International Journal of Entomology Research
ISSN: 2455-4758
Impact Factor: RJIF 5.24
www.entomologyjournals.com
Volume 3; Issue 2; March 2018; Page No. 96-103

Molecular studies on Indian mosquitoes (Diptera: Culicidae): A review

Devinder Singh, Navneet Kaur Rai, Divya Vashist*
Department of Zoology and Environmental Sciences, Punjabi University, Patiala, Punjab, India

Abstract
Molecular studies are recognized as a useful tool in species identification and sequence disparity. Nuclear region and mitochondrial genome has been analyzed and studied comprehensively all over the world. In this review, a detailed description of the molecular work done by several workers in India has been provided. It can be concluded that, the molecular studies on mosquitoes in India focused more on COI, ITS1 and ITS2 region and rest of the molecular markers are overlooked.

Keywords: mitochondrial DNA, nuclear DNA, COI, ITS1, ITS2

Introduction

Mosquitoes belonging to family Culicidae and order Diptera, are a large group of insects present throughout the temperate and tropical regions and even beyond the Arctic Circle of the world (Harbach, 2007) [30]. India is ranked fifth in terms of mosquito biodiversity after Brazil, Indonesia, Malaysia and Thailand (Foley *et al*., 2007) [23]. A total of 3,556 species of family Culicidae comprising of two subfamilies Culicinae and Anophelinae are currently recognized (Harbach, 2018) [31]. Indian mosquito fauna includes 393 species divided among 49 genera and 41 subgenera among which subfamily Anophelinae contains 61 species in one genus followed by subfamily Culicinae with 332 species in 11 tribes and 48 genera (Bhattacharyya *et al.*, 2014) [6].

Mitochondrial DNA and nuclear rDNA have widely been used in insect systematics (Otranto and Stevens, 2002) [53]. Recent studies on DNA-based approaches show a promising trend in the rapid description of biodiversity. In addition to that they are quite useful in determining sibling relationship between the species. The use of molecular markers has become an indispensable tool for the identification of cryptic species Collins and Paskewitz, 1996 [10]; Proft *et al*., 1999 [58]; Scott *et al*., 1993 [65]; Xu and Qu, 1997 [80].

Among molecular markers that are used in such studies, ribosomal DNA (rDNA), second internal transcribed spacer (ITS2), second and third domain (D2, D3) of rDNA 28S gene and cytochrome oxidase subunits I and II (COI, COII) of mitochondrial DNA are widely applied to mosquito species discrimination and phylogenetic reconstructions Chen *et al*., 2003 [9]; Collucci and Sallum, 2003 [11]; Garros *et al*., 2005a [24], b [25]; Kampen, 2005 [32]; Krzywinski *et al*., 2001 [38]; Ma and Xu, 2005 [44]; Marrelli *et al*., 2005 [49]; Sallum *et al*., 2002 [61]; Wilkerson *et al*., 2005 [79]. Mitochondrial DNA and nuclear ribosomal DNA are convenient targets due to conserved primer binding sequences and ease of amplification based on their typically high copy number (Reidenbach, 2009) [60].

In this article an attempt has been made to review the molecular research work that has been conducted on Indian species of mosquitoes.

Molecular studies of culicidae in India

Kshirsagar *et al.* (1997) [39] amplified mitochondrial 16S rRNA gene fragment from the cell line and larvae of known insect species and conducted heteroduplex analysis to detect the sequence variation. Shouche and Patole (2000) [70] analyzed a 450 bp hypervariable region of the mitochondrial 16S rRNA gene in three major genera of mosquitoes, *Aedes*, *Anopheles* and *Culex*. The sequences were found unusually A+T rich and a stretch of Ts found as distinguishing feature between *Aedes* and *Culex* from *Anopheles*. Manonmani *et al.* (2001) [46] used species-specific differences in the nucleotide sequences of rDNA ITS2 region to develop a diagnostic PCR assay for two sibling species of the *Anopheles fluviatilis* complex, members of which are major vectors of malaria in central and northern parts of India.

Manonmani *et al.* (2003) [45] subjected a total of 401 specimens of *Anopheles fluviatilis* to cytological identification and rDNA-ITS2 PCR assay and found that except for the small discrepancy in the identification results, both the techniques revealed the presence of only two species of the *Anopheles fluviatilis* complex in Orissa as has been observed earlier. Singh *et al.* (2004a) [73] studied differentiation of members of *Anopheles fluviatilis* species complex by an allele-specific polymerase chain reaction based on 28s rDNA sequences from India. The assay evaluated against chromosomally examined individuals from different localities with different sympatric associations and it could differentiate unambiguously all the members of the complex. Again, Singh *et al.* (2004b) [75] developed an allele-specific polymerase chain reaction to the D3 domain of 28S rDNA that discriminated *Anopheles culicifacies* species complex. The assay was also validated using chromosomally identified specimens of *Anopheles culicifacies* from different geographical regions of India representing different sympatric associations.

Goswami *et al.* (2005) [26] developed PCR-RFLP of mitochondrial COII and ITS2 of rDNA markers for the

identification of members of *Anopheles culicifacies* complex. Goswami *et al.* (2006) [27] conducted a study to identify all members of the *Anopheles culicifacies* complex using allele-specific polymerase chain reaction assays. The PCR assays developed from the D3 and ITS2 regions of rDNA failed to identify all the members of the *Anopheles culicifacies* complex while COII sequences showed single bp difference that have been used to differentiate all the members of this complex.

Prakash *et al.* (2006) [56] identified members of the *Anopheles minimus* and *Anopheles dirus* complexes from north eastern region of India using sequences for the ITS2 of rDNA. They differentiated *Anopheles minimus* (species A) of the *Anopheles minimus* complex and *Anopheles baimaii* (species D) of the *Anopheles dirus* complex from Arunachal Pradesh, Assam, Meghalaya and Nagaland. Alam *et al.* (2006) [2] reported sequence analysis of rDNA ITS2 and D3 regions of the four members of *Anopheles annularis* group- *Anopheles nivipes, Anopheles philippinensis, Anopheles annularis* and *Anopheles pallidus* for their molecular identification from Andaman and Nicobar Islands. They found that there were no intraspecific sequence variation among the specimens and the interspecific sequence differences were greater for ITS2 than the D3 regions.

Singh *et al.* (2006) [74] examined the conspecificity of *Anopheles fluviatilis* S and *Anopheles minimus* C by analysing the DNA sequences of nuclear ribosomal ITS2 and D2-D3 domain of 28S rDNA. Manonmani *et al.* (2007) [48] developed an rDNA-ITS2 PCR assay to group the five species of *Anopheles culicifacies* complex into the two categories (A and D and B, C and E) without the need of an intermediate RFLP assay, thus reducing the number of steps involved in the available identification assay and significantly cutting down the time and cost.

Kumar *et al.* (2007) [41] studied DNA barcodes for several species of mosquitoes belonging to 15 genera, prevalent in India, which included major vector species. However, two closely related species, *Ochlerotatus portonovoensis* and *Ochlerotatus wardi* could not be identified as separate species based on DNA barcode approach as their lineages indicated negligible genetic divergence. Alam *et al.* (2007a) [3] reported the sequence analysis and a method for their molecular identification of the rDNA ITS2 and D3 regions of the four members of the *Anopheles annularis* group- *Anopheles nivipes, Anopheles philippinensis, Anopheles annularis* and *Anopheles pallidus* from Assam and Andaman and Nicobar Islands. Alam *et al.* (2007b) [1] analyzed the distribution of *Anopheles annularis* complex collected from Sonapur (Assam), Jabalpur (Madhya Pradesh), Ranchi (Jharkhand), and Ghaziabad (Uttar Pradesh) and developed a molecular method using the rDNA ITS2 and Domain 3 for identification purposes.

Kohli and Choudhry (2007) [37] aimed at sequence analysis of mitochondrial 16S rDNA gene fragment in 2 wild populations of *Armigeres subalbatus*, carrier of Filariasis and Japanese encephalitis in humans. Alam *et al.* (2008) [4] described the sequencing of rDNA ITS2 and D3 loci of *Anopheles stephensi stephensi* (type form) and *Anopheles stephensi mysorensis*. They also revealed that these populations showed identical sequences at both rDNA loci.

Raghavendra *et al.* (2009) [59] examined sequence variations among members of the *Anopheles culicifacies* complex within the variable D2 domain of the 28S rDNA subunit to study phylogenetic relationship among them. The designed PCR assay was evaluated using DNA from specimens of the *Anopheles culicifacies* complex from different areas of India.

Mohanty *et al.* (2009) [50] inferred the phylogenetic relationships among the Anopheline species of subgenus *Cellia* using COI and COII genes, the rRNA gene particularly the D3 region, and ITS2 region. The study proved useful in properly defining species positions and resolving the ambiguity that normally arises due to morphological taxonomy.

Bora *et al.* (2009) [8] described variations in the three mitochondrial DNA markers viz., COI, COII and cytochrome b among *Anopheles sundaicus* populations from Andaman and Nicobar islands. Their study revealed that there were three different combined genotypes (genotypes I, II and III) in the populations of *Anopheles sundaicus*.

Sharma *et al.* (2009) [67] used RAPD to ascertain the genetic variability in *Culex quinquefasciatus* populations collected from various locations and proposed the usefulness of study to understand the population variation under different ecological conditions and development of effective vector management strategies. Singh *et al.* (2010) [76] determined the DNA sequences of morphologically identified *Anopheles minimus* s.l. and *Anopheles fluviatilis* s.l. collected from the Kamrup district in Assam for ITS2 and D3 domain of 28S rDNA.

Dhananjeyan *et al.* (2010) [19] successfully attempted molecular identification of *Aedes aegypti, Aedes albopictus* and *Culex vishnui* subgroup mosquitoes by using nuclear ribosomal ITS and extracting DNA from whole specimen, legs, wings and other alternate sources like larval and pupal exuviae. They further suggested that the ITS2 marker could very well differentiate *Aedes aegypti* and *Aedes albopictus* and ITS1 marker could distinguish 3 species of the *Culex vishnui* subgroup viz,. *Culex tritaeniorhynchus, Culex pseudovishnui* and *Culex vishnui.* Bhattacharyya *et al.* (2010) [5] collected house-frequenting female adults of the *Anopheles philippinensis– nivipes* species complex from 23 localities in the six north–eastern states. Each species were identified using morphological keys as well as molecular method using rDNA ITS2 gene.

Dixit *et al.* (2010) [20] inferred phylogenetic relationships and estimated divergence time among six malaria vector species belonging to subgenus *Cellia*, prevalent in India. The phylogenetic status of Indian malaria vectors followed the pattern based on morphological and cytological classifications that was reconfirmed with COII and ITS2 genetic regions.

Sharma and Choudhry (2010) [69] conducted sequence characterization of rDNA ITS1, ITS2 and COII gene as potential molecular markers for studying genetic relatedness and phylogenetic kinship among six important species of genus *Anopheles.*

Sharma *et al.* (2010) [68] examined the phylogenetic relationship among the *Culex quinquefasciatus* population collected from different geographical regions of India, using 16S rRNA gene sequences. Kaura *et al.* (2010) [34] conducted at the comparative study of sequence variations in ITS2 of seven allopatric populations (5 Indian, 2 Sri Lankan) of

Anopheles subpictus. Kohli *et al.* (2011a) [36] dealt with the ITS2 sequence based interspecific variations and molecular phylogenetics of five Oriental species of genus *Culex viz*: *Culex quinquefasciatus, Culex vishnui, Culex bitaeniorhynchus, Culex Tritaeniorhynchus* and *Culex mimeticus.* The length of ITS2 sequence varied from 323 to 410 bp with G-C content ranging from 50.7% to 66.5%.

Kohli *et al.* (2011b) [35] conducted sequence characterization of ITS2, COII gene and 16S gene fragments as potential molecular markers for studying genetic relatedness and phylogenetic kinship among five important species of genus *Culex*. Dixit *et al.* (2011) [21] designed EPIC (Exon Primed Intron Crossing) primers and successfully amplified and sequenced eight DNA fragments in an Indian population sample of *Anopheles minimus* and tested for their usability as genetic markers for population genetic studies to infer population structure and demography of this species.

Sarma *et al.* (2012a) [63] undertook a study to generate authentic information on distribution of *Anopheles philippinensis* and *Anopheles nivipes* in the north-east India using rDNA ITS2 region. Sarma *et al.* (2012b) [62] also studied genetic population structure of the malaria vector *Anopheles baimaii* in north-east India using COII gene from 176 individuals and found no genetic differentiation among populations with the exception of the Central 2 population in the Barail hill area that was significantly differentiated from other populations. Gupta and Preet (2012) [29] optimized a protocol for the genomic DNA extraction and RAPD-PCR in mosquito larvae collected from Agra. Das *et al.* (2012) [16] developed a PCR technique to differentiate the aquatic stages of species of *Aedes* viz., *Aedes aegypti, Aedes albopictus* and *Aedes vittatus* and to detect the most abundant species among them by using 18S rDNA marker. Their study revealed the presence of *Aedes albopictus* in the largest number followed by *Aedes aegypti* and *Aedes vittatus.*

Singh *et al*. (2012) [78] conducted molecular characterization of *Anopheles maculatus* complex species in north-east India and found no intraspecific differences in the sequences of ITS2 region of rDNA within north-east India while, few differences were detected in the sequences of *Anopheles dravidicus*, *Anopheles maculatus* and *Anopheles pseudowillmori* from north-east India with species from the neighbouring countries. Sathe and Jagtap (2012) [64] reported a new species *Anopheles krishnai* from India and described phylogenetic correlation of closely related species by neighbor joining method.

Sharma *et al.* (2013) [66] studied the phylogenetic relation among the four different mosquito species: *Anopheles stephensi, Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus* by using PCR-RFLP of COI gene. Paramasivan *et al.* (2013) [54] gave a preliminary report on DNA barcoding and phylogenetic relationships of certain public health important mosquito species recorded in rural areas of south India. The study concluded that the DNA-based barcoding would really be a simple and very useful method in identifying the mosquitoes and also immensely complement the conventional morphology based taxonomical procedures.

Nanda *et al.* (2013) [52] reported a new *Anopheles fluviatilis* population from villages under Laksar Community Health Centre, District Haridwar. Kumar *et al.* (2013) [43] studied COI gene for *Anopheles fluviatilis* species complex from India. Kumari *et al.* (2013) [43] studied the prevalence of *Anopheles subpictus* complex consisting of four sibling species A, B, C and D and their role in transmission of malaria in four districts *i.e.* Angul, Khurda, Cuttack and Puri of Odisha using COI gene. Das *et al.* (2013) [17] identified the *Anopheles culicifacies* sibling species complex by PCR using COII region and detected *Anopheles culicifacies* sibling species A, B, C, D and E in the malaria endemic regions of Odisha. They detected *Anopheles culicifacies* E for the first time in Odisha, which was further confirmed by molecular phylogenetics. They further found that *Anopheles culicifacies* B was the most abundant species, followed by *Anopheles culicifacies* C and E.

Daravath *et al.* (2013) [13] conducted molecular identification of *Aedes albopictus* and quantitative analysis of COI gene in South-Indian populations. Manonmani *et al.* (2013) [47] evaluated COII region based species specific assay for identifying members of the *Anopheles culicifacies* species complex in various parts of India. Zomuanpuii *et al.* (2013) [81] developed a diagnostic PCR assay for distinguishing members of subgenus *Cellia* species based on the interspecific ITS2 variation among 10 Anopheline species prevalent in Mizoram.

Das *et al.* (2014) [15] determined the vectorial capacity and assessed the genetic diversity of *Anopheles annularis* collected from different endemic regions of Odisha by using PCR-RFLP and sequencing of D3 region of 28S rDNA. Dutta *et al.* (2014) [22] studied mitochondrial genetic variation for the first time among *Anopheles minimus* species from two malaria endemic states of North East India using COII gene. They analyzed 546 bp of COII sequence and identified mutations at 6 of the 546 variable sites.

Bindu and Sebastian (2014) [7] studied COI gene sequence of *Armigeres subalbatus* to evaluate its relationship with the different species of mosquitoes and to generate a database for its molecular barcoding. Daravath *et al.* (2014) [12] studied DNA sequence comparisons and phylogenetic relationship of COI gene for *Aedes albopictus* in order to inspect the homology of the sequences and evolutionary divergence with that of Hyderabad species. Kaura *et al.* (2014) [33] developed a PCR-RFLP based method for the differentiation of allopatric populations of *Anopheles stephensi* by using ITS1 and ITS2. They acknowledged the fact that ITS1 and ITS2 PCR-RFLP assays had a potential which could be useful for rapid and accurate identification of large numbers of mosquitoes, and might also assist in carrying out extensive epidemiologic and vector incrimination studies.

Murgan *et al.* (2015) [51] used complementary approach of CO1 gene based identification while analysing 10 species of mosquito vectors belonging to three genera, *Aedes, Culex* and *Anopheles* from India namely; *Aedes aegypti, Aedes albopictus, Aedes vittatus, Anopheles culicifacies, Anopheles niligricus, Anopheles stephensi, Anopheles vagus, Culex quinquefasciatus, Culex pseudovishnui* and *Culex vishnui.* They also observed that the congeneric divergence was three times higher than the conspecifics which indicated ample nucleotide variations. Paul *et al.* (2015) [55] conducted a survey to know the seasonal abundance as well as morphological variation and molecular characterization in two *Anopheles* species *i.e. Anopheles subpictus* and *Anopheles vagus* by amplifying ITS2 region. They observed that these species were closely related on the basis of GC content and repeat

sequence but at the same time the interspecific variation in ITS2 length was well marked between them.

Daravath *et al.* (2015) [14] conducted multiple sequence alignment of *Culex quinquefasciatus* from different geographical regions to check the sequence similarity. Sequence alignment studies elucidated the similarity and differences among different species in India along with other parts of the world. The sequence alignments of Hyderabad *Culex* species exhibited decent alignment with UK species and concluded that COI gene could be used as DNA barcode to identify the organism.

Gupta *et al.* (2016) [28] examined different DNA sequences of ITS2 and COI gene from globally distributed *Aedes* species and found that the identified Indian *Aedes* mosquito species showed significant variation in the DNA sequence when compared with *Aedes aegypti* from other geographical regions. Das *et al.* (2016) [18] conducted molecular identification of the most abundant species of *Aedes* vectors collected from different breeding spots of Sonitpur district, Assam and established the phylogenetic relationship with same species reported earlier from other parts of Southeast Asia.

Kumar *et al.* (2017) [40] developed a PCR methodology to distinguish members of *Culex vishnui* subgroup based on DNA barcoding from different geographical regions of country. Preet and Gupta (2017) [57] focused on the analysis of population genetic structure of *Culex quinquefasciatus* from various larval habitats in Agra using RAPD- PCR. Singh and Vashist (2017) [71] studied and analysed interspecific and intraspecific variations among four species of *Anopheles* using COII gene from Punjab state and concluded that identification on the basis of COII gene was accurate and helpful in differentiating the species. Singh *et al.* (2017) [72] studied genetic profile of five different mosquito species *Aedes vittatus, Aedes aegypti, Culex quinquefasciatus, Culex bitaeniorhynchus and Culex vishnui* using COII gene to understand phylogenetic relationship among them from Punjab and adjoining states.

Conclusion

On the basis of literature surveyed it is quite clear that a lot of molecular work has been done on COI (barcode gene), ITS1 and ITS2 (nuclear region) while few studies have been conducted on other mitochondrial and nuclear genes. The work has been summarized in Table 1. An important limitation of molecular work in India is that researchers have focused more on COI, ITS1 and ITS2 region thereby neglecting rest of the molecular markers which could also prove valuable in determining the other vast and diverse objectives of the research work. In terms of geographical region, the mosquito fauna of the northeast and southern part has been better explored by researchers in comparison to the north and northwest part of the country. Hence it is a need of the hour that upcoming research work should focus more on the diverse fauna of these overlooked regions to explore the biodiversity and generate molecular data for the species occurring in this region.

Table 1: Molecular studies on Indian mosquitoes.

S.No	Author's Name	State
1.	Manonmani *et al.* (2001), (2003)	Odisha.
	Manonmani *et al*. (2007)	Odisha, Rajasthan, Jharkhand, Tamil Nadu.
	Manonmani *et al*. (2013)	Odisha, Rajasthan, Tamil Nadu.
2.	Singh *et al.* (2004a)	Uttaranchal, Odisha, Karnataka.
	Singh *et al.* (2004b)	Rajasthan, Uttaranchal, Haryana, Gujarat, Uttar Pradesh, Madhya Pradesh, Jharkhand, Karnataka, Tamil Nadu.
3.	Goswami *et al.* (2005)	Uttar Pradesh, Madhya Pradesh, Tamil Nadu and Haryana.
	Goswami *et al*. (2006)	North India, South India, West India, East India, Central India.
4.	Prakash *et al*. (2006)	Arunachal Pradesh, Assam, Meghalaya, Nagaland
5.	Alam *et al.* (2006)	Andaman and Nicobar islands.
	Alam *et al.* (2007a)	Assam and Andaman and Nicobar islands.
	Alam *et al*. (2007b)	Assam, Uttar Pradesh, Madhya Pradesh, Jharkhand.
	Alam *et al*. (2008)	Chennai, Karnataka, Odisha, Pondicherry
6.	Singh *et al*. (2006)	Odisha
7.	Kohli and Choudhary (2007)	Chandigarh, Himachal Pradesh
8.	Kumar *et al.* (2007)	Pondicherry, Karnataka, Andhra Pradesh, Tamil Nadu, Odisha, Goa, Andaman and Nicobar islands.
9.	Raghavendra *et al.* (2009)	Rajasthan, Haryana, Odisha, Gujarat, Uttar Pradesh, Madhya Pradesh
10.	Mohanty *et al*. (2009)	Odisha
11.	Bora *et al*. (2009)	Andaman and Nicobar islands
	Sharma *et al.* (2009)	Gujarat, Rajasthan, Punjab, Assam Jammu and Kashmir
12.	Singh *et al*. (2010)	Assam
13.	Dhananjeyan *et al.* (2010)	Tamil Nadu
14.	Dixit *et al.* (2010)	Assam, Andaman and Nicobar, Uttaranchal, Chhattisgarh
15.	Sharma and Choudhry (2010)	Punjab, Haryana
16.	Sharma *et al.* (2010)	Jammu and Kashmir, Punjab, Rajasthan, Gujarat, Madhya Pradesh.
17.	Kaura *et al.* (2010)	Chandigarh, Punjab
18.	Bhattacharyya *et al.* (2010)	Assam, Arunachal Pradesh, Meghalaya, Mizoram, Nagaland, Tripura.
19.	Kohli *et al.* (2011a, 2011b)	Haryana, Punjab, Himachal Pradesh and Chandigarh
20.	Dixit *et al.* (2011)	West Bengal
21.	Sarma *et al.* (2012a, 2012b)	Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland, Tripura.
22.	Gupta and Preet (2012)	Uttar Pradesh

23.	Das *et al.* (2012), (2014)	Odisha
24.	Singh *et al.* (2012)	Arunachal Pradesh, Assam, Meghalaya, Sikkim, Tripura, Mizoram, Manipur, Nagaland
25.	Paramasivan *et al.* (2013)	Tamil Nadu
26.	Nanda *et al.* (2013)	Uttar Pradesh
27.	Kumar *et al.* (2013)	Odisha, Jharkhand
28.	Kumari *et al.* (2013)	Odisha
29.	Das *et al.* (2013)	Odisha
30.	Zomuanpuii *et al.* (2013)	Mizoram
31.	Dutta *et al.* (2014)	Assam, Arunachal Pradesh
32.	Bindu and Sebastian (2014)	Kerala
33.	Daravath *et al.* (2013), (2014), (2015)	Hyderabad
34.	Kaura *et al.* (2014)	Punjab
35.	Murgan *et al.* (2015)	Tamil Nadu
36.	Paul *et al.* (2015)	West Bengal
37.	Das *et al.* (2016)	Assam
38.	Gupta *et al.* (2016)	Rajasthan
39.	Preet and Gupta (2017)	Uttar Pradesh
40.	Singh and Vashist (2017)	Punjab
41.	Singh *et al.* (2017)	Jammu and Kashmir, Punjab

References

1. Alam MT, Das MK, Dev V, Ansari MA and Sharma YD. Identification of two cryptic species in the *Anopheles (Cellia) annularis* complex using ribosomal DNA PCR-RFLP. Parasitology Reseach 2007b; 100: 943-948.
2. Alam MT, Das MK, Dev V, Ansari MA, Sharma YD. PCR-RFLP method for the identification of four members of the *Anopheles annularis* group of mosquitoes (Diptera: Culicidae). Transactions of the Royal Society of Tropical Medicine and Hygiene 2006; 101:239-244.
3. Alam MT, Das MK, Dev V, Ansari MA, Sharma YD. PCR-RFLP method for the identification of four members of the *Anopheles annularis* group of mosquitoes (Diptera: Culicidae). Transactions of the Royal Society of Tropical Medicine and Hygiene, 2007a; 101:239-244.
4. Alam MT, Bora H, Das MK, Sharma YD. The type and mysorensis forms of the *Anopheles stephensi* (Diptera: Culicidae) in India exhibit identical ribosomal DNA ITS2 and domain-3 sequences. Parasitology Research 2008; 103:943-948.
5. Bhattacharyya DR, Prakash A, Sarma NP, Mohapatra PK, Singh S, Sarma DK *et al*. Molecular evidence for the involvement of Anopheles nivipes (Diptera: Culicidae) in the transmission of Plasmodium falciparum in north-eastern India. Annals of Tropical Medicine & Parasitology. 2010; 104(4):331-336.
6. Bhattacharyya DR, Rajavel AR, Natarajan R, Mohapatra PK, Jambulingam P, Mahanta J, *et al*. Faunal richness and the checklist of Indian mosquitoes (Diptera: Culicidae). Journal of species lists and distribution 2014; 10(6):1342-1358.
7. Bindu PU, Sebastian CD. Genetic structure of mitochondrial cytochrome oxidase subunit I gene of the mosquito, Armigeres subalbatus. International Journal of Research. 2014; 1(10):49-56.
8. Bora H, Das MK, Ahmed A, Sharma YD. Variations in the mitochondrial DNA markers in the *Anopheles (Cellia) sundaicus* population from the Andaman and Nicobar islands, India. Acta Tropica, 2009; 112:120-124.
9. Chen B, Butlin RK, Harbach RE. Molecular phylogenetics of the Oriental members of the Myzomyia series of Anopheles subgenus Cellia (Diptera: Culicidae) inferred from nuclear and mitochondrial DNA sequences. Systematic Entomology 2003; 28:57-69.
10. Collins FH, Paskewitz SM. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic Anopheles species. Insect Molecular Biology 1996; 5:1-9.
11. Collucci E, Sallum MAM. Phylogenetic analysis of the subgenus Kerteszia of Anopheles (Diptera: Culicidae: Anophelinae) based on morphological characters. Insect systematics & evolution, 2003; 34:361-372.
12. Daravath S, Setti A, Singh Y, Swarnagowreeswari G, Yadav MM, Pawar SC, *et al*. DNA barcode of COI genetic marker of the Indian *Aedes albopictus* (Skuse) (Insecta: Diptera: Culicidae), Medical Science 2014; 5(15):21-25.
13. Daravath S, Yadav MM, Chakrapani P, Naik BR. Molecular Identification of *Aedes albopictus* (Diptera: Culicidae) and quantitative analysis of COI gene in South-Indian species. International Journal of Current Microbiology and Applied Sciences. 2013; 2(8):102-109.
14. Daravath SS, Siddaiah M, ReddyaNaik B. Molecular characterization and phylogenetic analysis of *Culex quinquefasciatus* by DNA barcoding. Advances in Entomology, 2015; **3**:118-124.
15. Das B, Patra AP, Das M, Mahapatra N, Tripathy H, Kar SK, *et al*. Vectorial capacity and genetic diversity of *Anopheles annularis* (Diptera: Culicidae) mosquitoes in Odisha, India from 2009 to 2011. Acta Tropica 2014; 137:130-139.
16. 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. Tropical Medicine & International Health. 2012; 17(2):235-243.
17. Das M, Das B, Patra AP, Tripathy HK, Mohapatra N, Kar SK, *et al*. *Anopheles culicifacies* sibling species in Odisha, eastern India: First appearance of *Anopheles culicifacies* E and its vectorial role in malaria transmission. Tropical Medicine & International Health. 2013; 18(7):810-821.

18. Das M, Das MK, Dutta P. Genetic characterization and molecular phylogeny of *Aedes albopictus* (Skuse) species from Sonitpur district of Assam, India based on COI and ITS1 genes. Journal of Vector Borne Diseases, 2016; 53:240-247.
19. Dhananjeyan KJ, Paramasivan R, Tewari SC, Rajendran R, Thenmozhi V, Leo VJ, *et al*. Molecular identification of mosquito vectors using genomic DNA isolated from eggshells, larval and pupal exuvium. Tropical Biomedicine. 2010; 27(1):47-53.
20. Dixit J, Srivastava H, Sharma M, Das MK, Singh OP, Raghavendra K, *et al*. Phylogenetic inference of Indian malaria vectors from multilocus DNA sequences. Infection, Genetics and Evolution, 2010; 10:755-763.
21. Dixit J, Srivastava H, Singh OP, Saksena DN, Das A. Multilocus nuclear DNA markers and genetic parameters in an Indian *Anopheles minimus* population. Infection, Genetics and Evolution. 2011; 11(3):572-579.
22. Dutta P, Khan SA, Topno R, Chowdhury P, Baishya M, Chowdhury P, *et al*. Genetic diversity and gene structure of mitochondrial region of *Anopheles minimus* (Diptera: Culicidae) - major malaria vector of Northeast India. Asian Pacific Journal of Tropical Medicine. 2014; 7(12):952-955.
23. Foley DH, Rueda LM, Wilkerson RC. Insight into mosquito biogeography from country species records. Journal of Medical Entomology. 2007; 44(4):554-567.
24. Garros C, Harbach RE, Manguin S. Morphological assessment and molecular phylogenetics of the Funestus and Minimus groups of Anopheles (Cellia). Journal of Medical Entomology, 2005a; 42:522-536.
25. Garros C, Harbach RE, Manguin S. Systematics and biogeographical implications of the phylogenetic relationships between members of the *funestus* and *minimus* groups of *Anopheles* (Diptera: Culicidae). Journal of Medical Entomology, 2005b; 42:7-18.
26. Goswami G, Raghavendra K, Nanda N, Gakhar SK, Subbarao SK. PCR-RFLP of mitochondrial cytochrome oxidase subunit II and ITS2 of ribosomal DNA: Markers for the identification of members of the *Anopheles culicifacies* complex (Diptera: Culicidae). Acta Tropica 2005; 95:92-99.
27. Goswami G, Singh OP, Nanda N, Raghavendra K, Gakhar SK, Subbarao SK, *et al*. Identification of all members of the *Anopheles culicifacies* complex using allele-specific polymerase chain reaction assays. American Journal of Tropical Medicine and Hygiene. 2006; 75(3):454-460.
28. 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. Journal of Vector Borne Diseases, 2016, 149-155.
29. Gupta S, Preet S. Protocol optimization for genomic DNA extraction and RAPD-PCR in mosquito larvae (Diptera: Culicidae). Annals of Biological Research 2012; 3(3):1553-1561.
30. Harbach RE. The Culicidae (Diptera): a review of taxonomy, classification and phylogeny. Zootaxa 2007; 1668:591-638.
31. Harbach RE. http://mosquito-taxonomic-inventory.info. 26 February 2018.
32. Kampen H. The ITS2 ribosomal DNA of Anopheles beklemishevi and further remarks on the phylogenetic relationships within the *Anopheles maculipennis* group of species (Diptera: Culicidae). Parasitology Research 2005; 97:118-128.
33. Kaura T, Mewara A, Sharma A, Kumar R, Chaudhry S, Sehgal R, *et al*. Secondary structure and restriction based analysis of the ribosomal DNA internal transcribed spacer regions (ITS 1 and 2) of allopatric populations of *Anopheles stephensi* (Diptera: Culicidae). Journal of Entomology and Zoology Studies 2014; 2(6):183-190.
34. Kaura T, Sharma M, Chaudhry S, Chaudhry A. Sequence polymorphism in spacer ITS2 of *Anopheles (Cellia) subpictus* Grassi (Diptera: Culicidae). Caryologia 2010; 63(2):124-133.
35. Kohli R, Chaudhry S, Bedi G. Genomic Analysis of Some *Culex* Mosquitoes (Culicidae: Diptera). Electronic Journal of Biology. 2011b; 7(1):6-10.
36. Kohli R, Sharma M, Chaudhry S. DNA Fingerprinting and phylogenetics of five species of genus *Culex* using ITS2 sequence (Diptera: Culicidae). Caryologia 2011a; 64(1):3-9.
37. Kohli R, Chaudhry S. Sequence analysis of mitochondrial 16s ribosomal RNA gene fragment in the two populations of *Armigeres* (*Armigeres*) *subalbatus* (Culcidae: Diptera). Cytologia. 2007; 72(1):83-88.
38. Krzywinski J, Wilkerson RC, Besansky NJ. Evolution of mitochondrial and ribosomal gene sequences in Anophelinae (Diptera: Culicidae): implications for phylogeny reconstruction. Molecular Phylogenetics and Evolution, 2001; 18:479-487.
39. Kshirsagar SG, Patole MS, Shouche YS. Characterization of insect cell lines: heteroduplex analysis employing a mitochondrial 16S ribosomal RNA gene fragment. Analytical Biochemistry, 1997; 253:65-69.
40. Kumar NP, Rajavel AR, Jambulingam P. Development of PCR methodology to distinguish species members of *Culex vishnui* subgroup (Diptera: Culicidae) based on DNA barcodes. Insect Science 2017; 24:336-340.
41. Kumar NP, Rajavel AR, Natarajan R, Jambulingam P. DNA Barcodes Can Distinguish Species of Indian Mosquitoes (Diptera: Culicidae). Journal of Medical Entomology. 2007; 44(1):1-7.
42. Kumar NP, Krishnamoorthy N, Sahu SS, Rajavel AR, Sabesan S, Jambulingam P. DNA Barcodes indicate members of the *Anopheles fluviatilis* (Diptera: Culicidae) species complex to be conspecific in India. Molecular Ecology Resources. 2013; 13(3):354-361.
43. Kumari S, Das S, Mahapatra N. *Anopheles subpictus* B and its role in transmission of malaria in Odisha, India. Tropical Biomedicine. 2013; 30(4):710-717.
44. Ma Y, Xu J. The Hyrcanus group of Anopheles (Anopheles) in China (Diptera: Culicidae): species discrimination and phylogenetic relationships inferred by ribosomal DNA internal transcribed spacer 2 sequences. Journal of Medical Entomology, 2005; 42:610-619.
45. Manonmani A, Nanda N, Jambulingam P, Sahu S, Vijayakumar T, Vani JR, *et al*. Comparison of polymerase chain reaction assay and cytotaxonomy for

identification of sibling species of *Anopheles fluviatilis* (Diptera: Culicidae). Bulletin of Entomological Research 2003; 93:169-171.
46. Manonmani A, Townson H, Adeniran T, Jambulingam P, Sahu S, Vijayakumar T. rDNA-ITS2 polymerase chain reaction assay for the sibling species of Anopheles fluviatilis. Acta Tropica, 2001; 78:3-9.
47. Manonmani AM, Mathivanan AK, Sadanandane C, Jambulingam P. Evaluation of the mtDNA-COII region based species specific assay for identifying members of the *Anopheles culicifacies* species complex. Journal of Arthropod-Borne Diseases. 2013; 7(2):154-163.
48. Manonmani AM, Sadanandane C, Sahu SS, Mathivanan A, Jambulingam P. rDNA-ITS2-PCR assay for grouping the cryptic species of *Anopheles culicifacies* complex (Diptera: Culicidae). Acta Tropica 2007; 104:72-77.
49. Marrelli MT, Floeter-Winter LM, Malafronte RS, Tadei WP, Lourenco-de-Oliveira R, Flores-Mendoza C. Amazonian malaria vector anopheline relationships interpreted from ITS2 rDNA sequences. Medical and Veterinary Entomology 2005; 19:208-218.
50. Mohanty A, Swain S, Kar SK, Hazra RK. Analysis of the phylogenetic relationship of *Anopheles* species, subgenus *Cellia* (Diptera: Culicidae) and using it to define the relationship of morphologically similar species. Infection, Genetics and Evolution, 2009; 9:1204-1224.
51. Murgan K, Vadivalagan C, Karthika P, Panneerselvam C, Paulpandi M, Subramaniam J, *et al*. DNA barcoding and molecular evolution of mosquito vectors of medical and veterinary importance. Parasitology Research, 2015; 115:107-121.
52. Nanda N, Singh OP, Dua VK, Pandey AC, Nagpal BN, Adak T, *et al*. Population cytogenetic and molecular evidence for existence of a new species in *Anopheles fluviatilis* complex (Diptera: Culicidae). Infection, Genetics and Evolution, 2013; 13:218-223.
53. Otranto D, Stevens JR. Molecular approaches to the study of myiasis-causing larvae. International Journal for Parasitology. 2002; 32(11):1345-1360.
54. Paramasivan R, Dhananjeyan KJ, Pandian RS. A preliminary report on DNA barcoding and phylogenetic relationships of certain public health important mosquito species recorded in rural areas of south India. Journal of Vector Borne Diseases, 2013; 50:144-146.
55. Paul S, Chattopadhyay A, Banerjee PK. Studies on seasonal abundance & molecular characterization of *Anopheles subpictus* and *Anopheles vagus* based on ITS2 sequence variability. International Journal of Mosquito Research. 2015; 2(3):131-135.
56. Prakash A, Walton C, Bhattacharyya DR, Loughlin SO, Mohapatra PK, Mahanta J. Molecular characterization and species identification of the *Anopheles dirus* and *Anopheles minimus* complexes in north-east India using r-DNA ITS-2. Acta Tropica, 2006; 100:156-161.
57. Preet S, Gupta S. Genetic diversity studies on *Culex quinquefasciatus* from diverse larval habitats using RAPD-PCR marker. International Journal of Mosquito. 2017; 4(2):93-98.
58. Proft J, Maier WA, Kampen H. Identification of six sibling species of the Anopheles maculipennis complex (Diptera: Culicidae) by a polymerase chain reaction assay. Parasitology Research, 1999; 85:837-843.
59. Raghavendra K, Cornel AJ, Reddy BPN, Collins FH, Nanda N, Chandra D, *et al*. Multiplex PCR assay and phylogenetic analysis of sequences derived from D2 domain of 28S rDNA distinguished members of the *Anopheles culicifacies* complex into two groups, A/D and B/C/E. Infection, Genetics and Evolution. 2009; 9(2):271-277.
60. Reidenbach KR, Cook S, Bertone MA, Harbach RE, Wiegmann BM, Besansky NJ. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. Evolutionary Biology. 2009; 298(9):1-14.
61. Sallum MAM, Schultz TR, Foster PG, Aronstein K, Wirtz RA, Wilkerson RC. Phylogeny of Anophelinae (Diptera: Culicidae) based on nuclear ribosomal and mitochondrial DNA sequences. Systematic Entomology. 2002; 27:361-382.
62. Sarma DK, Prakash A, O'Loughlin SM, Bhattacharyya DR, Mohapatra PK, Bhattacharjee K, *et al*. Genetic population structure of the malaria vector *Anopheles baimaii* in north-east India using mitochondrial DNA. Malaria Journal, 2012b; 11:76.
63. Sarma NP, Prakasha A, Bhattacharyyaa DR, Kalita MC, Mohapatra PK, Singha S, *et al*. Spatial distribution and molecular characterization of *Anopheles nivipes* and *Anopheles philippinensis* (Diptera: Culicidae) in north-east India. Acta Tropica, 2012a; 122:247-254.
64. Sathe TV, Jagtap M. Taxonomy and molecular phylogeny of new species of the genus *Anopheles* (Cellia) Theobald (Culicidae: Diptera) from India. Journal of the Entomological Research Society. 2012; 36(1):71-75.
65. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction. The American Journal of Tropical Medicine and Hygiene. 1993; 49:520-529.
66. Sharma AK, Chandel K, Tyagi V, Mendki MJ, Tikar SN, Sukumaran D. Molecular phylogeny and evolutionary relationship among four mosquito (Diptera: Culicidae) species from India using PCR-RFLP. Journal of Mosquito Research. 2013; 3(8):58-64.
67. Sharma AK, Mendki MJ, Tikar SN, Chandel K, Sukumaran D, Parashar BD, *et al*. Genetic Variability in geographical populations of *Culex quinquefasciatus* Say (Diptera: Culicidae) from India based on random amplified polymorphic DNA analysis. Acta Tropica, 2009; **112**:71-76.
68. Sharma AK, Mendki MJ, Tikara SN, Kulkarni G, Veera V, Prakasha S, *et al*. Molecular phylogenetic study of *Culex quinquefasciatus* mosquito from different geographical regions of India using 16S rRNA gene sequences. Acta Tropica, 2010; 116:89-94.
69. Sharma M, Chaudhry S. Molecular Cytogenetics of some *Anopheles* mosquitoes (Culicidae: Diptera). Electronic Journal of Biology. 2010; 6(1):13-18.
70. Shouche Y, Patole MS. Sequence analysis of mitochondrial 16S ribosomal RNA gene fragment from seven mosquito species. Journal of Biosciences 2000; 25(4):361-366.

71. Singh D, Vashist D. Studies on some species of Indian Anopheles based on mitochondrial gene COII (Diptera: Culicidae: Anophelinae). Journal of Entomology and Zoology Studies. 2017; 5(3):1895-1899.
72. Singh D, Gill NK, Rai NK. Sequence analysis of mitochondrial COII gene fragment from five Culicinae mosquito species. Journal of Entomology and Zoology Studies. 2017; 5(4):1386-1390.
73. Singh OP, Chandra D, Nanda N, Raghavendra K, Sunil S, Sharma SK, *et al*. Differentiation of members of the *Anopheles fluviatilis* species complex by an allele-specific polymerase chain reaction based on 28s ribosomal DNA sequences. American Journal of Tropical Medicine and Hygiene. 2004a; 70(1):27-32.
74. Singh OP, Chandra D, Nanda N, Sharma SK, Htun PT, Adak T, *et al*. On the conspecificity of *Anopheles fluviatilis* species S with *Anopheles minimus* species C. Journal of Biosciences. 2006; 31(5):671-677.
75. Singh OP, Goswami G, Nanda N, Raghavendra K, Chandra D, Subbarao SK, *et al*. An allele-specific polymerase chain reaction assay for the differentiation of members of the *Anopheles culicifacies* complex. Journal of Biosciences. 2004b; 29:275-280.
76. Singh OP, Nanda N, Dev V, Bali P, Sohail M, Mehrunnisaa A, *et al*. Molecular evidence of misidentification of *Anopheles minimus* as *Anopheles fluviatilis* in Assam (India). Acta Tropica. 2010; 113:241-244.
77. Singh S, Chandra R, Kumar A. A study of prevalence of Anopheline mosquitoes from a periurban locality of Bareilly District. The Journal of Experimental Sciences. 2010; 1(5):17.
78. Singh S, Prakasha A, Yadav RNS, Mohapatraa PK, Sarmaa NP, Sarmaa DK, *et al*. Anopheles (Cellia) maculatus group: Its spatial distribution and molecular characterization of member species in north-east India. Acta Tropica. 2012; 124:62-70.
79. Wilkerson RC, Foster PG, Li C, Sallum MAM. Molecular phylogeny of neotropical Anopheles (Nyssorhynchus) albitarsis species complex (Diptera: Culicidae). Annals of the Entomological Society of America. 2005; 98:918-925.
80. Xu JN, Qu FY. Ribosomal DNA difference between species A and D of the Anopheles dirus complex of mosquitoes from China. Medical and Veterinary Entomology**.** 1997; 11:134-138.
81. Zomuanpuii R, Ringngheti L, Brindha S, Gurusubramanian G, Kumar NS. ITS2 characterization and *Anopheles* species identification of the subgenus *Cellia*. Acta tropica, 2013; 125:309-319.