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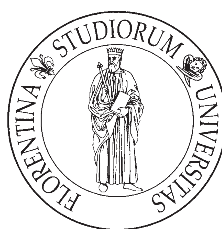

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DNA Fingerprinting and Phylogenetics of Five Species Of Genus *Culex* Using ITS2 Sequence (Diptera: Culicidae)

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Abstract — The present paper deals with the ITS2 sequence based interspecific variations and molecular phylogenetics of five Oriental species of genus *Culex viz*: *Cx. quinquefasciatus, Cx. vishnui, Cx. bitaeniorhynchus, Cx. tritaeniorhynchus* and *Cx. mimeticus.* The length of ITS2 sequence varied from 323 to 410 bp with G:C content ranging from 50.7% to 66.5%. The sequence carried indels at 221 positions while the transition to transversion (ts/tv) ratio ranged from 0.41 to 0.76 in which *Cx. tritaeniorhynchus* had the lowest ts/tv value of 0.04 while *Cx. mimeticus* had the highest value of 0.76. The most frequent transversions were of the A:T type. By using the sequence of *Anopheles stephensi* taken as an outgroup, the maximum parsimony based phylogenetic tree with threshold (considering only the transversions) showed that *An. stephensi* and *Cx. quinquefasciatus* were supported by 100% bootstrap value beyond which all other species got bifurcated into two clades in which one clade consisted of *Cx. tritaeniorhynchus* + *Cx. mimeticus* with a bootstrap value of 62.8% while the other consisted of *Cx. bitaeniorhynchus* + *Cx. vishnui* with a value of 19.5%.

Key words: five *Culex* species, ITS2, phylogenetics.

INTRODUCTION

In the recent years, investigations on the molecular cytogenetics of the mosquito vectors of malaria, filaria, dengue and yellow fever have become priority areas of research. The multilevel approach to the studies on the genomic analysis has become more demanding as some of the mosquito taxa are known to exist as groups of sibling species whose recognition needs more than one parameters of study (Narang *et al*. 1993a;b; Munstermann 1995; Subbarao 1996; Reinert *et al*. 1997; Chaudhry 1999; Ramirez and Dessen 2000 a;b; WHO/UNDP/World Bank 2003; Chaudhry *et al.* 2006). The correct identification of disease carrying species and their subspecific variants is also a first important step in co-relating their malariogenic activities with their correct taxonomic status. The phenomenon of insecticide resistance, genetics of resistance and genetics of vectorial capacity had also been important in developing various different means of their population suppression (Coluzzi and Kitzmiller 1975; Steiner *et al.* 1982; WHO 1984). In the last few years, molecular systematics of insects has undergone remarkable growth. Advances made in the methods of data generation and analysis have led to the accumulation of a fair amount of DNA sequence information from some of the major vectors of disease (Besansky *et al.* 1992; Wilkerson *et al.* 1995; Carlson 1997; Marinucci *et al.* 1999; Proft *et al.* 1999; Chaudhry *et al.* 2004; Neetu and Chaudhry 2005; Chaudhry and Kohli 2007; Kohli and Chaudhry 2007).

As compared to the mosquito species belonging to genus *Anopheles* very little progress has been made in accumulating the genomic information on different species of the genera *Culex* and *Aedes* which also include several species of epidemiological significance. Keeping this

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in view, species-specific PCR primers have also been designed for studying the sequence variations in the first and second internal transcribed spacers (ITS 1,2) of nuclear rDNA gene array of *Culex pipiens* complex as these are also important genetic markers (COLLINS *et al.* 1996; CORNEL *et al.* 1996; WALTON *et al.* 1999). In the light of the advances made in the DNA diagnostics of mosquitoes and the number of options to choose the genes of interest for phylogenetic studies, the present paper deals with the study of DNA fingerprinting and phylogenetics of five species of genus *Culex* using ITS2 sequence (Culicidae: Diptera). The aim of the study was to test the utility of these introns as potential sequences for studying the phylogeny of five species of the genus *Culex viz: Cx. quinquefasciatus, Cx. vishnui, Cx. bitaeniorhynchus, Cx. tritaeniorhynchus* and *Cx. mimeticus.*

MATERIALS AND METHODS

Larvae and adults of all the five species were collected from a village Nadasahib (Panchkula, Haryana), 20 Kms South-east of Chandigarh, Hamirpur (Himachal Pradesh), Patiala, (Punjab) and Sector 25 of Chandigarh ((30º43'N, 76º47'E). The DNA was extracted by following the phenol-chloroform extraction method of AUSUBEL *et al.* (1999) according to which, three specimens of freshly hatched unfed females each of the two populations were individually homogenized in 100ml of lysis buffer consisting of 10mM Tris-HCl, 1mM EDTA, 25 mM NaCl and 1% SDS for lysis of cell membranes. These contents were incubated at 37°C for 30 minutes in a water bath after which proteinase K was added to the tubes and the contents were incubated again at 65°C for 1h in a water bath. While the tubes were still warm, 3M sodium acetate was also added and again incubated on ice for 1 h. After this, the contents were ultracentrifuged at 4°C for 10 min at 10,000 rpm. The supernatant containing the dissolved DNA was transferred to fresh 1.5 ml eppendorf tube to which equal volumes of phenol-chloroform-isoamyl alcohol were added. These tubes were again centrifuged at 8,000 rpm for 10 min at 4°C after which the upper aqueous layer containing the DNA was transferred to another eppendorf tube and twice the volume of chilled ethanol (-20°C) was added before keeping the tubes overnight at -20°C. These tubes were again centrifuged for 10 min after which the aliquot was removed without disturbing the pellets containing the DNA. The contents of the pellets were washed gently with 70% ethanol and dried. Each pellet was then dissolved in 20 ml of Tris-EDTA (10 : 1) buffer for maintaining the pH before storage at 4°C.

Extraction and PCR amplification of DNA - Both forward and reverse primers used in the present *in vitro* DNA amplification of ITS2 were oligomers having a base sequence of FP 5'-TGTGAACTGCAGGACACAT-3' and RP 5'-TATGCTTAAATTCAGGGGGT-3' (PORTER and COLLINS 1991; WALTON *et al.* 1999). The amplification reactions were performed as per the modified protocol of WILLIAMS *et al.* (1990) according to which each of the 25 ml of reaction mixture was prepared by mixing 1-PCR buffer, 0.2 mM each of all the four deoxyribonucleotide triphosphates (dNTPs) *i.e.* dATP, dCTP, dGTP and dTTP, 20 pmol of Primer, 1 unit of Taq DNA polymerase and 10 ng of the genomic DNA of the mosquito. The amplification was initiated with one cycle of hot start at 94°C for 10 min for denaturation of DNA followed by repeated cycles of denaturation, annealing and extension of DNA at 94°C for 1 min, at 56°C for 45 s and 72°C for 1 min respectively for a total of 35 cycles. The final cycle consisted of one cycle of extension at 72°C for 10 min. In all such amplifications, a negative control consisting of all the components of reaction mixture except the DNA, was also processed for the authentication of results. All the experiments were performed in triplicate to rule out the experimental errors while the PCR end products were electrophoresed in 1.2% agarose gel for getting the desired DNA bands.

RESULTS AND DISCUSSION

Sequence Analysis - The PCR amplification of ITS2 sequence from the five species yielded DNA bands ranging from 390-477 base pairs (Fig. 1). For the purpose of sequence based cladistic analysis of species *An. stephensi* was taken as an outgroup whose sequence was used during sequence alignment. The detailed sequence analysis of ITS2 revealed marginally higher percentage of 51.8% of G:C content in each species.

MARRELI *et al.* (2005) studied as many as sixteen anopheline species from the Amazon Basin, Brazil. In all of them the length of ITS2 varied from 323 to 410 bp, with G:C content ranging from 50.7% to 66.5% while sequence homologies ranged from 25% to 99%. In the overall as-

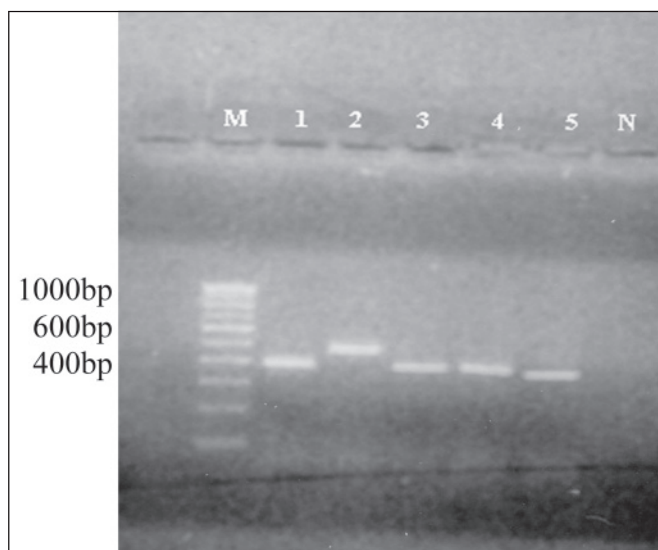

Fig. 1 — PCR generated DNA bands of rDNA ITS2 sequence. lane M: Gene Ruler (DNA ladder), lane 1: *Culex bitaeniorhynchus,* lane 2: *Culex quinquefasciatus,* lane 3: *Culex vishnui*; lane 4: *Culex tritaeniorhynchus,* lane 5: *Culex mimeticus*; lane N: negative control.

sessment of A:T and G:C ratios of the present five species of genus *Culex,* it was observed that A:T and G:C regions were not uncommon in the populations of *Cx. quinquefasciatus*. These results are in agreement with MARRELLI *et al.* (2005) who also studied the A:T and G:C contents in sixteen different populations of this species from across the world including Brazil, Mexico and Florida. This exhaustive survey also revealed that majority of the subspecies and biotypes of this taxon overlapped geographically in many locations in North America, South America, Australia, Europe, Africa and in the Middle and far East.

In the multiple sequence alignment the loci marked with asterisk (*) shows those regions where base sequences are identical in all the species while dashes (–) indicate the loci differing due to insertions/deletions (indels) (Fig. 2). Accordingly, the sequence carried indels at 221 positions while the transition to transversion (ts/tv) ratio ranged from 0.41 to 0.76 in which *Cx. tritaeniorhynchus* had the lowest ts/tv value of 0.04 while *Cx. mimeticus* had the highest ts/tv value of 0.76. The most frequent transversions were of the A:T type. An interesting feature of the sequences was the base repeat of A (in bold) from base 387 to 399 in the sequence of *Cx. quinquefasciatus* (Fig. 3).

Phylogenetic analysis - Genetic distance among the species was estimated by the application of Kimura-2 parameter model using MEGA 3.1 software. Sites containing alignment gaps were not used in the distance analysis and were treated as missing information. Maximum Parsimony (MP), Neighbor Joining (NJ) and Maximum Likelihood (ML) Methods were used for the construction of phylogenetic trees by using PAUP version 4.0 beta 10 (Swofford, 2001). According to the Maximum Parsimony based phylogenetic tree with threshold (considering the transversions) showed that *An. stephensi* and *Cx. quinquefasciatus* were supported by 100% bootstrap value beyond which all other species got bifurcated into two clades in which one clade consisted of *Cx. tritaeniorhynchus*+ *Cx. mimeticus* with a bootstrap value of 62.8% while the other consisted of *Cx. bitaeniorhynchus* + *Cx. vishnui* with a value of 19.5% (Fig. 4).

For understanding the usefulness of different weightings, the relative frequencies of transitions and transversions were also estimated using Kimura-2 parameter distance method (K2P) which is meant for estimating the evolutionary rate of base substitutions through comparative studies of nucleotide sequences. With this, the data was re-analysed by giving two times more weightage to transversions than to transitions as this is the minimum value registerd in the software programme. The resultant weighted tree showed 100% bootstrap value for *An. stephensi* and *Cx. quinquefasciatus,* whereas *Cx. bitaeniorhynchus* and *Cx. vishnui* were supported by considerably lesser values of 29% and 37% respectively. In the same way *Cx. tritaeniorhynchus* and *Cx. mimeticus* got bifurcated into one clade with bootstrap value of 76% (Fig. 5).

Finally, the phylogenetic tree was constructed using Maximum Likelihood (ML) Method with molecular clock that helps in calculating the molecular substitution rates and also detects heterogeneity in those substitutions which

```
bitaeniorhynchus     ---------------GTACCTTGCATCTTGACC---TGCATATTG--CACATCGT----- 35
quinquefasciatus     --------------CGTACCTTGCAG-TTGAT----TACATATTG--CACATCCGCTCTA 39
vishnui              ---------------GTAACCGGCCG-TTGAAC---TACATATTG--CACATCCCC---- 35
tritaeniorhynchus    CAATTGTTTTTGTTAGGACCCGGAAACACCAAC---AACA-ACAA--CAAGTAGTGCTCA 54
mimeticus            ---------------GTGGCGGGCAG--TGTAT---TGTGAACTG--CAGGACACATG-A 37
Anopheles            --------TGTGTGACTGCCAGGACACATGAACACCGACACGTTGAACGCATGATGGATG 52
                                         *  *                         *

bitaeniorhynchus     ACAACAGTA-----CGATT-GT--ACACATTTTTGAGTGCCTA------TATTTATCTAT 81
quinquefasciatus     GCAACTATGTTAGCCGATTAGT--ACACATTTTTGAGTGCCTA------TATTTATCTAT 91
vishnui              ACAATTGTA-----CGATT-GT--ACACATTTTTGAGTGCCTA------TATTTATCTAT 81
tritaeniorhynchus    TCCTCCACCTA---CAACAGGA--AGACGTTCTCGAGTTTGTA------TATTTATATTT 103
mimeticus            ACACCGATA-------AGTTGA--ACGCATATTGGCAT-CGGA------CGTTCA---AC 78
Anopheles            AACTCGGTTTCACCCCGACCGATGACACATCCTTGAGTGCCTACCAAGTTATCGATATAC 112
                                         *   *  * *  * *  *    *         *  *

bitaeniorhynchus     TC--AACT----GTGTATGCGTGCTCACGCGCGTATACACGCAGCATGGTGT-----TTT 130
quinquefasciatus     TC--AACT----GTGCACACACGC-------------ACGCAAAATGGTGT-----TTT 126
vishnui              TC--AACT----GTGTGTGCTCTCT--CGGGGGCACGCACGCAGCATGGTGT-----TTT 128
tritaeniorhynchus    TT--AAATTCATGAGTTCTTCCTCTCGGGGGGGCACGCACGCAGCATGGTGT-----TTT 156
mimeticus            CC--GACCG---ATGCACACATGGGGGGGGCCCCCCCAGTTTGTTTTGTGTAGG-ACGC 132
Anopheles            TCCTACCAGACTGACTGTCCCATCCCCGCGATGGGCTGTCGCAGAATGGCGTGCTCGGAC 172
                                                                  * * **

bitaeniorhynchus     GCTGCCTTCGGTGGCTGG--CAAAACATTTAAGATACTCC-CGGTTCGGG---------T 178
quinquefasciatus     GCTGCCTTCGGTGGCTGG--GAAAACATTCAAGACGCTCAGCGGCTCGGGGTTTTCGTTC 184
vishnui              ACTGCCTTCGGTGGCTGG--TAAAACATTTAAGATACTCC-CGGTTCGGG---------T 176
tritaeniorhynchus    ACTGCCTTCGGTGGCGGG--TAAAACATCTAAGATACTCC-CGGTTCGGG---------- 203
mimeticus            ACCAGCCACACCAACAAGAACAAATCATACACATCATACAACAGCACGAA---------- 182
Anopheles            CCCGTATGTGGGACCGTGGGCGCTGAAAGTGAGAGTGCTATTAACAAGGTGGTACGCAAG 232
                      *             * *        *                 *

bitaeniorhynchus     GCG-ACGACCATCACTGGCGCGCG---ACACG-AC-------------------GTGAA 213
quinquefasciatus     GCGGACGGCCA-CACTGGTGCGCACGCACGCG-ACTGAACGAATGAACAACGAGAGAAAA 242
vishnui              GCG-ACGACCATCACTGGCGCGCG---ACACG-AC-------------------GTGAG 211
tritaeniorhynchus    GGGGGCGACCATCACCGGCGCGCG---ACACG-AC-------------------GTGAG 239
mimeticus            GAACACAACGTTCACGGGTGAGAGTATTTATT-TATACATAGTGGTGTGTTTTTTGTGGG 241
Anopheles            GCGAGAGATGA--ACGGGCGCGCGTCAAGTCGCACGGTTCGACCTCCAGTATCAACTAGG 290
                     *             ** ** * *

bitaeniorhynchus     AATACATCCCACACACCAACCTGGGTTGGGCCCCCAAGGAAACTCTC-----CCAAGCAA 268
quinquefasciatus     AATCCCTCCCACCCACCTGCCTGGGTTGGGCATGGATGTTCTCTCTCTCCCCCCCCGCCT 302
vishnui              AATACATCCCACACACCAACCTGGCTTGGGCGCCGATGTAAACTCTC------TCAGTCA 265
tritaeniorhynchus    AATACATCCCACACACCAACATGGTTTGGGGGCCGATGTAAAATCTT------TCAGTCA 293
mimeticus            GGGAGACCACACGCACC-GCGTTGTTTTGCTTTACGTAGAGGGCAACACAACACAGATAA 300
Anopheles            GATGAAACCCCGCAGCCTAACAGATTAACACCAGGCGCTAGCAAAGGGGTCCCCGGTTG 350
                            * * * ** *      * **

bitaeniorhynchus     CCGGTTGCCGGGGGGC-----CGTTTGACC----CCCCCCCAAAACCGA----AAAAAAA 315
quinquefasciatus     CCCACACTCGTTCGTT-----CGTTCATCCGCCGCCCTCTCGCGCCCCCGTCCAAAAAAA 357
vishnui              TCCGATGTCGCGGTGC-----CG--CGTCCGC--CCACCATATGAACCGGATAAACACTC 316
tritaeniorhynchus    TCCGATGTCG--CGGT-----GGGGCGTCCGC--CCACCACATGAAAAGGCTAAACACTC 344
mimeticus            TACTCGCGCGGGGGGG-----GCGACCAGCACCTCCGCGGCGCGCGACGAGATGAGACAT 355
Anopheles            GCTCGGGTCGAGTAACACTTGCGGCCCAACGCGCCCGTCAC-CATCTGCTCGCCATTCTC 409
                             **                   *     **                *

bitaeniorhynchus     AGAAATATTTCTCAAAAAAAGGGGGACACA------------CCCCCCAATAAAAAATAA 363
quinquefasciatus     AAAACCACCCCACAAACAAGGAGTGGGATAAAAAAAAAAAAACCCCCACGCCTCCCATAA 417
vishnui              CCATGTAGGCCTCAAATAATGTGTGAC-TAC-----------CCCCTGAAATTTAAGCAT 364
tritaeniorhynchus    CCATGTAGGCCATCAAATAAGTGTGGCAG------------------------------ 373
mimeticus            CACACCCCACACCACCCCGGGGGGGGCGCGGAGGGAAAACCCAGCCCTCATCAGCCGTGG 415
Anopheles            TCAAGTAGGCGTCAAGTGATGTGTGACGACC-----------CCCTGAAATTTAAAGCAT 458
                                         * * *

bitaeniorhynchus     AGGGGTTGTGTCCTGCACTT-CACAAAA-------------------------------- 390
quinquefasciatus     AGAGTGGGTGCCCCCCCCCT-TTAATTATAAATAACCTGAAAATAAAAAAA--------- 467
vishnui              AAGTCATGTGTCTTGCAGTT-CACAAA--------------------------------- 390
tritaeniorhynchus    ------------------------------------------------------------
mimeticus            CGGAGTGACGTGCACGACTTGTGAAATGCAGTGAACATTAGCGTGCCACAAAGCGTGATG 475
Anopheles            ------------------------------------------------------------

bitaeniorhynchus     --
quinquefasciatus     --
vishnui              --
tritaeniorhynchus    --
mimeticus            TG 477
Anopheles            --
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Fig. 2 — Multiple sequence alignment of ITS2 sequence in five species of genus Culex (* Identical bases, - Insertion/ Deletion (indels).

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bitaeniorhynchus     AGAAATATTTCTCAAAAAAAGGGGGACACA------------CCCCCCAATAAAAAATAA
quinquefasciatus     AAAACCACCCCACAAACAAGGAGTGGGATAAAAAAAAAAAAACCCCCACGCCTCCCATAA
vishnui              CCATGTAGGCCTCAAATAATGTGTGAC-TAC-----------CCCCTGAAATTTAAGCAT
tritaeniorhynchus    CCATGTAGGCCATCAAATAAGTGTGGCAG-------------------------------
mimeticus            CACACCCCACACCACCCCGGGGGGGGCGCGGAGGGAAAACCCAGCCCTCATCAGCCGTGG
Anopheles            TCAAGTAGGCGTCAAGTGATGTGTGACGACC-----------CCCTGAAATTTAAAGCAT
                                               * * *
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Fig. 3 — Repeats of adenine (**AAA**…) in ITS2 sequence of *Cx. quinquefasciatus.*

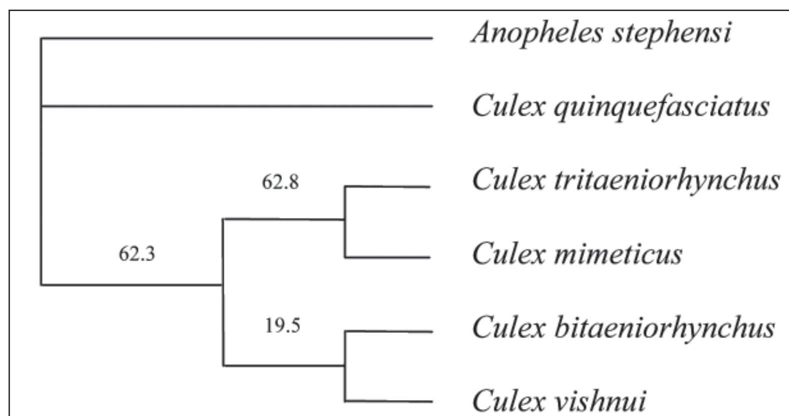

Fig. 4 — Maximum Parsimony based tree showing phylogenetic relatedness among five *Culex* species and *Anopheles stephensi* outgroup.

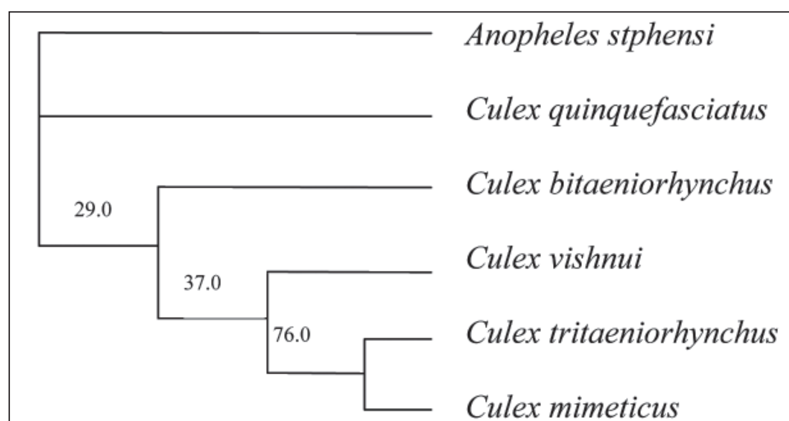

Fig. 5 — Distance Matrix based tree showing phylogenetic relatedness among five *Culex* species and *Anopheles stephensi* outgroup.

showed a close phylogenetic evolution between *An. stephensi* and *Cx. quinquefasciatus* with 100% bootstrap value. Strong bootstrap values generated a clade for *Cx. mimeticus* and *Cx. tritaeniorhynchus* while *Cx. vishnui* and *Cx. bitaeniorhynchus* fell under a different clade due to bootstrap value of 64% (Fig. 6).

On the basis of the ITS sequences, MARRELLI *et al.* (2005) found *Cx. quinquefasciatus* and *Cx. pipiens* to be polyphyletic. They also found that populations located in or near the zone of hybridization contained some individuals which were morphologically *Cx. pipiens* but had genotype characters of *Cx. quinquefasciatus*. According to the earlier studies of SIRIVANAKARN (1970, 1975), CHAUDHRY (1981) and SAGANDEEP *et al.* (1994) it has been established that the *Culex vishnui* complex is a group of subspecific variants which are similar in the adults and male genitalia but are chiefly characterized by the differences in the larval stages. CHAUDHRY (1981) made a beginning in the study of genomic studies of *Cx. vishnui* by providing a much needed larval salivary polytene chromosome map. As per its present status *Cx. vishnui* is comprised of five sibling species *viz: vishnui, pseudovishnui, perplexus, annulus, aliens* and *tritaeniorhynchus*. Out of these, *vishnui, pseudovishnui* and *tritaeniorhynchus* are more common and can be segregated on the basis of their morphotaxonomic characters while the remaining two species in this group are apparently rare and seldom encountered in the field collec-

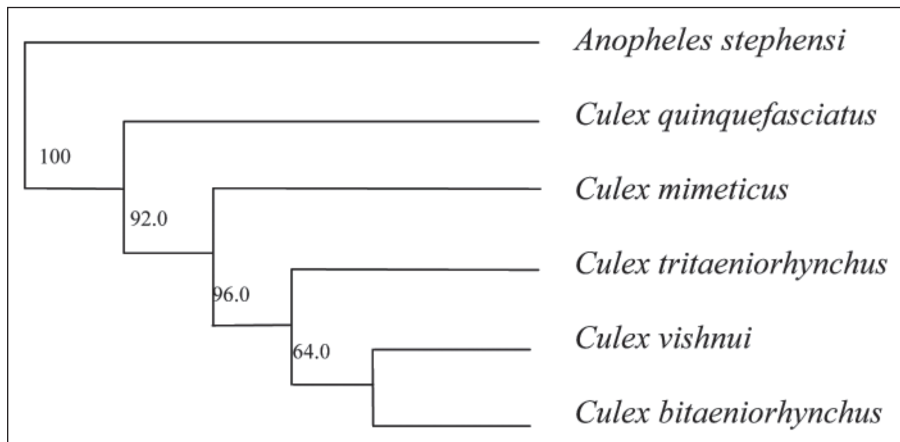

Fig. 6 — Maximum Likelihood based tree showing phylogenetic relatedness among five *Culex* species and *Anopheles stephensi* outgroup.

tions. Within this complex *tritaeniorhynchus* and its infraspecific form *summorosus* are currently treated as a single variable species as both show slight differences in the male phallosomes (SAGANDEEP *et al.* 1994). Recently, CHAUDHRY and KOHLI (2007) have extended their studies to the sequence analysis of mitochondrial 16S ribosomal RNA gene fragment of two populations of *Culex quinquefasciatus* from North-west India with some valuable comments on the sequence characteristics of this important taxon.

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