from the state was dissected through the morphometric and molecular approaches for the first time. In the present studies two species *Anopheles longirostris*, *An. jamesii* and *Aedes aegypti* was undertaken but the molecular work was done only on *An. longirostris*.

MATERIALS AND METHODS

Manipur lies in the North eastern state of India between 23.83° N to 25.68° N Latitudes and 93.03° E to 94.78° E Longitudes. The valley lies at an elevation of approximately 790m above the sea level with the area covered of 22,327 km². The state belongs to subtropical to temperate climate with an annual rainfall of about 913mm (June to September) which made the favourable ecosystem for mosquito diversity. Present study begins with the collection of specimens using aerial nets from the foot hill sides and around the Loushi pat (Lake) of Tekcham Maning Leikai, Thoubal district Manipur, India with GPS Tag of Lat 24.553479°; Long 93.99151° and few adults and larvae from other parts of Manipur valley (Table 1). Some of the collected specimens were preserved as dry stuff to preserve for long term preservation and also to retain the morphological integrity. While few were fixed using fixative (1 glacial acetic acid: 3 alcohol) for cytological studies. Fifteen of the selected specimens were kept in absolute alcohol for molecular analysis.

For morphological identification and descriptions pictorial keys were taken as references. To identify from adult mosquito species, the insects collected by hand swiping of net were sorted into mosquito and non-mosquito species. Only the mosquitoes of the concerned were collected in collection chambers while the rest were released on spot. The collected

mosquitoes were differentiated and group into male and female then morphological characteristics were observed to differentiate up to Genera and sub-genus. Lastly base on the all-morphological features, and following keys of Narayan *et al.* (2024), Walter Reed Biosystematics Unit (WRBU), Rueda (2004), WHO, Huang (1977, 1980), the mosquitoes were identified to group, complex or species level.

For molecular analysis four groups 15 specimens each which were morphologically identified were kept in separate centrifuge tube containing absolute alcohol with different serial number marked on it by permanent marker. DNA samples were extracted from 15 adult mosquitoes of each species by the standard phenol/chloroform method (Sambrook *et al.*, 1989) and stored at the Dhanamanjuri University, Imphal. PCR was performed using Taq DNA polymerase (GeNei Merck, Mumbai, India) and a model 2720 Thermal Cycler (Applied Biosystems). The complete mitochondrial Cytb coding sequence (470 bp) was amplified using the universal primers CYT BF and CYT BR (L20934) (Lyman, 1999). The amplified fragments were used as templates for sequence determination with the above universal primers and two internal primers (BioKart Bangalore). Both DNA strands of the PCR products were sequenced using an automated method with a BigDye terminator Cycle Sequence Kit (ver. 3.1; Applied Biosystems) and an automated sequencer (model 3130; Applied Biosystems). Sequence fragments obtained with different primers were assembled using MEGA5 software (Tamura *et al.*, 2011), and the sequences were aligned by Clustal W.

Constructed neighbour-joining (NJ) tree using MEGA5. In NJ analyses, the substitution model that better described the substitution patterns of the Cytb(HKY+G; Hasegawa *et al.*, 1985) data was determined by MEGA5. In ML analysis, the tree topology search was evaluated using simultaneous nearest-neighbour interchange (NNI). In NJ analysis, the Kimura two-parameter model (K2) was used. The reliability of nodes was assessed using 1000 bootstrap replicates.

Determined Cytb gene sequences (470 bp) in two (13 *Aedes* Manipur; 14 *Anopheles* Manipur)

mosquitoes from Manipur. Constructed NJ trees using the Cytb sequences, together with database sequences, to assess the phylogenetic relationships within and between species.

RESULTS AND DISCUSSION

The characteristics of adult *Ae. aegypti* areas follows-scutum has lyre-shaped silvery- white scales. Proboscis is dark in colour. Wing scales are

dark. Legs have white basal bands. In larva strong black hooks on sides of thorax is present. One row of comb scales with pitchfork- shaped is present. Pupa: paddles have stubble. Palps have silvery-white scales at the tips. Clypeus has white scales. All these features are in accordance with the other authors (Figs. 2, 3). The adults are predominantly found during daytime and very agile. The larvae are usually procured from unused utensil or storage water tank of any material with rain water deposits.



Fig1. Different sites of species collection from different habitats of valley districts



Fig. 2 *Aedes aegypti*, (A) dorsal view of male mosquito scutum with lyre-shaped silvery-white scales; (B) female adult with white basal bands on tarsomers; (C) wing with dark scales

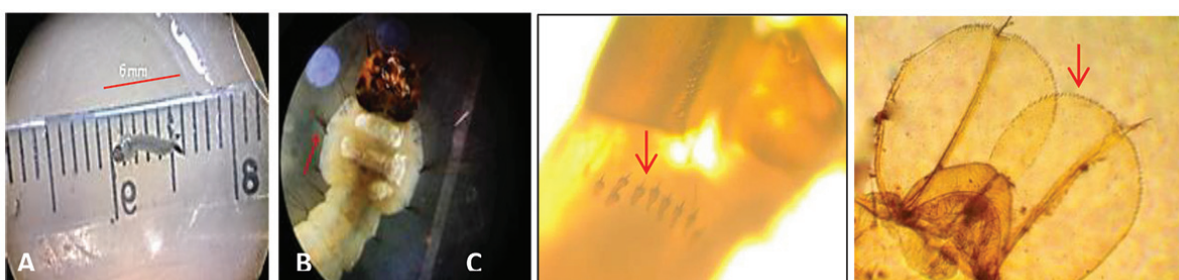


Fig. 3 Larva (*Ae. aegypti*) - (A) 4th instar larva is about 6mm in length; (B) larva with a strong black hook-on lateral side of thorax; (C) single row of comb scales with pitchfork-shaped; (D) Pupal paddles with stubble structure on the curve margin

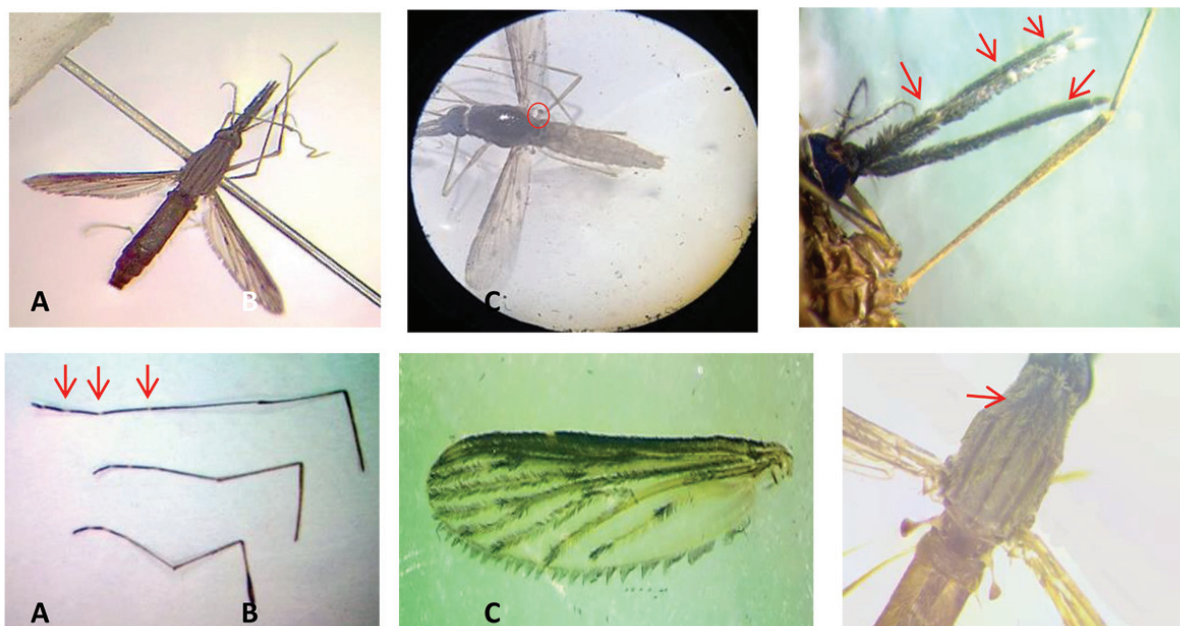


Fig. 4 (A) *Anopheles longirostris* (Adult); (B) Halter capitellum black-scaled dorsally; (C) Palps with distinctive pair of dark bands are separated by narrow pale band while Proboscis completely dark; (D) Legs with tarsi white spotted or bands; (E) Dark and pale spots on the wings; (F) Scutum with broad decumbent pale scales throughout

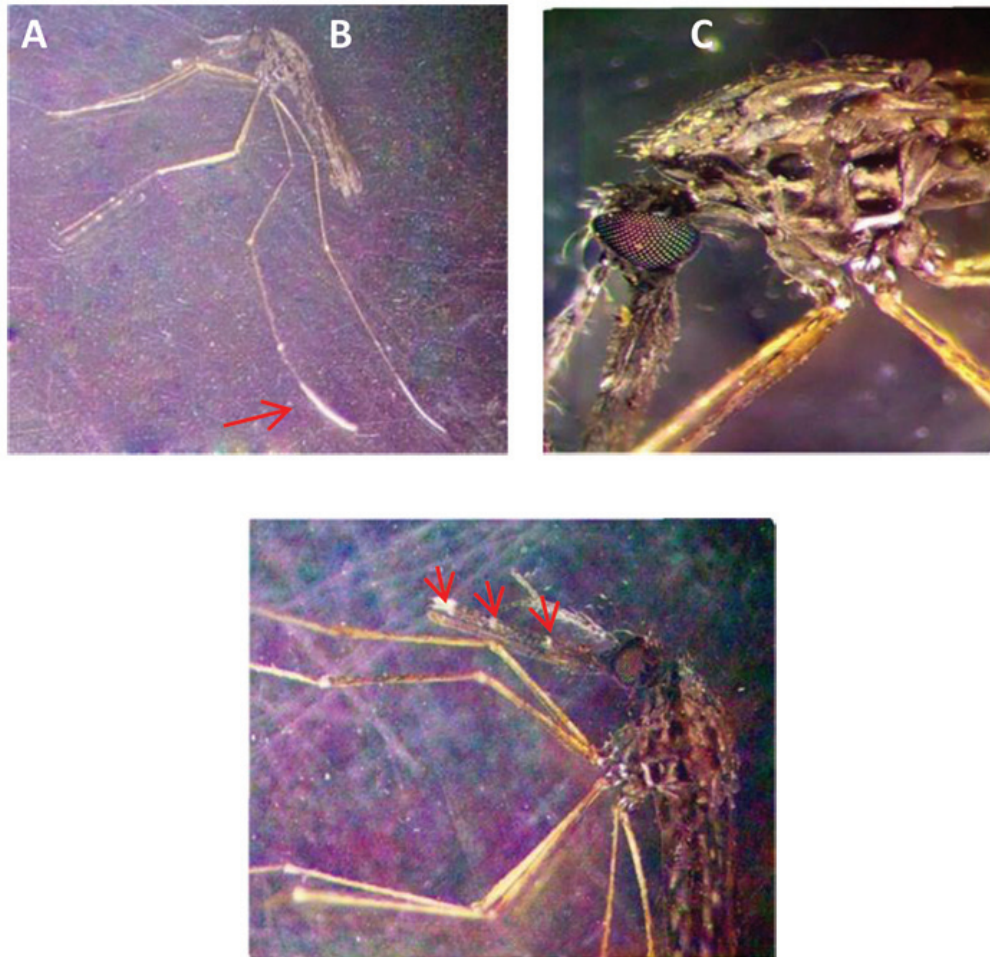


Fig. 5 *Anopheles jamesii*: A- adult female with tarsi 3, 4, 5 of hind limbs completely white; B- Lateral view of thorax with three distinct black patches below the scutum on post pronotal, pre alar and upper mesepimeral; C- Apical pale band nearly equal to the pre apical dark band on palps

Two *Anopheles* species are found in the same habitats. The habitat is the goat shed in the Tekcham, Thoubal district. First one is *An. jamesii* whose characteristics are: Pale area on costa and subcostal including vein 1, pale area greater than or equal to 4, Present of specking in the legs, Apical pale band nearly equal to the pre apical dark band, Hind tarsomeres 5, 4, 3 completely pale (Fig. 5A-C). The morphometrical features of this species are quite in accordance and confirmative keys. But other second features are blending of two species: *An. longirostris* and *An. frauti*. The characteristic features pointing towards *An. longirostris* are: Palpi and probosis are approximately equal in length, Palpi

are dark with pale bands, Apical pale band is about the same length as the pre-apical dark band, Wings have four dark spots on the costa, Dark and pale scales are arranged in a distinctive pattern with a pale fringe spots at vein tips, Covered in narrow dark scales, No distinct pale lines/stripes on the scutum, Dorsal side of the abdominal tergite without pale marking, Ventral side often with lighter bands. And characteristics that point towards *An. frauti* are Apical half of palp dorsal view has mostly 4 white and 3 black bands, Distinct black bands, separated by narrow pale band, 4th white band is reduced or completely dark, proboscis mostly dark but small apical pale band present (Fig. 4). The

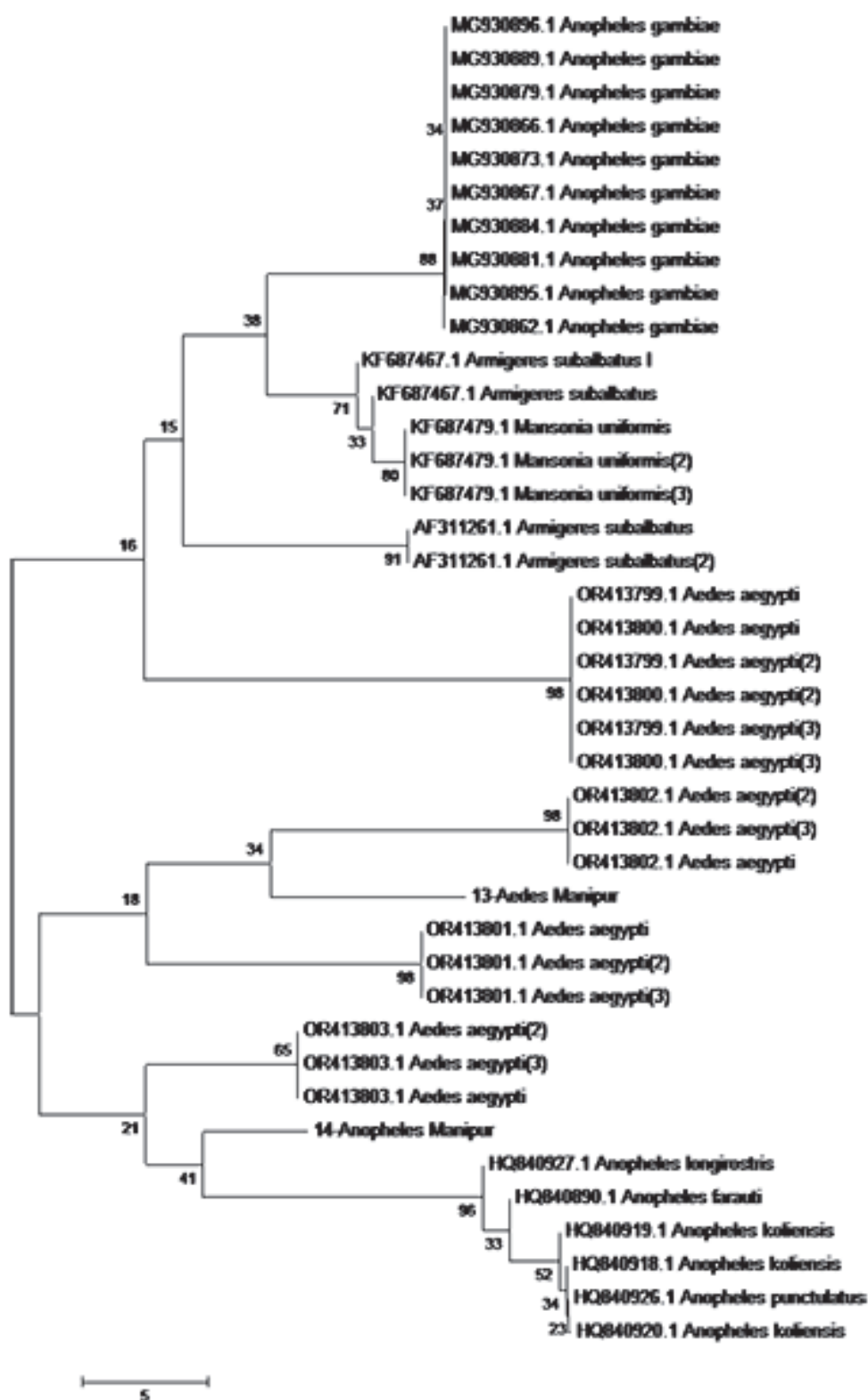


Fig. 6 Phylogenetic tree constructed by neighbour joining technique using the Cytb sequences, together with database sequences, to assess the phylogenetic relationships within and between species

molecular analysis details of *Ae. aegypti* and *An. longirostris* were ascertained using the cytochrome b genes (Cyt b). Though the characteristics are mixing and also in the phylogenetic tree also but the nearest being the *An. longirostris* (Fig. 6).

Dengue cases in different districts of Manipur had experienced a sudden surge from 2022/2023. The species collected from infected areas is *Ae. aegypti*. The larvae of *Ae. Aegypti* are collected from water tank that store rain water but hard to collect from drain/sewage water meaning the species is more adapted in the natural water source rather than the tap water or brackish water though reports come from brackish water (Ramasamy *et al.*, 2011). This might be the reason behind the surge of the disease monsoon. Female mosquitoes *Ae. aegypti* rested on cloth hangings and curtains, followed by furniture as reported by Dalpadado *et al.* (2022). So, after rain it is advisable to chlorinate the water tank or any other place where the larvae could harbour like unused tyre, utensils, plastic bottles or any other rain water accumulating materials as the female will lay eggs for spreading the disease in the crowded urban areas etc. Dengue is an arboviral disease. It is one of the eight neglected tropical disease (WHO, 2016). The South Asian (SEA) and the western Pacific represent 44

75% of the current global burden of dengue (Aguiar *et al.*, 2015). In the early 2000s, dengue was endemic in a few southern states of India (Maharashtra, Karnataka, Tamil Nadu and Pondicherry) and northern states (Delhi, Rajasthan, Haryana, Punjab and Chandigarh). It has recently spread too many states, including the union territories (Chakravarti, 2012). In Manipur, the disease assumed to be almost absent before 2020 though some cases of exotic origin like from Delhi through students. The cases of the disease are rising tremendously in last four years (Sanathoi *et al.*, 2024). This disease is causing health alert in valley areas particularly in Imphal East and West. As the vector is confirmed through morphology and molecular methods the future action plan is to study the biology and life cycle for effective control of this disease.

Another emerging vector is the *Anopheles* species which was assumed to be absent in the valley district though their presence from Moreh (Tengnoupal District) (Dutta *et al.*, 2005), and Jiribam district was evident from the climatic condition like hot and humid condition in these areas as reported by Ayekum *et al.* (2021). But it is very surprised to collect two species of *Anopheles* from Tekcham, Thoubal district. *Anopheles jamesii* is

Table 1. Sites of species collection from different habitats of valley districts alongwith coordinates

No.	Site	District	GPS Tag.	Specimen	Habitat
1.	NgakrabamLeirak	Imphalwest	Lat 24.786753°; Long 93.937369°	Adult <i>Aedes</i> sp.	Water tank
2.	KhuraiKonsamLeikai	Imphal east	Lat 24.834592°; Long 93.973789°	<i>Aedes aegypti</i>	Flower vase with watercontainer
3.	Ekop pat	thoubal	Lat 24.58777972°; Long 93.95153314°	Adult <i>Mansonia uniformis</i>	Bushes near water bodies
4	TekchamManingleikai	Thoubal	Lat 24.553117°; Long 93.991191	Adult <i>Mansonia uniformis</i>	Foot hills
5	D.M.U campus	Imphal	Lat 24.822097°; Long 93.941272°	Adult <i>Armigeres</i>	Foot of wall
6.	Loushipat	Thoubal (Lake)Tekcham	Lat 24.553479°; Long 93.99151°	<i>Anopheles longirostris</i>	Goat shed
7	Loushipat	Thoubal (Lake)Tekcham	Lat 24.553479°	<i>Anopheles jamesii</i> Long 93.99151°	Goat shed

quite distinct from the other on the basis of morphology. The species was reported from Meghalaya (Malaria, J., 2021) through molecular identification technique, from Coastal Karnataka (Gowthami *et al.*, 2024) the study highlighted its prevalence in the area during the declining phase of Malaria; Goa (Ajeetkumar *et al.*, 2024) study found it to be positive carrier of *Plasmodium* during longitudinal mosquito surveys. It was also reported from Nagaland (Knight and Stone, 1977, Reinert, 1975, 2001). The molecular approach not only identifies the species it also enables us to determine the age, time of diversification, more precisely the point of speciation. For instance, the two most important disease vectors in the *Ae.* subgenus *Stegomyia* that have become closely associated with humans in behavior, habitat preferences, and invasiveness, *Ae. (Stegomyia) aegypti* and *Ae. (Stegomyia) albopictus*, diverged from one another 31 MYA (Moureu *et al.*, 2015). In the subfamily Anophelinae, the lineages confined to what is now the Americas diverged first in the form of the genus *Chagasia* during the early Cretaceous, 142 MYA. Approximately 40 million years later, the separation of Gondwana is reflected in the divergence times of *Anopheles* lineages: the *Anopheles* subgenera associated exclusively with the Americas diverged from all other *Anopheles* 103 MYA (Soghigian *et al.*, 2023). The other species of *Anopheles* is assumed to be *An. longirostris* through molecular approach though the blending features with *An. farauti*. Out of the 17 species of *Anopheles* reported by Dutta *et al.* (2005) either *An. longirostris* or *An. farauti* is reported from Manipur. So, intense work is required in future to confirm and validate the occurrence of the species. Till date the occurrence of malaria from the Thoubal particularly Tekcham is not detected from the said area or not inspected thoroughly. If the vector is present sooner or later the disease might show up in near future so area should be informed about the potentiality of the occurrence of the disease in the area. The two fatal species viz., *Ae. aegypti* and *An. longirostris* are confirmed through morphologically and molecular methods in the present study. *Aedes aegypti* is responsible for dengue in the valley district while *An. longirostris* is assumed to be the vector for malaria. Till date

the occurrence of malaria from the Thoubal particularly Tekcham is not detected from the said area or not inspected thoroughly.

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