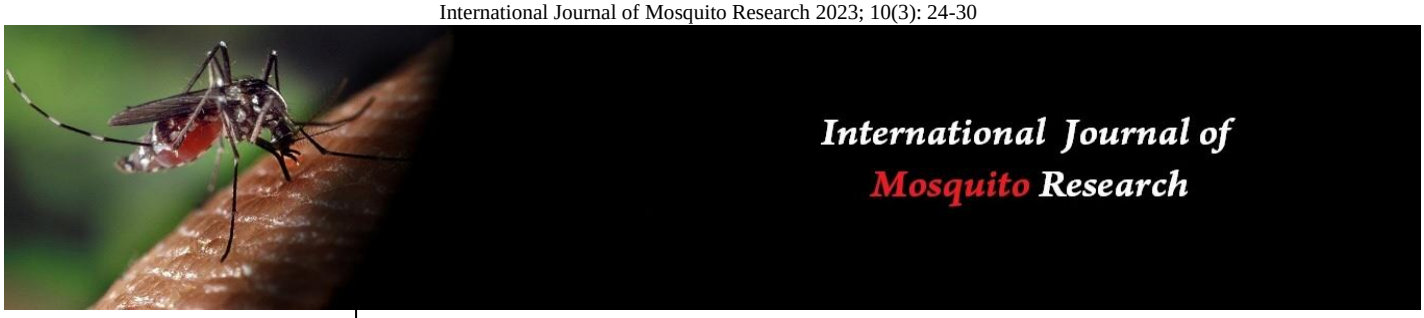

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2023; 10(3): 24-30

www.dipterajournal.com
Received: 08-02-2023
Accepted: 12-05-2023

Rishabh Sen
Vector Molecular Genetics Research Unit, Department of Zoology (UG & PG) Serampore College, Serampore, Hooghly, West Bengal, India

Anindita Datta
Vector Molecular Genetics Research Unit, Department of Zoology (UG & PG) Serampore College, Serampore, Hooghly, West Bengal, India

Pranab Kumar Banerjee
Vector Molecular Genetics Research Unit, Department of Zoology (UG & PG) Serampore College, Serampore, Hooghly, West Bengal, India

Corresponding Author:
Rishabh Sen
Vector Molecular Genetics Research Unit, Department of Zoology (UG & PG) Serampore College, Serampore, Hooghly, West Bengal, India

Molecular characterization of *Aedes aegypti* in West Bengal by using ITS2 Primer

Rishabh Sen, Anindita Datta and Pranab Kumar Banerjee

DOI: https://doi.org/10.22271/23487941.2023.v10.i3a.675

Abstract
Aedes aegypti is the primary vector for the spread of human arboviruses such as the chikungunya, dengue, and yellow fever viruses. Since there is no vaccination or antiviral medicine available, controlling this mosquito population is essential to prevent diseases. In this study molecular characterization based on ITS2 was carried out on *Aedes aegypti* isolated from five districts of West Bengal. The NCBI BLAST software was used for the bioinformatics analysis. These findings imply that these isolates from the five districts are related to other strains found on the Asian and American continents, possibly due to the same origin. Therefore, precise vector identification is a key component in the development of strategies that could effectively manage diseases that are transmitted by vectors.

Keywords: *Aedes aegypti*, ITS2, Molecular Characterization, NCBI, West Bengal

1. Introduction
Aedes aegypti mosquitoes are an important threat to public health since they spread several serotypes of viral infections that cause dengue, dengue haemorrhagic fever (DHF) and chikungunya in the human population [1]. In order to prevent these diseases, control of this *Aedes* mosquito population is necessary because there is no vaccine or antiviral medicine available. Previously (1996) the main dengue outbreak occurred in India, affecting different regions in Delhi and Lucknow [2, 3]. It has been reported [4] that Dengue is one of the main public health issues in West Bengal. These mosquito-borne diseases affect almost all districts of the state [5]. Thus the *Aedes* mosquito is a crucial target for disease surveillance programmes but at present the information regarding its distribution, density, disease transmission rate and seasonal prevalence is meagre. In order to identify which mosquito species are refractory or susceptible vectors, it is crucial to know its bionomics and the morphogenetic structure of these vectors [6]. Several lines of data [7-9] revealed the morphogenetic variation and gene flow among the same vector species of local population of *Aedes aegypti* in Southeast Asia, Africa, America and Latin America. Meena (2021) [10], reported about the genetic diversity and disease transmission potential of *Aedes aegypti* in different ecological environments in India. Several vector, including mosquitoes, frequently exhibit morphological and genetic variations within a species (intra-specific variation). Various lines of data [11, 12, 13] indicated that variations with a higher adaptive value are selected under specific environmental conditions. As a result, a study on intra-specific variations is required for vector species identification, which serves as a basis for proper implementation of the vector control programme. Nowadays species diversity in a particular population has been studied by using molecular methods. On that account, several specific molecular markers (such as mitochondrial Cytochrome Oxidase C subunit I and II (COI & COII), Internal Transcribed Spacers (ITSs) of ribosomal DNA genes, 28S rDNA gene, and Cytochrome Oxidase B have been generally used to know the molecular diversity of the *Aedes aegypti* [14]. Therefore, many scientists [15-17] have used ITS2 molecular marker. Furthermore in India several research works [3, 6, 13, 18] have been extended on this topic but very few research works have been done in West Bengal. Therefore, we have undertaken the research work by using ITS-2 primer to know the molecular diversity of *Aedes aegypti* from the five different districts (Howrah, Hooghly, Kolkata, North 24 Parganas and South 24 Parganas) of West Bengal.

2. Material and Method

2.1 Collection and Rearing of Larvae

Aedes aegypti larvae were collected from different breeding habitats such as plastic containers, coconut shells, tyres etc. from five different districts (Howrah, Hooghly, Kolkata, North 24 Parganas and South 24 Parganas) of West Bengal and were reared in the laboratory insectaries, supplied with 10% sucrose solution. The emerged adults were etherized and heat fixed for further experiments.

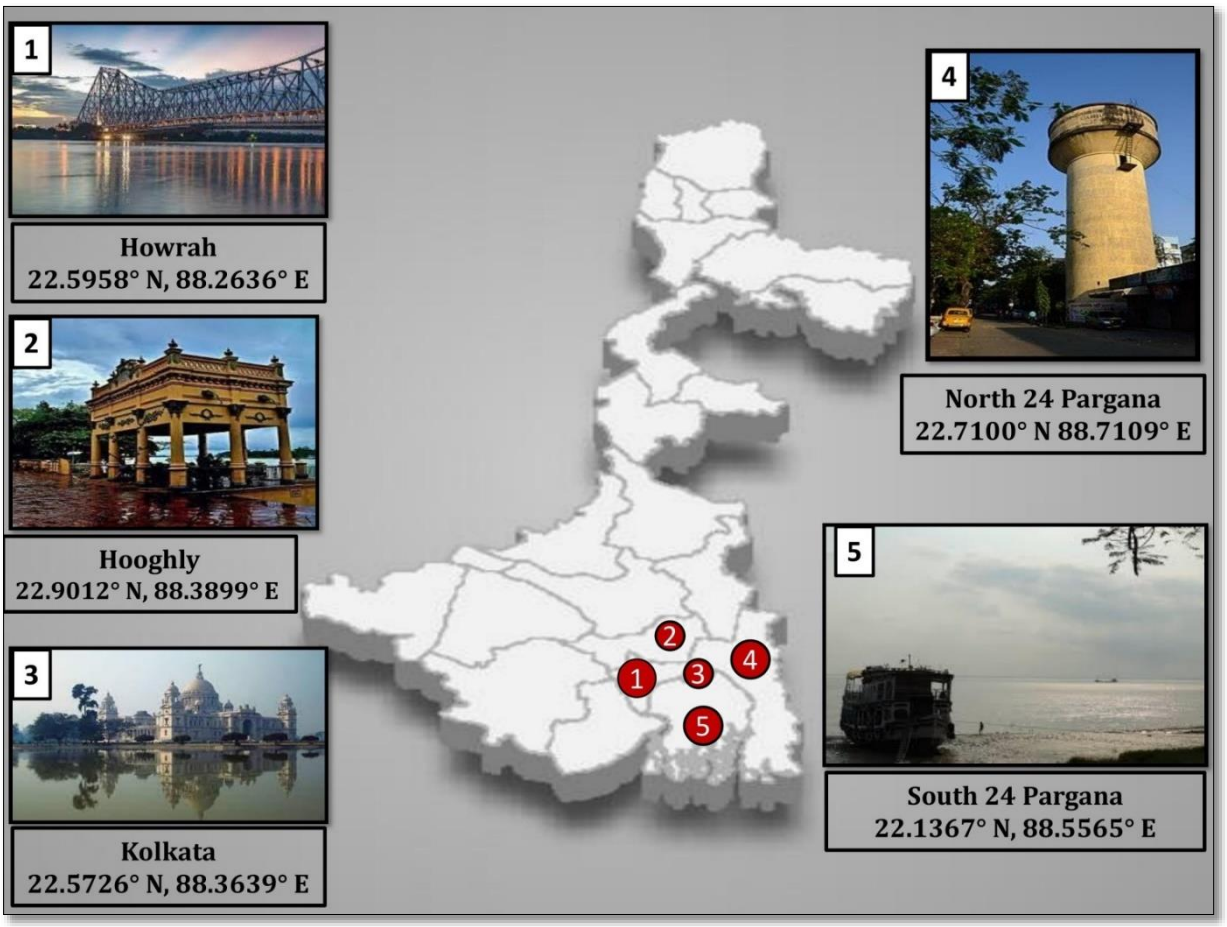

Fig 1: Study Areas

2.2 Morphological identification

Aedes aegypti species were identified with the help of Stereoscopic microscope in the Laboratory. Thorax of *Aedes aegypti* adult has white scales on the top and lyre shaped. Each hind region has white bands and abdomen dark brown to back to back. Molecular phylogeny of this species further confirms their identification.

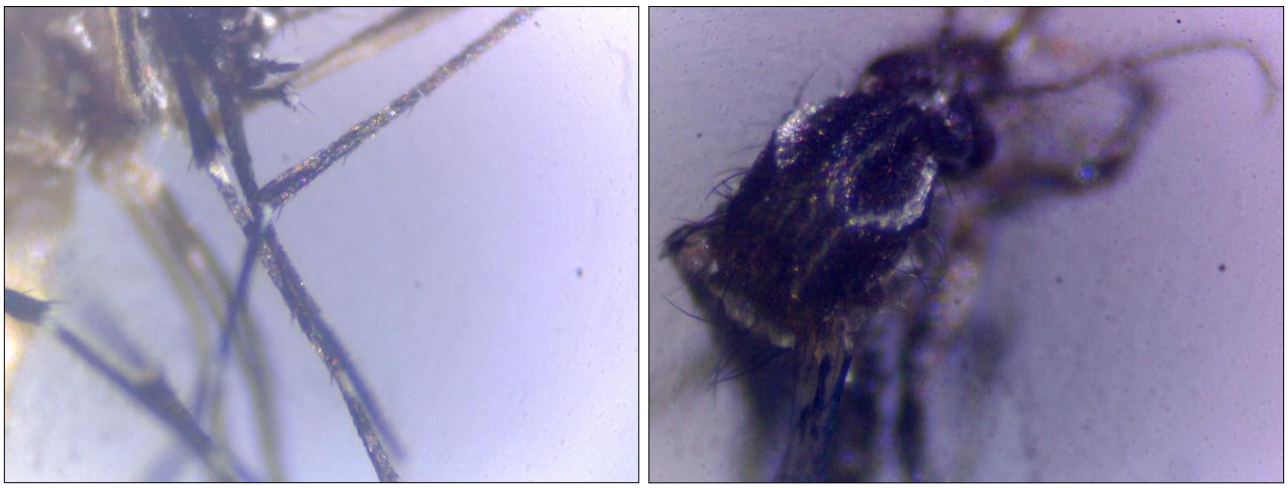

Fig 2: Wild type *Aedes aegypti*: (A) Leg (B) Thorax

2.3 DNA Isolation and PCR amplification

DNA was isolated from *Aedes aegypti* collected from the five districts. Its quality was evaluated on 1% agarose gel, a single band of high-molecular weight DNA has been observed. Fragment of ITS-2 region was amplified by PCR. The PCR amplicon was purified to remove contaminants. ITS-2 sequences of *Aedes aegypti* from different districts isolates, available at NCBI database, were aligned to retrieve the common conserved region with the help of universal primers. The forward primer was 5′-AGG ACA CAT GAA CAC CCA CA-3′ and reverse primer was 5′-CTC GCA GCT ACT CAG GGA AT-3′. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with ITS2F and ITS2R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of the PCR amplicon was generated from forward and reverse sequence data using aligner software.

3. Results and Discussion

3.1 Identification of *Aedes* species and sequence analysis

For molecular identification, ITS-2 region was amplified using gDNA isolated from mosquito samples from five districts. A single discrete PCR amplicon band of nearly 300-350 bp was observed when resolved on agarose gel for the entire samples. Amplification of 300-350 bp fragments in each case suggested that the mosquitoes belong to same species. The ITS-2 region sequence was used to carry out BLAST with the database of NCBI Genbank. The first 10 sequences were chosen and aligned using the multiple alignment programme Clustal W based on the maximum identity score. Thus, in combination with morphological characteristics of the field collected mosquito, molecular markers based analysis further confirmed the identity of the mosquito species as *Aedes aegypti*.

Table 1: Base pair length and NCBI retrieved sequences of ITS-2 of *Aedes aegypti* from five districts of West Bengal

Variant	Sequence length (BP)	Genbank Acession Number
Howrah	322 bp	KF471587.1
Hooghly	348 bp	KF471587.1
Kolkata	332 bp	KF471584.1
North 24 Pargana	313 bp	KY382418.1
South 24 Pargana	328 bp	KY382418.1

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
Aedes aegypti clone Aeaeg_USA_Rs3_2_1	562	562	100%	1e-155	100.00%	KF471587.1
Aedes aegypti clone Aeaeg_F_NC1_2_3	562	562	100%	1e-155	100.00%	KF471577.1
Aedes aegypti isolate UP/ZOO/KA003_005	556	556	100%	6e-154	99.67%	KY382418.1
Aedes aegypti isolate sb1	556	556	100%	6e-154	99.67%	OK632016.1
Aedes aegypti isolate AAV10	556	556	100%	6e-154	99.67%	MZ851347.1
Aedes aegypti isolate AAV9	556	556	100%	6e-154	99.67%	MZ851346.1
Aedes aegypti isolate AAV8	556	556	100%	6e-154	99.67%	MZ851345.1
Aedes aegypti isolate AAV5	556	556	100%	6e-154	99.67%	MZ851344.1
Aedes aegypti isolate AAV3	556	556	100%	6e-154	99.67%	MZ851342.1
Aedes aegypti isolate AAV2	556	556	100%	6e-154	99.67%	MZ851341.1

Fig 3: NCBI reports obtained for *Aedes aegypti* from Howrah

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Aedes aegypti clone Aeaeg_USA_Rs3 2 1	582	582	100%	1e-161	99.69%	KF471587.1
Aedes aegypti clone Aeaeg_F_NC1_2_3	582	582	100%	1e-161	99.69%	KF471577.1
Aedes aegypti isolate UP/ZOO/KA003_005	577	577	100%	5e-160	99.37%	KY382418.1
Aedes aegypti isolate sb1	577	577	100%	5e-160	99.37%	OK632016.1
Aedes aegypti isolate AAV10	577	577	100%	5e-160	99.37%	MZ851347.1
Aedes aegypti isolate AAV9	577	577	100%	5e-160	99.37%	MZ851346.1
Aedes aegypti isolate AAV8	577	577	100%	5e-160	99.37%	MZ851345.1
Aedes aegypti isolate AAV5	577	577	100%	5e-160	99.37%	MZ851344.1
Aedes aegypti isolate AAV3	577	577	100%	5e-160	99.37%	MZ851342.1
Aedes aegypti isolate AAV2	577	577	100%	5e-160	99.37%	MZ851341.1

Fig 4: NCBI reports obtained for Aedes aegypti from Hooghly

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Aedes aegypti clone Aeaeg_USA_Rs1_2_4	479	479	100%	1e-130	99.25%	KF471584.1
Aedes aegypti isolate ASS_03_DBR	479	479	100%	1e-130	99.25%	KP259840.1
Aedes aegypti isolate UP/ZOO/KA003_005	460	460	100%	4e-125	98.11%	KY382418.1
Aedes aegypti isolate aesb1	460	460	100%	4e-125	98.11%	ON652374.1
Aedes aegypti voucher HNDSPSITS29758SF.ab1	460	460	100%	4e-125	98.11%	ON118417.1
Aedes aegypti voucher HNDCHOLUTECAITS21458SF.ab1	460	460	100%	4e-125	98.11%	ON118415.1
Aedes aegypti voucher HNDSPSITS28758SF.ab1	460	460	100%	4e-125	98.11%	ON118414.1
Aedes aegypti voucher HNDCHOLUTECAITS23228SR.ab1	460	460	100%	4e-125	98.11%	ON118407.1
Aedes aegypti isolate sb1	460	460	100%	4e-125	98.11%	OK632016.1
Aedes aegypti isolate AAV10	460	460	100%	4e-125	98.11%	MZ851347.1

Fig 5: NCBI reports obtained for *Aedes aegypti* from Kolkata

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
Aedes aegypti isolate UP/ZOO/KA003_005	538	538	100%	2e-148	99.66%	KY382418.1
Aedes aegypti isolate aesb1	538	538	100%	2e-148	99.66%	ON652374.1
Aedes aegypti voucher HNDSPSITS28758SF.ab1	538	538	100%	2e-148	99.66%	ON118414.1
Aedes aegypti isolate sb1	538	538	100%	2e-148	99.66%	OK632016.1
Aedes aegypti isolate AAV10	538	538	100%	2e-148	99.66%	MZ851347.1
Aedes aegypti isolate AAV9	538	538	100%	2e-148	99.66%	MZ851346.1
Aedes aegypti isolate AAV8	538	538	100%	2e-148	99.66%	MZ851345.1
Aedes aegypti isolate AAV5	538	538	100%	2e-148	99.66%	MZ851344.1
Aedes aegypti isolate AAV3	538	538	100%	2e-148	99.66%	MZ851342.1
Aedes aegypti isolate AAV2	538	538	100%	2e-148	99.66%	MZ851341.1

Fig 6: NCBI reports obtained for *Aedes aegypti* from North 24 Pargana

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
Aedes aegypti isolate UP/200/KA003_005	424	424	100%	6e-114	91.05%	KY382418.l
Aedes aegypti isolate sb1	424	424	100%	6e ll4	91.05%	OK6320 6.1
Aedes aegypti isolate AAV1O	424	424	100%	6e-114	91.05%	MZ851347.1
Aedes aegypti isolate AAV9	424	424	100%	6e-114	91.05%	MZ851346.1
Aedes aegypti isolate MV8	424	424	100%	6e-114	91.05%	MZ851345.1
Aedes aegypti isolate AAV5	424	424	100%	6e-114	91.05%	MZ851344.1
Aedes aegypti isolate AAV3	424	424	100%	6e-114	91.05%	MZ851342.1
Aedes aegypti isolate MV2	424	424	100%	6e-114	91.05%	MZ85134 1.1
Aedes aegypti isolate AAV1	424	424	100%	6e·114	91.05%	MZ851340.1
Aedes aegypti HNOSPSITS28758SF.ab1	422	422	99%	2e-113	91.03%	ON118414.1

Fig 7: NCBI reports obtained for *Aedes aegypti* from South 24 Pargana

These findings suggest that these isolates from the five districts are similar to other Asian and American continent strains possibly due to the same origin. Thus, we can expand our knowledge regarding present vector surveillance and control programmes by better understanding the vectorial potential of these geographically diversified mosquito strains.

Table 2: GC content and No. of Restriction Enzyme Cutting site of ITS2 region of *Aedes aegypti* of five districts

Districts	GC Content (%)	No. of Restriction Enzyme Cutting site
Howrah	45%	26
Hooghly	51%	40
Kolkata	44%	23
North 24 Pargana	50%	36
South 24 Pargana	44%	26

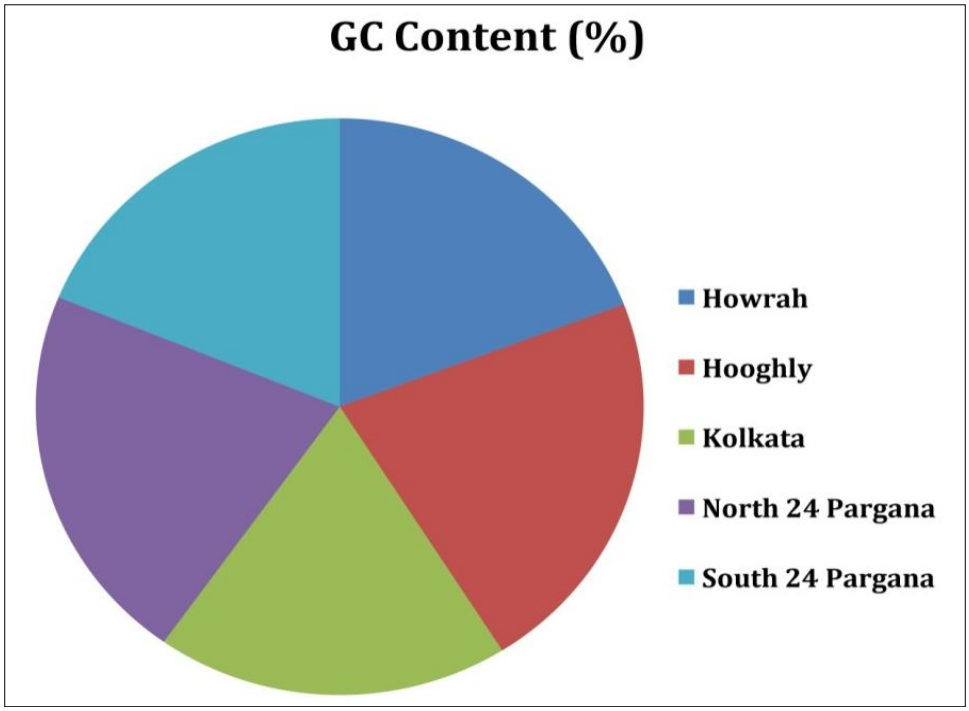

Fig 8: Comparison on GC content and of ITS2 region of *Aedes aegypti* of five districts

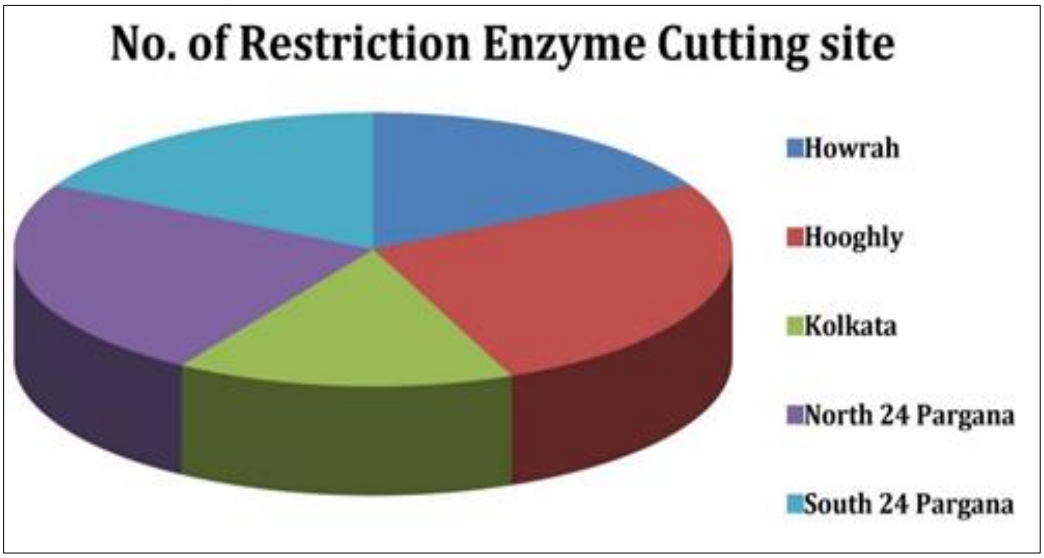

Fig 9: Comparison on no. of Restriction Enzyme Cutting site of ITS2 region of *Aedes aegypti* of five districts

Table 3: Total types of ITS2 repeat sequences of *Aedes aegypti* of five districts

Districts	Dimer	Trimer	Tetramer	Pentamer	Polymer
Howrah	16	47	60	29	19
Hooghly	16	54	81	24	14
Kolkata	16	47	56	34	20
North 24 Pargana	16	49	55	27	13
South 24 Pargana	16	47	60	30	17

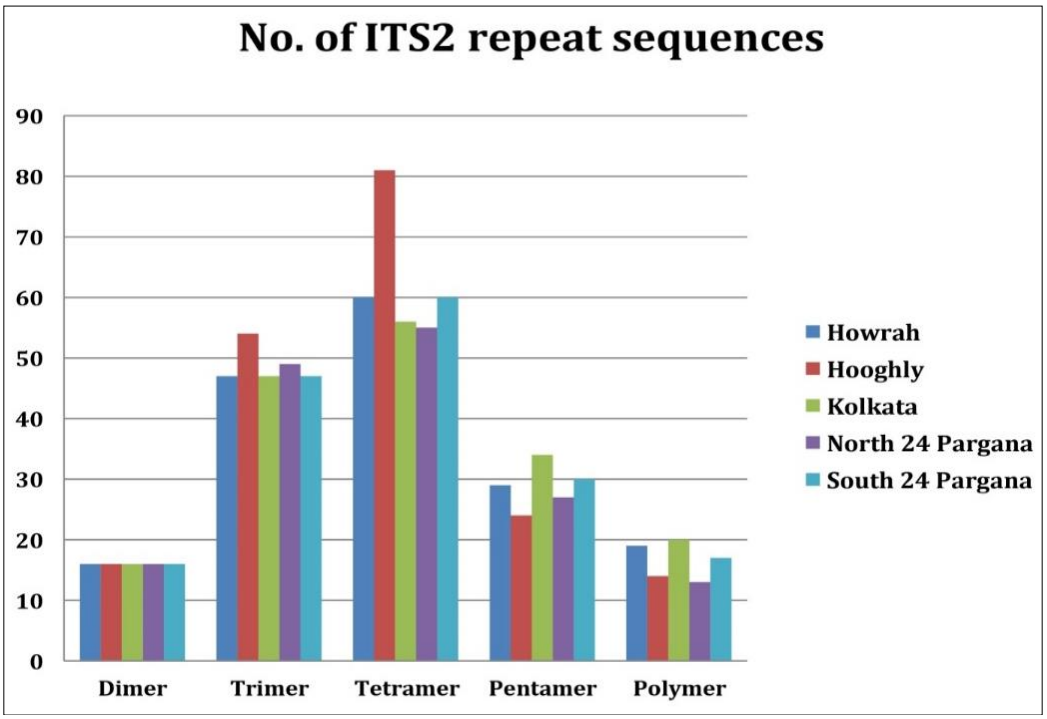

Fig 10: Total types of ITS2 repeat sequences of *Aedes aegypti* of five districts

3.2 Discussion

Several studies [6, 19, 20] have showed a relationship between the geographic origin of vector species and its vector competence and pesticide resistance. Studies on the molecular diversity of the *Aedes* species by using ITS2 marker provide more insight details at molecular levels and shows similarities with other strains all over the world. As the ITS2 rDNA is a non-coding region, it is subjected to high quantity of mutations, and this makes it a good option to study the phylogenetics of the closely related species [21]. Our investigation has also been forwarded on molecular characterisation by using molecular markers of ITS-2 of *Aedes aegypti* from different districts of West Bengal. Data of ITS2 sequence analysis (Table 1) of five districts; Howrah, Hooghly, Kolkata, North 24 parganas and South 24 Parganas of West Bengal indicated that all the samples have nearly

similar ITS2 amplicon length, with a range of 310-350 base pairs. The ITS2 sequences of five districts have highest similarities with this species of accession numbers KF471587.1, KF471587.1, KF471584.1, KY382418.1 and KY382418.1 respectively (Table 1). Our present observations reveal that *Aedes aegypti* samples collected from Howrah and Hooghly districts show more than 99% similarity with *Aedes aegypti* (KF471587.1) of USA. Furthermore, the samples of Kolkata show 99% similarity with *Aedes aegypti* strain (KF471584.1) of USA and the samples of North 24 parganas and South 24 Parganas show similarity with the Sri Lankan strains (KY382418.1) of *Aedes aegypti*. Studies on the basis of GC content and number of restriction enzyme cutting sites of observed *Aedes* species of Howrah, Kolkata and South 24 Pargana are closely related whereas Hooghly and North 24 Pargana show similarity (Table 2). Furthermore the observations of the ITS2 repeat sequences indicate that there is very few variation in the number of repeating sequences among the strains of *Aedes aegypti* of the abovementioned districts (Table 3).

4. Conclusion

Aedes aegypti is a significant disease vector that results in a significant number of fatalities annually, which requires the focus on its genetic diversity. Several genetic marker combinations have been used for studying the molecular diversity of *Aedes* mosquito species population. The geographic origins of mosquito populations have epidemiological significance hence these are a crucial part of management methods for vector-borne diseases. This has been demonstrated by many research studies where a relationship between the geographic origin of vectors and features like vector competence and pesticide resistance has been found. In the present study, sequences of molecular markers of ITS-2 of *Aedes aegypti* from West Bengal have been compared with their counterparts in the world. The species of *Aedes* that was subsequently colonised in the laoratory was identified using sequence analysis of ITS-2 from morphologically identified *Aedes aegypti*. Therefore, for the development of mosquito control strategies accurate vector identification is a crucial aspect.

5. Acknowledgement

The authors are thankful to the West Bengal Higher Education Science and Technology and Biotechnology (HESTBT) 2018-2019 (MRP), for funding the research program. A sincere gratitude is also expressed to the Principal and Head of the Department of Zoology, Serampore College for allowing us to use the Vector Biology laboratory unit and equipments to obtain the study objectives.

6. References

1. Higgs S, Vanlandingham D. Chikungunya virus and its mosquito vectors. Vector Borne Zoonotic Dis 2015;15(4):231-40.
2. Agarwal R, Kapoor S, Nagar R, Misra A, Tandon R, Mathur A. A clinical study of the patients with dengue hemorrhagic fever during the epidemic of 1996 at Lucknow, India. Southeast Asian J Trop Med Public Health. 1999;(4):735-40. PMID: 10928368.
3. Meena AR. Identification and confirmation of *Aedes albopictus* (Skuse, 1864) and *Aedes aegypti* (Linnaeus, 1762) using DNA Barcoding. Int J Mosq Res. 2022;9(2):51-57. DOI: https://doi.org/10.22271/23487941.2022.v9.i2a.601
4. Hati AK. Studies on dengue and dengue haemorrhagic fever (DHF) in West Bengal State, India. J Commun Dis. 2006;38:124 9.
5. Majhi Jitendra, Singh Ritesh, Yadav Vikas, Garg Vinay, Sengupta Paramita, Atul Pravin Kumar, *et al.* Dynamics of dengue outbreaks in gangetic West Bengal: A trend and time series analysis. Journal of Family Medicine and Primary Care. November 2020;9(11):5622-5628. | DOI: 10.4103/jfmpc.jfmpc_800_20
6. Gupta K, Dhawan R, Kajla M, Kumar S, Jnanasiddhy B, Singh NK, *et al.* Molecular identification of Aedes aegypti mosquitoes from Pilani region of Rajasthan, India. J Vector Borne Dis. 2016 Apr-Jun;53(2):149-55. PMID: 27353585.
7. Urdaneta-Marquez L, Failloux AB. Population genetic structure of *Aedes aegypti* the principal vector of dengue viruses. Infect Genet Evol. 2011;11(2):253–61.
8. Ryan SJ, Carlson CJ, Mordecai EA, Johnson LR. Global expansion and redistribution of *Aedes*-borne virus transmission risk with climate change. PLOS Neglected Tropical Diseases. 2019;13(3):e0007213. https://doi.org/10.1371/journal.pntd.0007213
9. Thabiani Aziz A. Distribution and mitochondrial CO1-based genetic diversity of *Aedes aegypti* L (Culicidae: Diptera) in Saudi Arabia. Saudi J Biol Sci. 2023 Mar;30(3):103566. DOI: 10.1016/j.sjbs.2023.103566. Epub 2023 Jan 23. PMID: 36748075; PMCID: PMC9898440.
10. Meena AR. Spatial Distribution of Dengue Vectors, India, Books clinic Publishing 07-Jul-2021
11. Andrews CA. Natural selection, genetic drift, and gene flow do not act in isolation in natural populations. Nat Educ Knowl. 2010;3(10):5.
12. Charlesworth D, Barton NH, Charlesworth B. The sources of adaptive variation. Proc R Soc B Biol Sci. 2017;284(1855):20162864
13. Kumar PN, Kalimuthu M, Kumar MS, Govindrajan R, Venkatesh A, Paramasivan R, *et al.* Morphological and molecular characterization of Aedes aegypti variant collected from Tamil Nadu, India. J Vector Borne Dis. 2022 Jan-Mar;59(1):22-28. PMID: 35708400. DOI: 10.4103/0972-9062.331413.
14. Dorzaban Hedayat, Soltani Aboozar, Alipour Hamzeh, Hatami Jafar, Hashemi Seyed, Shahriari-Namadi Marziae, *et al.* Morphological and molecular-based identification of Aedes aegypti (Diptera: Culicidae), a main vector of Dengue Fever, the first record in Iran after decades; c2020. 10.21203/rs.3.rs-20236/v1.
15. Patsoula Elina, Samanidou-Voyadjoglou Anna, Spanakos Gregory, Kremastinou Jenny, Nasioulas George, Vakalis Nicholas. Molecular and Morphological Characterization of Aedes albopictus in Northwestern Greece and Differentiation from Aedes cretinus and Aedes aegypti. Journal of medical entomology. 2006;43:40-54. 10.1603/0022-2585(2006)043[0040:MAMCOA]2.0.CO;2.
16. Surendran SN, Sarma DK, Jude PJ, *et al.* Molecular characterization and identification of members of the *Anopheles subpictus* complex in Sri Lanka. Malar J. 2013;12:304. https://doi.org/10.1186/1475-2875-12-304
17. Iyiola OA, Shaibu RD, Shittu O, Adelaja OJ, Ibrahi ATK,

Fadipe TO, *et al*. Genetic Diversity and Molecular Characterization of Mosquitoes (Diptera: Culicidae) In North-Central Nigeria Using Ribosomal DNA ITS2 and Mitochondrial 16S-DNA Sequences. The Iraqi Journal of Veterinary Medicine. 2022;44(2):78-91. (Original work Published December 28, 2020) https://doi.org/10.30539/ijvm.v44i2.985

18. Das B, Swain S, Patra A, Das M, Tripathy HK, Mohapatra N, *et al*. Development and evaluation of a single-step multiplex PCR to differentiate the aquatic stages of morphologically similar *Aedes* (subgenus: Stegomyia) species. Trop Med Int. Health. 2012 Feb; 17(2):235-43. DOI: 10.1111/j.1365-3156.2011.02899.x. EPUB 2011 Oct 31. PMID: 22040518.
19. Milam CD, Farris JL, Wilhide JD. Evaluating mosquito control pesticides for effect on target and non-target organisms. Arch. Environ. Con. Tox. 2000;39:324-328.
20. Lourenço-de-Oliveira R, Vazeille M, de Fillippis AM, Failloux AB. *Aedes aegypti* in Brazil: Genetically differentiated populations with high susceptibility to dengue and yellow fever viruses. Trans R Soc. Trop Med Hyg. 2004;98(1): 43–54.
21. Chattopadhyay A, Bakshi SD, Betal S, Banerjee PK. Molecular identification and control of Culex mosquito by *Citrus limon* in West Bengal, India. Int J Mosq Res. 2021;8(1):06-10.