Low prevalence of natural *Wolbachia* in major malarial vectors *Anopheles culicifacies* s.l., and *Anopheles stephensi*: a first report

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Abstract

Wolbachia is an alpha-proteobacteria present in several arthropods. The present study focussed on the identification of *Wolbachia* in *Anopheles culicifacies* s.l., and *Anopheles stephensi*, which are wild malarial vector mosquitoes in India. A total of four hundred and eighty seven adult female mosquito samples were collected using taxonomic keys and confirmed by molecular analysis where 48.25% was *Anopheles culicifacies* s.l., and 51.75% was *Anopheles stephensi*. The presence of *Wolbachia* was identified using *16S* rRNA, *wsp* and *FtsZ* genes, where nested PCR of *16S* rRNA alone was successful and sequenced. Only seven mosquitoes (1.4%) were positive for *Wolbachia*. *In silico* and restriction digestion of *16S* rRNA gene product using RsaI enzyme showed that the identified *Wolbachia* belongs to supergroup B, which is further supported by phylogenetic analysis. Low prevalence rate of natural *Wolbachia* was observed in *An. culicifacies* s.l. (1.7%) and *An. stephensi* (1.2%). This is the first report on the presence of *Wolbachia* in *Anopheles culicifacies* s.l. and *Anopheles stephensi*.

Keywords: Anopheles culicifacies s.l., *Anopheles stephensi, Wolbachia,* malarial vector, *16S* rRNA, endosymbiont.

Introduction

Wolbachia is an intracellular alpha-proteobacteria found in a wide range of arthropods. It was first discovered by Hertig and Wolbach in 1924 considered to be the abundant endosymbiont found in invertebrates [1] and cause reproductive abnormalities (cytoplasmic incompatibility, male killing, parthenogenesis and feminization) in the host [2,3]. This endosymbiotic proteobacteria naturally infect 65% of insect species [2,4,5] including the family *Culicidae* [6,7]. So far *Wolbachia* wildtype is reported in the following mosquito species: *Culex* (*Cx.*) *pipiens* [1], *Cx. quinquefasciatus* [8], *Aedes* (*Ae.*) *albopictus* [9,10], *Ae. aegypti* [11] and few *Anopheles* species [12,13].

The natural occurrence of *Wolbachia* in *Anopheles* species has not been extensively studied. The *Anopheles* genera of *Culicidae* consists of 537 species [14], where 41 species are dominant vector species (DVS) responsible for the transmission of malaria [15]. Among the 41 DVS, 19 species/species complex were found within Asian-Pacific region [16]. As per WHO, 228 million malaria cases occurred worldwide in 2018; where India is one among the twenty countries that carries 85% of the global malarial burden [17]. In India, *An. baimaii*, *An. fluviatilis*, *An. minimus*, *An. sundaicus*, *An. culicifacies* complex species (A, B, C, D & E) and *An. stephensi* are primary vectors in transmitting malaria, with *An. culicifacies* complex and *An. stephensi* as major contributors [18]. *An. culicifaciess. s.l.* is widely distributed in rural, semi-urban and forest areas [18-20] and, *An. stephensi* in peri-, semi- and urban areas [18,19].

In Tamil Nadu, *An. stephensi* [21-23] and *An. culicifacies* [22,24,25] are the main malaria vectors. Interestingly, few reports on natural *Wolbachia* endosymbionts in major malarial vector *Anopheles* species are reported including *An. gambiae*, *An. coluzzii* [12,13], *An. arabiensis* [26] and *An. moucheti* [27,28]. The present study aimed at the investigation of *Wolbachia* infection in wild mosquito population collected from different geographical locations in Tamilnadu.

Materials and Methods

Mosquito collection and taxonomy

Adult female mosquito samples were collected using nets and aspirators, from the 5 different locations along the foothills of the Western Ghats, Southern India (Fig.1) and identified

initially by taxonomic keys [29,30] and later verified by DNA barcoding. During collection, the mosquitoes bearing ectoparasites mites has been discarded and not been used in any experiments. In the field, each samples was washed twice in 95% ethanol to avoid external contamination [31], and transferred in vial containing 95% ethanol for preservation [32] and brought to the lab for further experiments.

DNA extraction and species identification

DNA isolation from individual mosquito was carried out using the Qiagen Blood and Tissue kit (Qiagen) with slight modification. The initial lysis step post homogenization in PBS was carried-out with proteinase K and lysis buffer at 56°C for 3 hr. Genomic DNA extracted was subjected to *COI* (cytochrome C oxidase subunit I) gene amplification using primers reported by Folmer and colleagues [33] for identification of the mosquito species.

***Wolbachia* detection by PCR**

For *Wolbachia* detection, three different sets of primers targeting conserved genes namely *16S* rRNA gene [34], *Wolbachia* surface protein (*wsp*) gene [35] and *FtsZ* cell cycle gene [36] were used for screening. *Cx. quinquefasciatus* been used as positive control. In addition, for low infection detection, a nested PCR using internal primers targeting 412 bp of *16S* rRNA gene was used [37]. Multilocus strain typing (MLST) of *Wolbachia* was done by targeting five conserved genes *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA* as described earlier [38]. The primer details are given in Table S1.

Molecular phylogenetic studies

The *Wolbachia* positive samples were Sanger sequenced (Barcode Biosciences Pvt. Ltd, Bangalore). The sequencing was carried out from the PCR products of *16S* rRNA (nested PCR) and *COI* from *Wolbachia* and *Anopheles* respectively. The contig assembly was done using MEGA7. The assembled sequences were submitted to GenBank, ENA Database and accession numbers were obtained. Additional mosquito and *Wolbachia* sp. sequences were collected from GenBank to clarify the interspecies relationship. The phylogenetic tree was constructed by maximum likelihood (ML) analysis in MEGA7 software. The tree inference options were set as follows: Heuristic Method Nearest-Neighbor-Interchange (NNI) with the very strong branch

swap filter with 1000 bootstrap replicates, gaps were treated as missing. The annotation of phylogenetic tree was performed using iTOL [39]. The number of restriction sites for RsaI was studied using multiple sequence alignment (MSA, Clustal Omega) to find out the supergroup of *Wolbachia* strains reported from this study.

Results:

Mosquito samples were collected from five different study sites **(**Fig.1**)**. Four hundred and eighty-seven adult female mosquito samples belong to the genus *Anopheles* was screened for the presence of *Wolbachia*. The overall study shows *An. stephensi* (51.75%) population was higher in comparison to *An. culicifacies*. The population of *An. stephensi* was higher in Srivilliputtur (70%), while *An. culicifacies* was higher in Cumbum (69.79%) (Table 1).

To identify the presence of *Wolbachia* endosymbiont in mosquito, the genes *wsp* and *FtsZ* genes (Table S1) were amplified; however, no positive results were obtained. MLST by standard primers and protocols did not yield any positive results in all the samples tested (data not shown). Interestingly, nested *16S* PCR amplification targeting the inner region of the *16S* rDNA gene results in positive outcome indicating the presence of *Wolbachia* endosymbiont in *Anopheles* mosquitoes. Out of 487 samples only seven samples i.e., 1.4% were positive for *Wolbachia* endosymbiont, where 3 are from *An. culicifacies* (MN268747, MN268748, MN268749) and 4 from *An. stephensi* (MN268743, MN268744, MN268746 and MN268750) (Table 2). MSA of *Wolbachia* strains reported the presence of four restriction sites (GTAC) for RsaI in agarose gel electrophoresis (data not shown) and *in silico* (Fig.2), indicating the isolates belong to supergroup B.

The phylogenetic analysis was done using *Wolbachia* sequences belonging to known Supergroup A, B, C & D. The analysis showed all the *Wolbachia* strains reported from this study (Table S2) were grouped under Supergroup B and non-monophyletic (Fig.3). The *Wolbachia* isolates from this study showed 97.85% - 99.04% sequence similarity with the reference sequences from *Wolbachia* belonging to Supergroup B [CAGB01000162 (wAlbB, France), NC_010981 (wPip, Srilanka), NC021084 (wNo, Sweden), NZ_CP021120 (wMeg, Brazil), NZ_CP034334 (wMau, USA) and NZ_CP034335 (wMau, USA); Table S4]. The genetic

diversity within group of Supergroup B (0.2033±0.2139) was higher than supergroup A (0.0012±0.0010) (Table S5). The diversity between supergroup B and other Supergroup A, C & D was highly divergent that ranges from 0.132629±0.1318681 to 0.1225491±0.1292306 (Table S5a).

Based on the divergence, Supergroup B has been subdivided into 6 clades (Fig.3). The isolates from *An. culicifacies* (TS1 & TS2) and *An. stephensi* (AE4, AE5 & AE6), formed a separate clade (Clade I) within supergroup B with the reference strains reported from *Culex quinquefascitus* (NC010981, Srilanka) and *Chrysomya megacephala* (NZ_CP021120, Brazil) (Fig.3). The isolates also shares 98-99% sequence similarity with the two reference sequences (Table S4) and less diverse (0.0018±0.0013; Table S6). The sequence of TS3 is closely related to sequence of *Wolbachia* from *An. minimus* (MT449019; Thailand) with less divergence (0.0014±0.0025) and distinct from other sequence represented as Clade II (Fig.3; Table S6). The sequence of TS4 in Clade VI is closely related with *Wolbachia* sequence reported from *An. gambiae* (KJ728741, Burkina Faso) (Fig.3) with 98.71% sequence similarity and, less diverse (0.0007±0.0010) within Clade VI (Table S6).

All the seven *Wolbachia* positive mosquitoes were amplified using insect *cytochrome C oxidase I* (*COI*) gene (Table S3) and confirmed at species level. The phylogenetic analysis shows there were two clades *An. stephensi* and *An. culicifacies* and, both were supported by 99% bootstrap value (Fig.4). Among the seven *Wolbachia* positive samples, four belongs to *An. stephensi* (LR736010, LR736012, LR736013 and LR736014) and three belongs to *An. culicifacies* s.l. (LR736007 - LR736009) (Table 2). *An. stephensi* showed highest similarity with KX467337, MH538704, KF406680 and NC_028223 (Fig.4) with a high in-group diversity of 0.052±0.01 (Table S7). Interestingly, LR736012 has shown to be divergent from other *An. stephensi* reported. *An. culicifacies* showed high similarity with other *An. culicifacies* strains (KR732656, KJ010898, DQ424962, KJ010896) and supported with 92% bootstrap value (Fig.4) and has low diversity (0.035±0.007) within the analyzed group (Table S7). The diversity between *An. stephensi* and *An. culicifacies* was high (0.16±0.03, Table S8).

Discussion:

The prevalence of *Wolbachia* has been reported in several arthropods including the orders *Coleoptera*, *Diptera*, *Hemiptera*, *Homoptera*, *Hymenoptera*, *Lepidoptera* and *Orthoptera* [5,7]. Among the *Diptera*, *Wolbachia* is been reported in several *Culicidae* including *Aedes*, *Culex* and *Coquillettidia* [7,40]. Novel *Wolbachia* infection in *Anopheles* species was least reported [7,40,41]. In this study, we report the occurrence of natural *Wolbachia* endosymbiont in wild *An. stephensi* and *An. culicifacies* for the first time.

Diversity of isolated *Wolbachia*:

Wolbachia was identified using *16S* rRNA gene [42,43]; however fine-scale phylogeny was not possible due to low evolutionary divergence of *16S* rRNA, thus *FtsZ* [44] and *wsp* [35] genes were used. Several studies reported amplification with *FtsZ* and *wsp* was unsuccessful [12,45,46] and, our results are similar. Nested PCR amplification of the inner region of *16S* rRNA was used in case of low-intensity *Wolbachia* infection [37] and proved to beneficial in identifying *Wolbachia* infection in *Anopheles* mosquitoes [26,47,37,45]; similarly in our study we detected *Wolbachia* from *An. stephensi* and *An. culicifacies*.

Baldo and colleagues [38] proposed genotyping *Wolbachia* using MLST (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*). In some cases, MLST in *Wolbachia* were not successful [38] possibly due to primer sequence divergence [13] and low infection densities [27]. Bleidorn and Gerth [48] have pointed out MLST loci are not suitable markers to study either genome-wide divergence rate or strain identification. However in our study, MLST amplification was unsuccessful and, it might be due to low infection density or primer sequence divergence or both.

Werren and colleagues [44] have reported *16S* rRNA can be used to distinguish between A and B supergroup due to the absence of RsaI restriction site in *Wolbachia* belonging to group A. Interestingly, Pourali and colleagues [49] have shown the presence of RsaI restriction site in supergroup A; however they have shown there was more RsaI site in B than A. We have performed *in silico* RsaI restriction site search in *16S* rRNA gene on reported *Wolbachia* supergroup A (EU096232, NC_002978.6, NC_012416, KP089991), supergroup B (NC_021984, CAGB01000162, MH967031) and our isolates; supergroup A has two restriction sites for all strains except KP089991 (*Wolbachia* from *An. coluzzii* [50]) that has three restriction sites,

which is possible due to recombination; our isolates and supergroup B shows four restriction sites (Fig.2).

The *16S* rDNA phylogeny shows all the *Wolbachia* isolates from this study belongs to supergroup B and it's non-monophyletic. Similar to our observation, recent reports [13,51,45] show supergroup B is polyphyletic. The genetic diversity in the clades of supergroup B was less with the excemption of clade III that composed of *Wolbachia* sequence from *Cx. quinquefasciatus* (Fig.3; Table S6). This shows that the nucleotide substitution or recombination was less in *16S* rRNA gene of *Wolbachia* from *Anopheles* mosquitoes.

Diversity of *Anopheles* mosquitoes and *Wolbachia* prevalence:

The genus *Anopheles* belongs to the family *Culicidae* that comprises of 465 species further divided into seven subgenera. *Anopheles* is one among the subgenera consists of 182 species [14,52]. The molecular phylogeny of *Anopheles* was limited to lower level classification respective to malarial vector and, morphologically defined groups found to be monophyletic [52]. Similarly in our study, *An. culicifacies* and *An. stephensi* was identified morphologically; later by *COI* gene amplification and, observed to be monophyletic within their respective species.

To till date, the prevalence of *Wolbachia* has been reported in 20 species of wild *Anopheles* mosquitoes. Baldini and colleagues [12] for the first time reported *Wolbachia* in wild *An. gambiae* and *An. coluzzii* from Burkina Faso. Later *Wolbachia* was reported in other wild *Anopheles* mosquitoes including *An. gambiae* (different from Burkina Faso [13]), *An. arabiensis* [26,27], *An. demeilloni* (previously *An*. species X) [27], *An. moucheti* [27,28], *An. funestus* [47], *An. melas* [53], *An. nili*, *An. coustani* [28], *An. maculatus* (*s.s.*), *An. sawadwongporni*, *An. pseudowillmori*, *An. dirus* (*s.s.*), *An. baimaii* [51], *An. carnevalei*, *An. hancocki*, *An. implexus*, *An. jebudensis*, *An. marshallii*, *An. minimus*, *An. nigeriensis*, *An. paludis*, *An. vinckei* [28], *An. balabacensis*, *An. latens*, *An. introlatus*, *An. macarthuri*, *An. barbirostris*, *An. hyrcanus* and *An. sinensis* [45] with our report on additional two species of *Anopheles*, totalling 32 species.

The natural prevalence rate is nil or low in *Anopheles* in comparison to other mosquitoes [41,45,54]. However within *Anopheles* species, the natural *Wolbachia* infection is variable. *An. arabiensis* [26,27], *An. coluzzii* [27,28], *An. funestus* [28,47], *An. melas* [53], *An. maculatus* (*s.s.*), *An. sawadwongporni*, *An. pseudowillmori*, *An. dirus* (*s.s.*), *An. baimaii* [51], *An. balabacensis*, *An. introlatus*, *An. macarthuri* [45] shows lesser natural prevalence rate (see Table S9). Higher prevalence rate has been reported from an *An. demeilloni* [27,55,56], *An. moucheti* [27,28], *An. latens*, *An. hyrcanus* and *An. barbirostris* [45] (see Table S9). *An. gambiae* shows variable prevalence rate [13,27,50] (see Table S9) and, possibly spatial population dynamics [50] may play a role in it. We have lesser prevalence of *Wolbachia* in *An. stephensi* (1.19%) and *An. culicifacies* (1.70%) in comparison to all other reported species.

The less prevalence rate of *Wolbachia* in *Anopheles* mosquitoes has raised several questions. Maternal transmission of *Wolbachia* was observed in natural population of *An. gambiae*, *An. coluzzii* and *An. arabiensis* [12,13,50,37]. Experimental evidence points out horizontal transfer of *Wolbachia* in *An. gambiae* [57-59], *An. stephensi* [58,60,61] and *An. coluzzii* [37] is possible; however some results in transient infection as in *An. gambiae* [59] than permanent (maternal transmission) as seen in *An. stephensi* [61,60]. Evidence suggests that native microbiome in *Anopheles* mosquitoes impedes the vertical transmission of *Wolbachia* [27,58,62]. *Asaia*, an acetic acid bacterium inhibits *Wolbachia* maternal transmission [58], mutual exclusion of *Wolbachia* in gonads [63] and co-infection of *Asaia* and *Wolbachia* has also been reported [55]. Interestingly, we have also checked for *Asaia* infection using PCR (Table S1) in *Anopheles* mosquitoes including *Wolbachia* positive; but found none (data not shown). *Variovorax*, a beta-proteobacteria has been observed in *Wolbachia* negative *An. coluzzii* [62], which warrants further research as a competitor to *Wolbachia*. The different *Wolbachia* strains can also differ in their interaction with the host [57] probably a reason for lesser prevalence rate. Taken together, prevalence of *Wolbachia* in *Anopheles* mosquito might be subjected to 1) native microbiota interference and 2) *Wolbachia*-host interaction. Since we studied on *Anopheles*, it is possible that native microbiota could interfere in *Wolbachia* colonizing this species leading to lesser prevalence rate; further research will be carried out in the future.

Lateral gene transfer (LGT) of *Wolbachia* genome was observed in *Callosobruchus chinensis* [64,65], *Onchocerca volvulus* [66] and, *D. ananassae* [67]. Incase of mosquitoes, LGT has been observed in *Ae. aegypti*, *Ae. mascarensis* [68] and *An. gambiae* [69]. Salivary gland surface (*SGS*) genes from *An. gambiae* and *Ae. aegypti* genome is said to be transferred from *Wolbachia* via LGT [69], where these genes are particularly found in female salivary gland; it's expression increase with age, after blood feeding and, facilitates *Plasmodium* sporozoite invasion [70,69]. However, there is no study on SGS relationship with *Wolbachia* infection. Endogenous bornavirus-like nucleoprotein, a functional protein homologous to Borna virus *nucleoprotein* integrated into *Ictidomys tridecemlineatus* ~8.5 MYA [71] inhibits exogenous Borna virus *in vivo* [72]. Similarly we hypothesis, *SGS* gene might interfere with *Wolbachia* infection in *Anopheles* mosquitoes leads to less prevalence rate which needs further research.

Is *Wolbachia* a contaminant in *Anopheles* mosquitoes?

According to Chrostek and Gerth [73], *Wolbachia* in *An. gambiae* could be from contamination through several sources – 1) *Wolbachia* present in ectoparasitic mites or midges and endoparasitic nematodes, 2) *Wolbachia* contamination from plants, where *Wolbachia* infested insects feed on the plants might transfer to uninfected insects reared on the same plants and 3) cohabitating insect with *Wolbachia* contaminating the water bodies, thus infecting other mosquito species. However, recent report evidently shows *Wolbachia* as an endosymbiont in *Anopheles* species – *An. gambiae, An. melas* [55], *An. moucheti* and *An. demeilloni* [56].

In our case, we would like to clarify the *Wolbachia* is from *Anopheles* mosquitoes and not from other sources. 1) Only adult female *An. culicifacies* and *An. stephansi* with no ectoparasitic mites or midges were collected from the study sites. In case of contaminant from filarial nematode, the *Wolbachia* should be from other Supergroup such as C and D. According to Ayala and colleagues [28], *Wolbachia* strains from *An. coustani* belongs to Supergroup B & C; further investigation shows *Wolbachia* belonging to Supergroup C (MK755772) is from nematode *Dirofilaria immitis* infected *An. coustani*. Our observation on the sequence (MK755772) shows the same that it belongs to Supergroup C with a 94% bootstrap (see Fig.3). This indicates *Wolbachia* from filarial parasites is distinct and belong to Supergroup C/D [74-76] and can be used as an indicator of *Wolbachia* from filarial endoparasites. In our study,

Wolbachia are from Supergroup B and none from Supergroup C/D (see Fig.3), indicating all the *Wolbachia* strains are from *Anopheles* mosquitoes than from filarial nematodes. Thus it eliminates the possibility of *Wolbachia* from ecto- and endo-parasites. 2) Adult female *An. culicifacies* and *An. stephensi* usually feed on blood meal [77,78]; the collected mosquitoes were surface sterilized and preserved in 95% ethanol and, no laboratory breeding was performed indicating *Wolbachia* is not from infected plants or other environmental contaminants. 3) The majority of co-habitating mosquito with *An. stephensi* and *An. culicifacies* is *Cx. quinquefaciatus* (personal communication). Few studies from India reported *Wolbachia* in *Cx. quinquefaciatus* [79-81]. We have included *Cx. quinquefaciatus* as positive control in our PCR experiments and were positive for *Wolbachia* (data not shown). When the *Wolbachia* sequences of *Cx. quequefasciatus* (AF397409, AF397410) from India was phylogenetically analyzed it forms a distinctive separate clade and not closer to our isolates (Fig.3). Even if *Wolbachia* is from *Cx. quinquefasciatus*, it can only consider as infection rather than 'contamination'; the infection might happened at the larval stages and the co-existence in the adult stage (our isolates are from adult female mosquitoes) should be consider as symbiotic relationship. In future, studies on cohabitation and *Wolbachia* transmission will be carried. Based on the above facts, we confirm *Wolbachia* in our study is from *An. stephensi* and *An. culicifacies* and not from ecto- or endo-parasites or other sources.

Chrostek and Gerth [73] pointed out that true symbiosis has to be established by demonstrating intercellular bacterial cells and intraovarian transmission. To till date, the available techniques are limited and thus addressing the above said factors in 'wild mosquitoes' is quite a challenge, but the future might hold a better way to prove the above said factors in elucidating true symbiosis of *Wolbachia* in wild mosquitoes.

Conclusion:

The current study has shown *Wolbachia* for the first time in *Anopheles* mosquitoes namely *An. culicifacies* and *An. stephensi* from Tamil Nadu, India. Nested *16S* rRNA PCR amplification is helpful in identification than *wsp*, *FtsZ* and MLST loci genes. The prevalence is lesser compared to other mosquitoes, which may be due to inhibition by native microbiota, host and *Wolbachia* interaction, and/or inhibition by endogenous gene product that result from LGT; these factors

will be analyzed in our future research. Future studies will also include the different organs of the mosquito showing *Wolbachia* by FISH method.

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Declarations:

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Author contribution

Concept, study design and methodology: SGS and APA. Performed lab experiments: TM and APA. Phylogenetic design and analysis: TM, SGS and APA. Pre-draft written: TM, SGS and APA. Critical revision of final version: SGS and APA. All authors read and approved the final manuscript.

Conflicts of interest/competing interests

The authors declare that they have no conflicts of interest/competing interests.

Availability of data and materials

The data are provided in the supplementary section and the sequence data from this study is available in GenBank.

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Figure legends:

Fig.1: Study site from Tamil Nadu

The map shows the study site at 5 different locations along the foothills of the Western Ghats, Southern India. The five locations were Coimbatore, Pollachi, Palani, Cumbum, Sirvilliputhur and Tenkasi. *An. stephensi* and *An. culicifacies* was populated at all the sites (red circle). The presence of *Wolbachia* was marked in yellow star for *An. stephensi* and green diamond for *An. culicifacies*.

Fig.2: Multiple sequence alignment of *Wolbachia 16S* rRNA sequence

The *Wolbachia 16S* rRNA sequence was aligned using Clustal O at EMBL-EBI server and edited in JalView. The restriction site of RsaI was highlighted in black. The sequences used are known *Wolbachia* supergroup A (EU096232, NC_002978.6, NC_012416, KP089991), supergroup B (NC_021984, CAGB01000162, MH967031), outgroup *Ricketssia* (CP003319, NR_074459.2) and our isolates. The restriction sites show our isolates belong to supergroup B with four restriction sites. Supergroup A consists of two restriction sites and, *Ricketssia* consists of a single restriction site.

Fig.3: Molecular phylogenetic analysis of *Wolbachia*

The phylogeny was inferred from the nucleotide dataset of *16S* rRNA gene by using the ML method. The sequences from this study were represented in red font. The tree with the highest log likelihood (-1044.31) is shown. The analysis involved 102 nucleotide sequences. There were a total of 339 positions in the final dataset. Scale bar 0.02 represents nucleotides substitution per position. The annotation was carried out using iTOL online software.

Fig.4: Molecular phylogenetic analysis of *Anopheles* mosquitoes

The phylogeny was inferred from the nucleotide dataset of *COI* gene by using the ML method. The sequences from this study were represented as green diamond. The tree with the highest log likelihood (-1429.44) is shown. The analysis involved 19 nucleotide sequences. There were a total of 396 positions in the final dataset. Scale bar 0.05 represents nucleotides substitution per position.

Table 1: Collection of mosquito samples from different locations

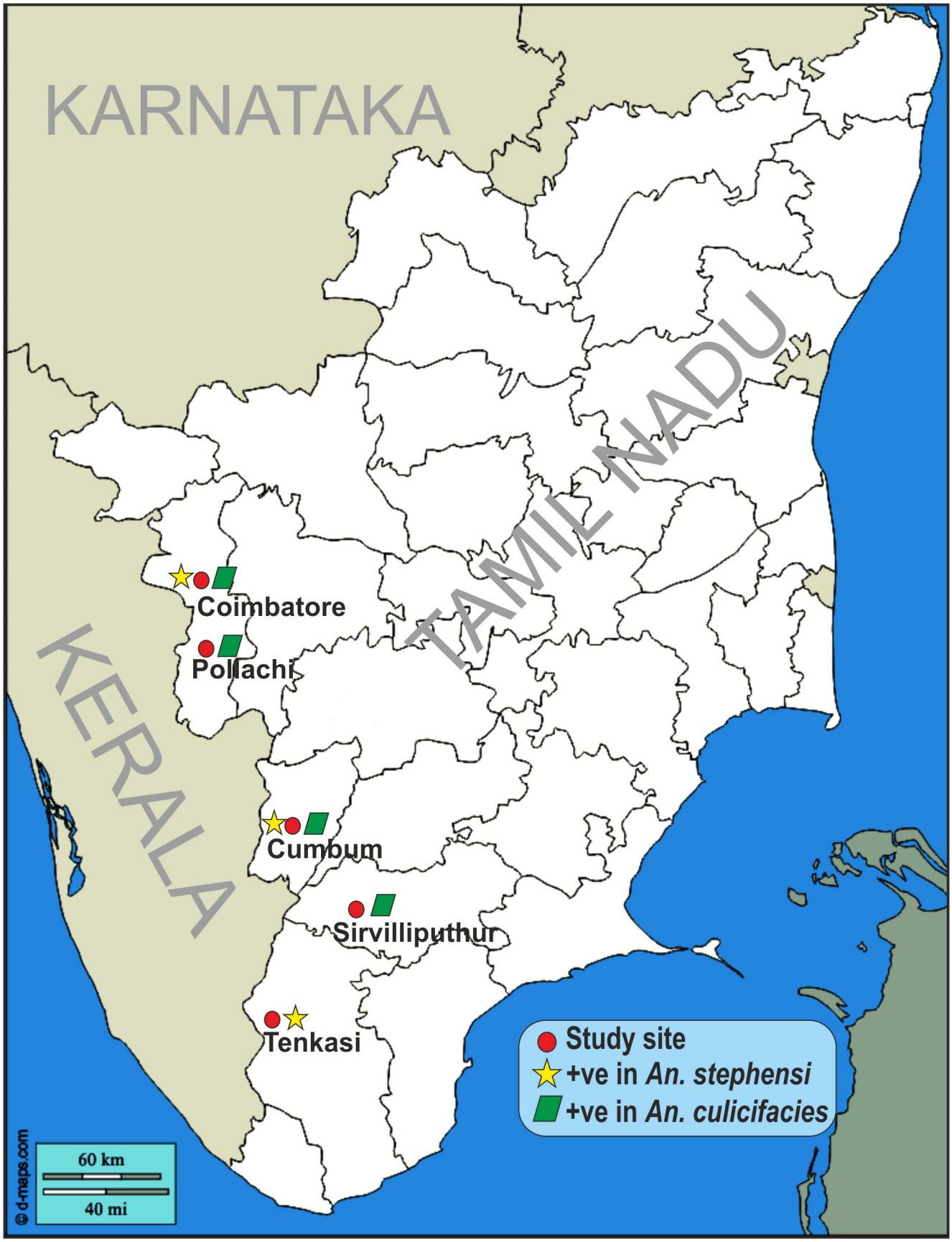
Place	***An. culicifacies***		***An. stephensi***	
	Collected (%*)	***Wolbachia* positive (%#)**	**Collected (%*)**	***Wolbachia* positive (%#)**
Coimabtore	38 (45.24)	1 (2.63)	46 (54.76)	1 (2.17)
Pollachi	48 (38.71)	1 (2.08)	76 (61.29)	0
Cumbum	67 (69.79)	1 (1.49)	29 (30.21)	1 (3.44)
Srivillputtur	21 (30.00)	1 (4.76)	49 (70.00)	0
Tenkasi	61 (53.98)	0	52 (46.02)	1 (1.92)
Total	235 (48.25)	4 (1.70)	252 (51.75)	3 (1.19)

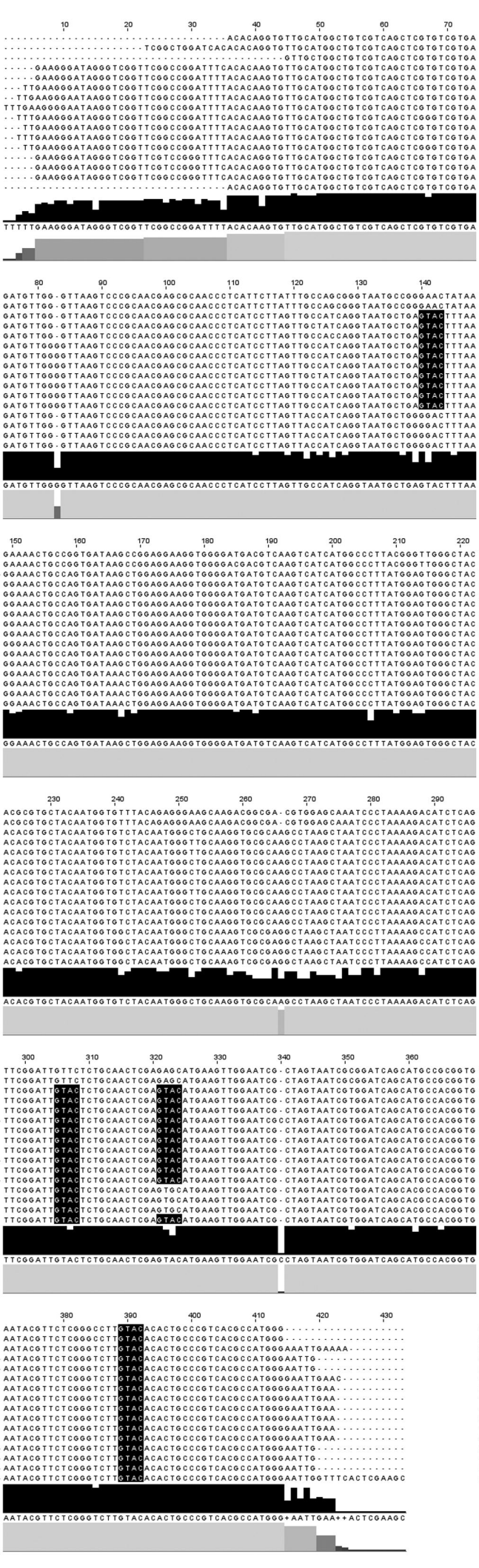
*Percentage from total mosquitoes collected

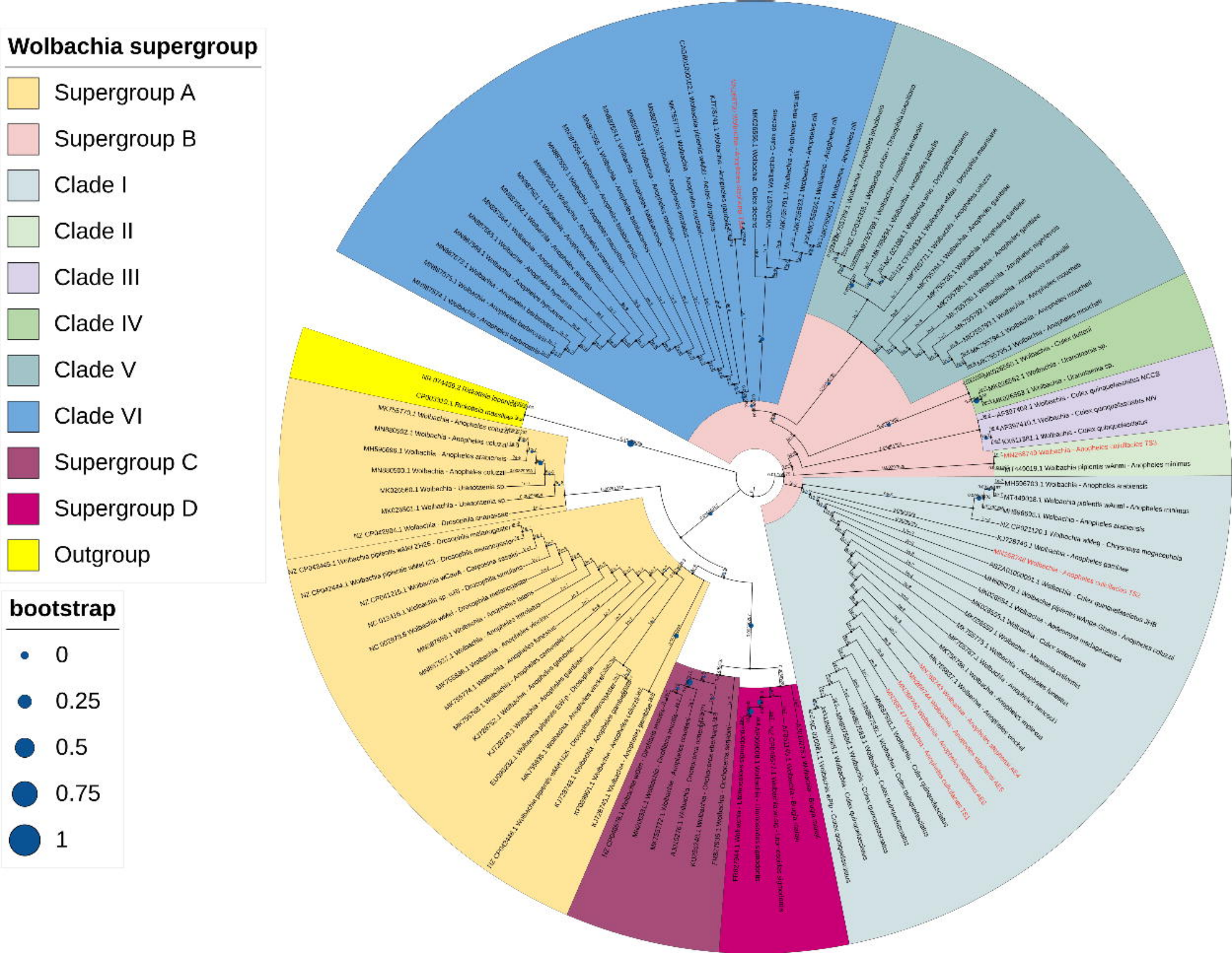
#Percentage of *Wolbachia* positive within species

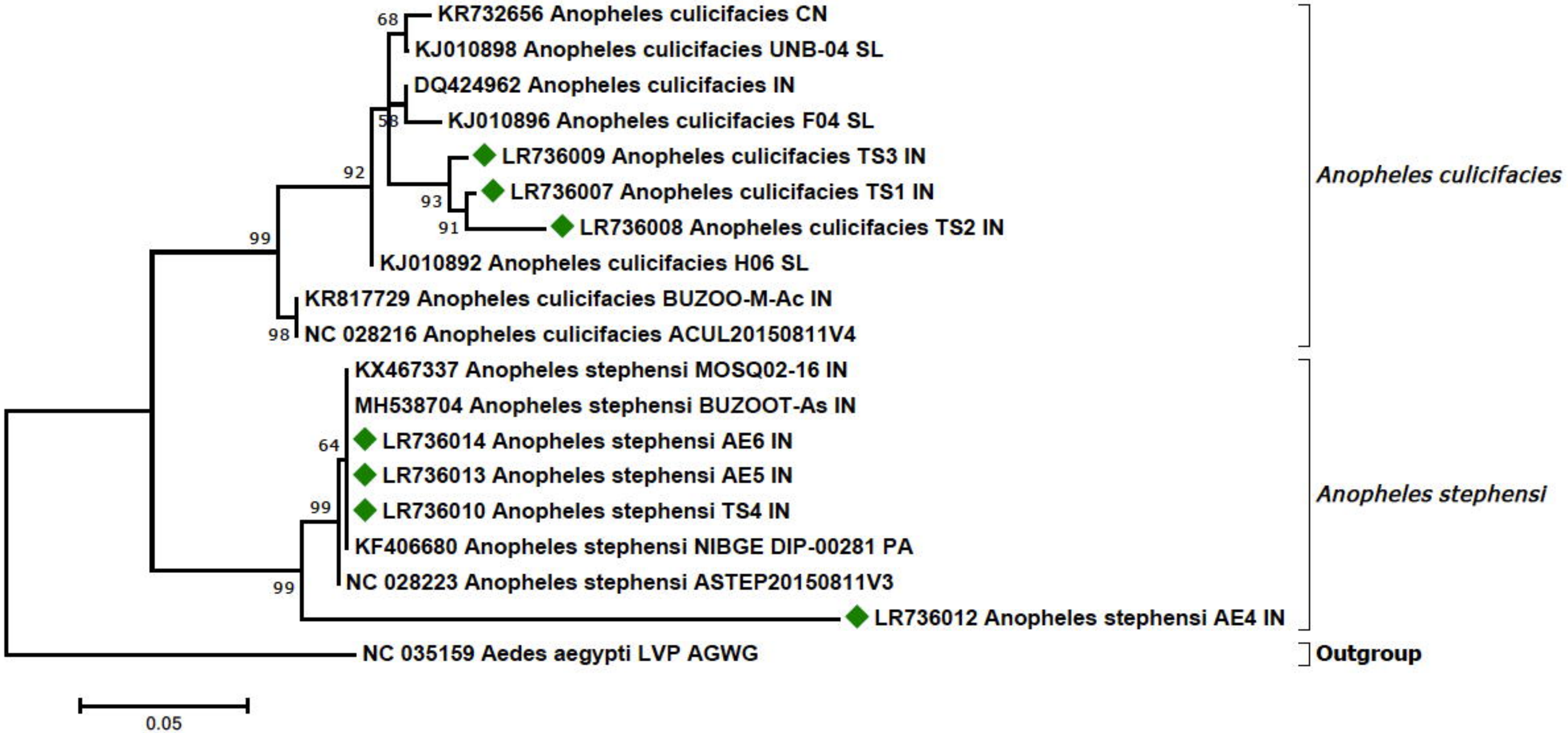
Table 2: The host and the *Wolbachia* endosymbiont isolate ID and sequence accession number

Host	**Isolate ID**	**Mosquito sequence Accession No.**	***Wolbachia* sequence Accession No.**
Anopheles culicifacies	TS1	LR736007	MN268747
Anopheles culicifacies	TS2	LR736008	MN268748
Anopheles culicifacies	TS3	LR736009	MN268749
Anopheles stephensi	TS4	LR736010	MN268750
Anopheles stephensi	AE4	LR736012	MN268743
Anopheles stephensi	AE5	LR736013	MN268744
Anopheles stephensi	AE6	LR736014	MN268746


KARNATAKA
TAMIL NADU
KERALA
Coimbatore
Pollachi
Cumbum
Sirvilliputhur
Tenkasi
Study site
+ve in *An. stephensi*
+ve in *An. culicifacies*
60 km
40 mi
© d-maps.com


CP003319.1_Rickettsia_massiliae/1-376
NR_074459.2_Rickettsia_japonica/1-389
MH596703.1_Wolbachia_-_Anopheles_arabiensis/1-378
CAGB01000162.1_Wolbachia_pipientis_wAlbB_-_Aedes_albopictus/1-412
NC_021084.1_Wolbachia_wNo_-_Drosophila_simulans/1-412
MN268747_Wolbachia_-_Anopheles_culicifacies_TS1/1-420
MN268746_Wolbachia_-_Anopheles_stephensi_AE6/1-419
MN268743_Wolbachia_-_Anopheles_stephensi_AE4/1-421
MN268750_Wolbachia_-_Anopheles_stephensi_TS4/1-419
MN268749_Wolbachia_-_Anopheles_culicifacies_TS3/1-418
MN268744_Wolbachia_-_Anopheles_stephensi_AE5/1-419
MN268748_Wolbachia_-_Anopheles_culicifacies_TS2/1-418
EU096232.1_Wolbachia_pipientis_EW-p_-_Drosophila/1-412
NC_002978.6_Wolbachia_wMel_-_Drosophila_melanogaster/1-412
NC_012416.1_Wolbachia_sp._wRi_-_Drosophila_simulans/1-412
KP089991.1_Wolbachia_-_Anopheles_coluzzii/1-396
Consensus
Occupancy


Wolbachia supergroup
Supergroup A
Supergroup B
Clade I
Clade II
Clade III
Clade IV
Clade V
Clade VI
Supergroup C
Supergroup D
Outgroup
bootstrap
0
0.25
0.5
0.75
1


KR732656 Anopheles culicifacies CN
KJ010898 Anopheles culicifacies UNB-04 SL
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KR817729 Anopheles culicifacies BUZOO-M-Ac IN
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68
58
92
93
91
99
98
64
99
99
Anopheles culicifacies
Anopheles stephensi
Outgroup
0.05