First report on the presence of natural Wolbachia population from major malarial vector mosquitoes Anopheles culicifacies s.l., and Anopheles stephensi from Tamil Nadu, India

S. Gowri Sankar1$, T. Mowna Sundari2,3$, A. Alwin Prem Anand2$*

1ICMR-Vector Control Research Centre - Field Station, Madurai - 625002, Tamil Nadu, India.
2DBT - BIF Centre (Under DBT BTISNet Scheme), Lady Doak College, Madurai - 625002, Tamil Nadu, India.
3Department of Biotechnology, Lady Doak College, Madurai - 625002, Tamil Nadu, India.

$Equally contributed

*Corresponding authors
A. Alwin Prem Anand
DBT - BIF Centre (Under DBT BTISNet Scheme)
Lady Doak College
Madurai - 625002
Tamil Nadu
India.
E-mail: alwinprem@gmail.com

T. Mowna Sundari
DBT - BIF Centre (Under DBT BTISNet Scheme)
Lady Doak College
Madurai - 625002
Tamil Nadu
India.
E-mail: mownasundari@ldc.edu.in
Abstract

Wolbachia is an alpha-proteobacteria present in several arthropods. The present study focussed on the identification of Wolbachia in wild malarial vector mosquitoes. This was achieved by molecular identification of Wolbachia from collected mosquitoes. A total of four hundred and eight seven mosquito samples were collected. Morphometric and molecular analysis revealed that they belong to Anopheles culicifacies s.l., (48.25%) and Anopheles stephensi (51.75%). The presence of Wolbachia was identified using 16S rRNA, wsp and FtsZ genes, where nested PCR of 16S rRNA alone was successful and then 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. The prevalence rate of natural Wolbachia was lesser in native malarial vector An. culicifacies s.l. and An. stephensi was about 1.7% and 1.2%, respectively. 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 (Hertig & Wolbach 1924) and cause reproductive abnormalities (cytoplasmic incompatibility, male killing, parthenogenesis and feminization) in the host (Werren 1997, Werren et al 2008). This endosymbiotic proteobacteria naturally infect 65% of insect species (Hilgenboecker et al 2008, Werren 1997, Werren et al 1995a) including the family Culicidae (de Oliveira et al 2015, Sicard et al 2019). So far Wolbachia wildtype is reported in the following mosquito species: Culex (Cx.) pipiens (Hertig & Wolbach 1924), Cx. quinquefasciatus (Mahilum et al 2003) Aedes (Ae.) albopictus (Dutton & Sinkins 2004, Sinkins et al 1995), Ae. aegypti (Coon et al 2016) and few Anopheles species (Baldini et al 2014, Gomes et al 2017).

The natural occurrence of Wolbachia in Anopheles species has not been extensively studied. The Anopheles genera of Culicidae consists of 537 species (Harbach 2013), where 41 species are dominant vector species (DVS) responsible for the transmission of malaria (Hay et al 2010). Among the 41 DVS, 19 species/species complex were found within Asian-Pacific region (Sinka et al 2011). 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 (WHO 2019). 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 (Subbaarao et al 2019). An. culicifacies s.s.l. is widely distributed in rural, semi-urban and forest areas (Dev & Sharma 2013, Goswami et al 2006, Subbarao et al 2019) and, An. stephensi in peri-, semi- and urban areas (Dev & Sharma 2013, Subbarao et al 2019).

In Tamil Nadu, An. stephensi (Arjunan et al 2015, Sharma & Hamzakoya 2001, Surendran et al 2019) and An. culicifacies (Arjunan et al 2015, Kar et al 1999, Suguna et al 1983) 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 (Baldini et al 2014, Gomes et al 2017), An. arabiensis (Baldini et al 2018) and An. moucheti (Ayala et al 2019, Jeffries et al 2018). The present study aimed at the investigation of
*Wolbachia* infection in wild mosquitoes population collected from different geographical locations in Tamilnadu.

**Materials and Methods**

**Mosquito collection and taxonomy**

Mosquito samples were collected from the 5 different locations along the foothills of the Western Ghats, Southern India (Fig.1). Adults were collected using nets and aspirators. Mosquitoes were identified initially by taxonomic keys (Christophers 1933, Das et al 1990) and later verified by DNA barcoding.

**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 (1994) 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 (Werren & Windsor 2000), *Wolbachia* surface protein (*wsp*) gene (Zhou et al 1998) and *FtsZ* cell cycle gene (Werren & Jaenike 1995) were used for screening. In addition, for low infection detection, a nested PCR using internal primers targeting 412 bp of *16S* rRNA gene was used (Shaw et al 2016). Multilocus strain typing (MLST) of *Wolbachia* was done by targeting five conserved genes *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA* as described earlier (Baldo et al 2006). The primer details are given in Supplementary Table 1.

**Molecular phylogenetic studies**

The *Wolbachia* positive samples were further sequenced. The sequencing was carried out from the PCR products of *16S* rRNA (nested PCR) and *COI* from *Wolbachia* and *Anopheles* respectively and, Sanger sequenced (Barcode Biosciences Pvt. Ltd, Bangalore). 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 number of restriction sites for RsaI was studied using multiple sequence alignment (MSA, Clustal Omega) to find out the supergroups of *Wolbachia* strains reported from this study.

**Results:**

Mosquito samples were collected from five different study sites (Fig.1). Four hundred and eighty-seven 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 (Supplementary Table 1) 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 showed that all the *Wolbachia* strains reported from this study (Table 3) were grouped under Supergroup B and non-monophyletic (Fig.3). The *Wolbachia* isolates reported from this study showed high sequence similarity within (99.05% to 99.76%)
and also with the reference sequences used in the study. The isolate TS3 (MN268749) formed a separate clade (Clade I) within supergroup B with three other strains reported from *Drosophila simulans* (NC021084.1) and *Drosophila mauritiana* (NZ_CP034334 and NZ_CP034335) supported with 65% bootstrap value (Fig.3). It also shares 98.55% sequence similarity with all the three reference sequences (Supplementary Table 2) and diverse (0.005±0.005; Supplementary Table 3). The sequence of TS4 forms a distinct clade (Clade II) supported by 64% bootstrap value with *Wolbachia* sequence reported from *Aedes albopictus* (CAGB01000162) and *Culex decens* (MK026556 and MK026557) (Fig.3) and, share 99.04% and 98.99% sequence similarity respectively (Supplementary Table 2). No genetic divergence was observed in Clade II (Supplementary Table 3). Rest of the isolates forms a separate clade (Clade III, Fig.3). They shared high similarity ranging from 99.28-98.57%. *Wolbachia* sequence from *Chrysomya megacephala* (NZ_CP021120.1) (Supplementary Table 2) and were less diverse (0.003±0.002) compared to within group Clade I (Supplementary Table 3). The genetic diversity within group of supergroup B was less (0.005±0.003) and there was no significant diversity observed within supergroup A (Supplementary Table 3). The genetic diversity between supergroup A and supergroup B was 0.02±0.01 (Supplementary Table 4a).

All the seven *Wolbachia* positive mosquitoes were amplified using insect *cytochrome C oxidase I* (COI) gene (Table 4) 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 (Supplementary Table 5). 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 (Supplementary Table 5). The diversity between *An. stephensi* and *An. culicifacies* was high (0.16±0.03, Supplementary Table 6).
Discussion:

The prevalence of Wolbachia has been reported in several arthropods including the orders Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera and Orthoptera (de Oliveira et al 2015, Werren et al 1995a). Among the Diptera, Wolbachia is been reported in several Culicidae including Aedes, Culex and Coquillettidia (de Oliveira et al 2015, Ricci et al 2002). Novel Wolbachia infection in Anopheles species was least reported (de Oliveira et al 2015, Kittayapong et al 2000, Ricci et al 2002). 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 (O'Neill et al 1992, Rousset et al 1992); however fine-scale phylogeny was not possible due to low evolutionary divergence of 16S rRNA, thus FtsZ (Werren et al 1995b) and wsp (Zhou et al 1998) genes were used. Several studies reported that FtsZ and wsp was unsuccessful (Baldini et al 2014, Marcon et al 2011, Wong et al 2020) and, our results are similar where amplification using wsp and FtsZ primers yielded no positive results. Nested PCR amplification of the inner region of 16S rRNA was used in case of low-intensity Wolbachia infection (Shaw et al 2016) and proved to beneficial in identifying Wolbachia infection in Anopheles mosquitoes (Baldini et al 2018, Niang et al 2018, Shaw et al 2016, Wong et al 2020); similarly in our study we detected Wolbachia from An. stephensi and An. culicifacies.

Baldo and colleagues (2006) proposed genotyping Wolbachia using MLST (gatB, coxA, hcpA, ftsZ and fbpA). In some cases, MLST in Wolbachia were not successful (Baldo et al 2006) possibly due to primer sequence divergence (Gomes et al 2017) and low infection densities (Jeffries et al 2018). Bleidorn and Gerth (2018) 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 (1995b) 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 (2009) 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 (Buck et al 2016)) 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 (Gomes et al 2017, Sawasdichai et al 2019, Wong et al 2020) show supergroup B is polyphyletic. Though our samples are in separate clades, the genetic diversity within the group (Supplementary Table 3) and clades of supergroup B was less (Supplementary Table 4b). This shows that the nucleotide substitution or recombination was minimum in 16S rRNA gene.

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 (Harbach 2004, Harbach 2013). The molecular phylogeny of Anopheles was limited to lower level classification respective to malarial vector and, morphologically defined groups found to be monophyletic (Harbach 2004). Similarly in our study, An. culicifacies and An. stephensi was first identified morphologically; later identified 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 (2014) 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 (Gomes et al 2017)), An. arabiensis (Baldini et al 2018), An. moucheti, (Ayala et al 2019, Jeffries et al 2018), An. funestus (Niang et al 2018), An. melas (Jeffries et al 2019), An. nili, An. coustani (Ayala et al
2019), An. maculatus (s.s.), An. sawadwongporni, An. pseudowillmori, An. dirus (s.s.), An. baimaii (Sawasdichai et al 2019), An. balabacensis, An. latens, An. introlatus, An. macarthuri, An. barbirostris, An. hyrcanus and An. sinensis (Wong et al 2020) with our report on additional two species of Anopheles, totalling 22 species.

The natural prevalence rate is nil or low in Anopheles in comparison to other mosquitoes (Kittayapong et al 2000, Rasgon & Scott 2004, Wong et al 2020). Within Anopheles species, the natural Wolbachia infection is variable. An. arabiensis (Baldini et al 2018, Jeffries et al 2018), An. coluzzii (Jeffries et al 2018), An. funestus (Niang et al 2018), An. melas (Jeffries et al 2019), An. maculatus (s.s.), An. sawadwongporni, An. pseudowillmori, An. dirus (s.s.), An. baimaii (Sawasdichai et al 2019), An. balabacensis, An. introlatus, An. macarthuri (Wong et al 2020) shows lesser natural prevalence rate. Higher prevalence rate has been reported from an unknown Anopheles species from Sub-Saharan Africa (Jeffries et al 2018), An. moucheti (Ayala et al 2019, Jeffries et al 2018), An. latens, An. hyrcanus and An. barbirostris (Wong et al 2020). An. gambiae shows variable prevalence rate (Buck et al 2016, Gomes et al 2017, Jeffries et al 2018) and, possibly spatial population dynamics (Buck et al 2016) 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 (Baldini et al 2014, Buck et al 2016, Gomes et al 2017, Shaw et al 2016). Experimental evidence points out horizontal transfer of Wolbachia in An. gambiae (Hughes et al 2014, Hughes et al 2011, Hughes et al 2012), An. stephensi (Bian et al 2013, Hughes et al 2014, Joshi et al 2017) and An. coluzzii (Shaw et al 2016) is possible; however some results in transient infection as in An. gambiae (Hughes et al 2011) than permanent (maternal transmission) as seen in An. stephensi (Bian et al 2013, Joshi et al 2017). Evidence suggests that native microbiome in Anopheles mosquitoes impedes the vertical transmission of Wolbachia (Hughes et al 2014, Jeffries et al 2018, Straub et al 2020). Asaia, an acetic acid bacterium inhibits Wolbachia maternal transmission (Hughes et al 2014). Variovorax, a beta-proteobacteria has been observed in Wolbachia negative An. coluzzii (Straub et al 2020),
which warrants further research as a competitor to Wolbachia. The different Wolbachia strains can also differ in their interaction with the host (Hughes et al 2012) 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 (Kondo et al 2002, Nikoh et al 2008), Onchocerca volvulus (Fenn et al 2006) and, D. ananassae (Hotopp et al 2007). Incase of mosquitoes, LGT has been observed in Ae. aegypti, Ae. mascarensis (Klasson et al 2009) and An. gambiae (Korochkina et al 2006). Salivary gland surface (SGS) genes from An. gambiae and Ae. aegypti genome is said to be transferred from Wolbachia via LGT (Korochkina et al 2006), where these genes are particularly found in female salivary gland; it’s expression increase with age, after blood feeding and, facilitates Plasmodium sporozoite invasion (King et al 2011, Korochkina et al 2006). 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 (Suzuki et al 2014) inhibits exogenous Borna virus in vivo (Fujino et al 2014). Similarly we hypothesis, SGS gene might interfere with Wolbachia infection in Anopheles mosquitoes leads to less prevalence rate which needs further research.

Chrostek and Gerth (2019) 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.

Acknowledgements:
SGS would like to acknowledge DST-SERB (YSS/2015/001847) and DHR-HRD (DHR/HRD/YS-14-2015-16) fellowships. The authors acknowledge DBT-BIF Centre, Lady Doak College for providing bioinformatics facility for data analysis.

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.

Reference:
Arjunan NK, Kadarkarai M, Kumar S, Pari M, Thiyagarajan N, et al. 2015. Factors influencing the spatial distribution of Anopheles larvae in Coimbatore District, Tamil Nadu, India. Acta tropica 152: 121-30
Ayala D, Akone-Ella O, Rahola N, Kengne P, Ngangue MF, et al. 2019. Natural Wolbachia infections are common in the major malaria vectors in Central Africa. Evolutionary Applications 12: 1583-94
Baldini F, Rouge J, Kreppel K, Mkandawile G, Mapua SA, et al. 2018. First report of natural Wolbachia infection in the malaria mosquito Anopheles arabiensis in Tanzania. Parasites & vectors 11: 635
Baldini F, Segata N, Pompon J, Marcenac P, Shaw WR, et al. 2014. Evidence of natural Wolbachia infections in field populations of Anopheles gambiae. Nature communications 5: 3985
Baldo L, Dunning Hotopp JC, Jolley KA, Bordenstein SR, Biber SA, et al. 2006. Multilocus sequence typing system for the endosymbiont Wolbachia pipientis. Applied and environmental microbiology 72: 7098-110
Bian G, Joshi D, Dong Y, Lu P, Zhou G, et al. 2013. Wolbachia invades Anopheles stephensi populations and induces refractoriness to Plasmodium infection. Science 340: 748-51
Bleidorn C, Gerth M. 2018. A critical re-evaluation of multilocus sequence typing (MLST) efforts in Wolbachia. FEMS Microbiol Ecol 94
Buck M, Nilsson LK, Brunius C, Dabiré RK, Hopkins R, Terenius O. 2016. Bacterial associations reveal spatial population dynamics in Anopheles gambiae mosquitoes. Sci Rep 6: 22806
Christophers SR. 1933. The fauna of British India, including Ceylon and Burma. Diptera. Vol. IV. Family Culicidae. Tribe Anophelini. London: Taylor and Francis. vi [vii], 317pp. + 3 pls pp.
Chrostek E, Gerth M. 2019. Is Anopheles gambiae a Natural Host of Wolbachia? mBio 10
Coon KL, Brown MR, Strand MR. 2016. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Molecular ecology 25: 5806-26
Das BP, Rajagopal R, Akiyama J. 1990. Pictorial key to the species of Indian anopheline mosquitoes. Journal of Pure and Applied Zoology 2: 131-62
de Oliveira CD, Goncalves DS, Baton LA, Shimabukuro PH, Carvalho FD, Moreira LA. 2015. Broader prevalence of Wolbachia in insects including potential human disease vectors. Bulletin of entomological research 105: 305-15
Dev V, Sharma VP. 2013. The dominant mosquito vectors of human malaria in India In Anopheles mosquitoes - New insights into malaria vectors, ed. S Manguin: IntechOpen
Dutton TJ, Sinkins SP. 2004. Strain-specific quantification of Wolbachia density in Aedes albopictus and effects of larval rearing conditions. Insect molecular biology 13: 317-22
Fenn K, Conlon C, Jones M, Quail MA, Holroyd NE, et al. 2006. Phylogenetic Relationships of the Wolbachia of Nematodes and Arthropods. PLoS pathogens 2: e94
Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular marine biology and biotechnology 3: 294-9
Fujino K, Horie M, Honda T, Merriman DK, Tomonaga K. 2014. Inhibition of Borna disease virus replication by an endogenous bornavirus-like element in the ground squirrel genome. Proceedings of the National Academy of Sciences 111: 13175-80
Gomes FM, Hixson BL, Tyner MDW, Ramirez JL, Canepa GE, et al. 2017. Effect of naturally occurring Wolbachia in Anopheles gambiae s.l. mosquitoes from Mali on Plasmodium falciparum malaria transmission. Proceedings of the National Academy of Sciences of the United States of America 114: 12566-71
Goswami G, Singh OP, Nanda N, Raghavendra K, Gakhar SK, Subbarao SK. 2006. Identification of all members of the anophelines culicifacies complex using allele-specific polymerase chain reaction assays. The American journal of tropical medicine and hygiene 75: 454-60
Harbach RE. 2004. The classification of genus Anopheles (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bulletin of entomological research 94: 537-53
Harbach RE. 2013. The Phylogeny and Classification of Anopheles In Anopheles mosquitoes - New insights into malaria vectors, ed. S Manguin: IntechOpen
Hay SI, Sinka ME, Okara RM, Kabaria CW, Mbithi PM, et al. 2010. Developing global maps of the dominant anophelines vectors of human malaria. PLoS medicine 7: e1000209
Hertig M, Wollbach SB. 1924. Studies on Rickettsia-Like Micro-Organisms in Insects. The Journal of medical research 44: 329-74
Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. 2008. How many species are infected with Wolbachia?—A statistical analysis of current data. FEMS microbiology letters 281: 215-20
Hotopp JCD, Clark ME, Oliveira DCSG, Foster JM, Fischer P, et al. 2007. Widespread Lateral Gene Transfer from Intracellular Bacteria to Multicellular Eukaryotes. Science 317: 1753-56
Hughes GL, Dodson BL, Johnson RM, Murdock CC, Tsujimoto H, et al. 2014. Native microbiome impedes vertical transmission of Wolbachia in Anopheles mosquitoes. Proceedings of the National Academy of Sciences of the United States of America 111: 12498-503
Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL. 2011. Wolbachia infections are virulent and inhibit the human malaria parasite Plasmodium falciparum in Anopheles gambiae. PLoS pathogens 7: e1002043
Hughes GL, Vega-Rodriguez J, Xue P, Rasgon JL. 2012. Wolbachia strain wAlbB enhances infection by the rodent malaria parasite Plasmodium berghei in Anopheles gambiae mosquitoes. *Applied and environmental microbiology* 78: 1491-5

Jeffries CL, Cansado-Utrilla C, Stica C, Walker T. 2019. High density Novel Wolbachia strains in Anopheles species from Guinea. *bioRxiv*: 772855

Jeffries CL, Lawrence GG, Golovko G, Kristan M, Orsborne J, et al. 2018. Novel Wolbachia strains in Anopheles malaria vectors from Sub-Saharan Africa. *Wellcome open research* 3: 113

Joshi D, Pan X, McFadden MJ, Bevins D, Liang X, et al. 2017. The maternally inheritable Wolbachia wAlbB induces refractoriness to Plasmodium berghei in Anopheles stephensi. *Frontiers in microbiology* 8

Jeffries CL, Lawrence GG, Golovko G, Kristan M, Orsborne J, et al. 2018. Novel Wolbachia strains in Anopheles malaria vectors from Sub-Saharan Africa. *Wellcome open research* 3: 113

Joshi D, Pan X, McFadden MJ, Bevins D, Liang X, et al. 2017. The maternally inheritable Wolbachia wAlbB induces refractoriness to Plasmodium berghei in Anopheles stephensi. *Frontiers in microbiology* 8

Kar I, Subbarao SK, Eapen A, Ravindran J, Satyanarayana TS, et al. 1999. Evidence for a new malaria vector species, species E, within the Anopheles culicifacies complex (Diptera: Culicidae). *Journal of medical entomology* 36: 595-600

King JG, Vernick KD, Hillyer JF. 2011. Members of the salivary gland surface protein (SGS) family are major immunogenic components of mosquito saliva. *The Journal of biological chemistry* 286: 40824-34

Kittayapong P, Baisley KJ, Baimai V, O'Neill SL. 2000. Distribution and diversity of Wolbachia infections in Southeast Asian mosquitoes (Diptera: Culicidae). *Journal of medical entomology* 37: 340-5

Klasson L, Kambris Z, Cook PE, Walker T, Sinkins SP. 2009. Horizontal gene transfer between Wolbachia and the mosquito Aedes aegypti. *BMC Genomics* 10: 33

Kondo N, Nikoh N, Ijichi N, Shimada M, Fukatsu T. 2002. Genome fragment of Wolbachia endosymbiont transferred to X chromosome of host insect. *Proceedings of the National Academy of Sciences of the United States of America* 99: 14280-5

Korochkina S, Barreau C, Pradel G, Jeffery E, Li J, et al. 2006. A mosquito-specific protein family includes candidate receptors for malaria sporozoite invasion of salivary glands. *Cellular Microbiology* 8: 163-75

Mahilum MM, Storch V, Becker N. 2003. Molecular and electron microscopic identification of Wolbachia in Culex pipiens complex populations from the Upper Rhine Valley, Germany, and Cebu City, Philippines. *Journal of the American Mosquito Control Association* 19: 206-10

Marcon HS, Coscrato VE, Selivon D, Perondini AL, Marino CL. 2011. Variations in the sensitivity of different primers for detecting Wolbachia in Anastrepha (diptera: tephritidae). *Brazilian journal of microbiology*: [publication of the Brazilian Society for Microbiology] 42: 778-85

Niang EHA, Bassene H, Makoundou P, Fenollar F, Weill M, Mediannikov O. 2018. First report of natural Wolbachia infection in wild Anopheles funestus population in Senegal. *Malaria journal* 17: 408

Nikoh N, Tanaka K, Shibata F, Kondo N, Hizume M, et al. 2008. Wolbachia genome integrated in an insect chromosome: Evolution and fate of laterally transferred endosymbiont genes. *Genome Research* 18: 272-80

O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences of the United States of America* 89: 2699-702

Pourali P, Roayaei Ardakani M, Jolodar A, Razi Jalali MH. 2009. PCR screening of the Wolbachia in some arthropods and nematodes in Khuzestan province. *Iranian Journal of Veterinary Research* 10: 216-22

Rasgon JL, Scott TW. 2004. An initial survey for Wolbachia (Rickettsiales: Rickettsiaceae) infections in selected California mosquitoes (Diptera: Culicidae). *Journal of medical entomology* 41: 255-7

Ricci I, Cancrini G, Gabrielli S, D'Amelio S, Favi G. 2002. Searching for Wolbachia (Rickettsiales: Rickettsiaceae) in mosquitoes (Diptera: Culicidae): large polymerase chain reaction survey and new identifications. *Journal of medical entomology* 39: 562-7
Rousset F, Vautrin D, Solignac M. 1992. Molecular identification of Wolbachia, the agent of cytoplasmic incompatibility in Drosophila simulans, and variability in relation with host mitochondrial types. *Proceedings. Biological sciences* 247: 163-8

Sawasdichai S, Chaumeau V, Dah T, Kulakkee T, Kaarechiwa L, et al. 2019. Detection of diverse Wolbachia 16S rRNA sequences at low titers from malaria vectors in Kayin state, Myanmar. *Welcome open research* 4: 11

Sharma SK, Hamzakoya KK. 2001. Geographical Spread of Anopheles stephensi Vector of Urban Malaria, and Aedes aegypti, Vector of Dengue/DHF, in the Arabian Sea Islands of Lakshadweep, India. *Dengue Bulletin* 25: 88-91

Shaw WR, Marcenac P, Childs LM, Buckee CO, Baldini F, et al. 2016. Wolbachia infections in natural Anopheles populations affect egg laying and negatively correlate with Plasmodium development. *Nature communications* 7: 11772

Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, et al. 2011. The dominant Anopheles vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic precis. *Parasites & vectors* 4: 89

Sinkins SP, Braig HR, O'Neill SL. 1995. Wolbachia pipientis: bacterial density and unidirectional cytoplasmic incompatibility between infected populations of Aedes albopictus. *Experimental parasitology* 81: 284-91

Straub TJ, Shaw WR, Marcenac P, Sawadogo SP, Dabire RK, et al. 2020. The Anopheles coluzzi microbiome and its interaction with the intracellular parasite Wolbachia. *Sci Rep* 10: 13847

Subbarao SK, Nanda N, Rahi M, Raghavendra K. 2019. Biology and bionomics of malaria vectors in India: existing information and what more needs to be known for strategizing elimination of malaria. *Malaria journal* 18: 396

Suguna SG, Tewar SC, Mani TR, Hiriyan J, Reuben R. 1983. Anopheles culicifacies species complex in Thenpennaiyar riverine tract, Tamil Nadu. *The Indian journal of medical research* 77: 455-9

Surendran SN, Sivabalakrishnan K, Sivasingham A, Jayadas TTP, Karvannan K, et al. 2019. Anthropogenic factors driving recent range expansion of the malaria vector Anopheles stephensi. *Frontiers in public health* 7: 53

Suzuki Y, Kobayashi Y, Horie M, Tomonaga K. 2014. Origin of an endogenous bornavirus-like nucleoprotein element in thirteen-lined ground squirrels. *Genes & genetic systems* 89: 143-8

Werren JH. 1997. Biology of Wolbachia. *Annual review of entomology* 42: 587-609

Werren JH, Baldo L, Clark ME. 2008. Wolbachia: master manipulators of invertebrate biology. *Nature reviews. Microbiology* 6: 741-51

Werren JH, Jaenike J. 1995. Wolbachia and cytoplasmic incompatibility in mycophagous Drosophila and their relatives. *Heredity* 75 (Pt 3): 320-6

Werren JH, Windsor D, Guo LR. 1995a. Distribution of *Wolbachia* among neotropical arthropods. *Proc Royal Soc Lond B* 262: 197-204

Werren JH, Windsor DM. 2000. Wolbachia infection frequencies in insects: evidence of a global equilibrium? *Proceedings. Biological sciences* 267: 1277-85

WHO. 2019. *World malaria report 2019*. pp. 232. Geneva: World Health Organization.

WHO. 2019. *World malaria report 2019*. pp. 232. Geneva: World Health Organization.

Wong ML, Liew JWK, Wong WK, Pramasivan S, Mohamed Hassan N, et al. 2020. Natural Wolbachia infection in field-collected Anopheles and other mosquito species from Malaysia. *Parasites & vectors* 13: 414
Zhou W, Rousset F, O'Neil S. 1998. Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. Proceedings. Biological sciences 265: 509-15
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 Rsal 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 (-290.81) is shown. The analysis involved 44 nucleotide sequences. There were a total of 120 positions in the final dataset. Scale bar 0.02 represents nucleotides substitution per position.

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                         |
Table 3: Sequences used for Wolbachia phylogeny

| S. No | Accession No  | Source organism | Isolate /Strain Id | Used sequence       | Year of isolation | Country         |
|-------|---------------|-----------------|-------------------|---------------------|-------------------|-----------------|
| 1.    | CAGB01000162  | *Aedes albopictus* | wAlbB          | Whole genome        | 2011              | France          |
| 2.    | CP003319      | *Rickettsia massiliae* | AZT80      | Complete genome     | 2012              | USA             |
| 3.    | EU096232      | *Drosophila*     | EW-p            | 16S rRNA partial sequence | 2007              | South Korea     |
| 4.    | KP089991      | *Anopheles coluzzii* | VK5_8.1.1  | 16S rRNA partial sequence | 2012              | Burkina Faso    |
| 5.    | MH596693      | *Anopheles arabiensis* | isolate 3   | 16S rRNA partial sequence | 2018              | Tanzania         |
| 6.    | MH596694      | *Anopheles arabiensis* | isolate 7   | 16S rRNA partial sequence | 2018              | Tanzania         |
| 7.    | MH596695      | *Anopheles arabiensis* | isolate 13  | 16S rRNA partial sequence | 2018              | Tanzania         |
| 8.    | MH596696      | *Anopheles arabiensis* | isolate 15  | 16S rRNA partial sequence | 2018              | Tanzania         |
| 9.    | MH596697      | *Anopheles arabiensis* | isolate 19  | 16S rRNA partial sequence | 2018              | Tanzania         |
| 10.   | MH596698      | *Anopheles arabiensis* | isolate 40  | 16S rRNA partial sequence | 2018              | Tanzania         |
| 11.   | MH596699      | *Anopheles arabiensis* | isolate 55  | 16S rRNA partial sequence | 2018              | Tanzania         |
| 12.   | MH596700      | *Anopheles arabiensis* | isolate 63  | 16S rRNA partial sequence | 2018              | Tanzania         |
| 13.   | MH596701      | *Anopheles arabiensis* | isolate 137 | 16S rRNA partial sequence | 2018              | Tanzania         |
| 14.   | MH596702      | *Anopheles arabiensis* | isolate 138 | 16S rRNA partial sequence | 2018              | Tanzania         |
| 15.   | MH596703      | *Anopheles arabiensis* | isolate 140 | 16S rRNA partial sequence | 2018              | Tanzania         |
| 16.   | MK026554      | *Aedoeomyia madagascarica* | TSA-AMAD-1 | 16S rRNA partial sequence | 2016              | Madagascar       |
| 17.   | MK026555      | *Culex antennatus* | TSA-CANT-1      | 16S rRNA partial sequence | 2016              | Madagascar       |
| 18.   | MK026556      | *Culex decens*   | TSA-CDEC-1      | 16S rRNA partial sequence | 2016              | Madagascar       |
| 19.   | MK026557      | *Culex decens*   | TSA-CDEC-2      | 16S rRNA partial sequence | 2016              | Madagascar       |
| 20.   | MK026558      | *Culex duttoni*  | TSA-CDUT-1      | 16S rRNA partial sequence | 2016              | Madagascar       |
| 21.   | MK026559      | *Mansonia uniformis* | TSA-MUNI-1    | 16S rRNA partial sequence | 2016              | Madagascar       |
| 22.   | MK026560      | *Uranotaenia sp.* |ANI-USP1-1   | 16S rRNA partial sequence | 2016              | Madagascar       |
| 23.   | MK026561      | *Uranotaenia sp.* | TSA-USP1-1     | 16S rRNA partial sequence | 2016              | Madagascar       |
| 24.   | MK026562      | *Uranotaenia sp.* | TSA-USP2-1     | 16S rRNA partial sequence | 2016              | Madagascar       |
| 25.   | MK026563      | *Uranotaenia sp.* | TSA-USP2-2     | 16S rRNA partial sequence | 2016              | Madagascar       |
| 26.   | MN268743      | *Anopheles stephensi* | AE4          | 16S rRNA partial sequence | 2017              | India*          |
| 27.   | MN268744      | *Anopheles stephensi* | AE5          | 16S rRNA partial sequence | 2017              | India*          |
|   | Accession Number | Species                  | Genome/Sequence Type                      | Year | Location   |
|---|-----------------|--------------------------|-------------------------------------------|------|------------|
|28.| MN268746        | Anopheles stephensi      | AE6 16S rRNA partial sequence             | 2017 | India*     |
|29.| MN268747        | Anopheles culicifacies   | TS1 16S rRNA partial sequence             | 2017 | India*     |
|30.| MN268748        | Anopheles culicifacies   | TS2 16S rRNA partial sequence             | 2017 | India*     |
|31.| MN268749        | Anopheles culicifacies   | TS3 16S rRNA partial sequence             | 2017 | India*     |
|32.| MN268750        | Anopheles stephensi      | TS4 16S rRNA partial sequence             | 2017 | India*     |
|33.| NC_002978       | Drosophila melanogaster  | wMel Complete genome                      | 2003 | USA        |
|34.| NC_012416       | Drosophila simulans      | wRi Complete genome                      | 2009 | Sweden     |
|35.| NC_021084       | Drosophila simulans      | wNo Complete genome                      | 2012 | Sweden     |
|36.| NR_074459       | Rickettsia japonica      | YH 16S rRNA partial sequence             | 2013 | USA        |
|37.| NZ_CP021120     | Chrysomyamegacephala     | wMeg Complete genome                      | 2015 | Brazil     |
|38.| NZ_CP034334     | Drosophila mauritiana    | wMau Complete genome                      | 2018 | USA        |
|39.| NZ_CP034335     | Drosophila mauritiana    | wMau Complete genome                      | 2018 | USA        |
|40.| NZ_CP041215     | Carposina sasakii        | wCauA Complete genome                     | 2017 | China      |
|41.| NZ_CP042444     | Drosophila melanogaster  | wMel_I23 Complete genome                  | 2019 | USA        |
|42.| NZ_CP042445     | Drosophila melanogaster  | wMel_ZH26 Complete genome                 | 2019 | USA        |
|43.| NZ_CP042446     | Drosophila melanogaster  | wMel_N25 Complete genome                  | 2019 | USA        |
|44.| NZ_CP042904     | Drosophila ananassae     | W2.1 Complete genome                      | 2019 | USA        |

*this study
Table 4: Sequences used for *Anopheles* mosquito phylogeny

| S.No. | Accession No. | Source organism | Isolate/Strain Id | Used sequence | Year of isolation | Country |
|-------|---------------|----------------|------------------|---------------|------------------|---------|
| 1.    | LR736007      | *Anopheles culicifacies* | TS1              | COI gene, partial cds; mitochondrial | 2017     | India*       |
| 2.    | LR736008      | *Anopheles culicifacies* | TS2              | COI gene, partial cds; mitochondrial | 2017     | India*       |
| 3.    | LR736009      | *Anopheles culicifacies* | TS3              | COI gene, partial cds; mitochondrial | 2017     | India*       |
| 4.    | LR736010      | *Anopheles stephensi*  | TS4              | COI gene, partial cds; mitochondrial | 2017     | India*       |
| 5.    | LR736012      | *Anopheles stephensi*  | AE4              | COI gene, partial cds; mitochondrial | 2017     | India*       |
| 6.    | LR736013      | *Anopheles stephensi*  | AE5              | COI gene, partial cds; mitochondrial | 2017     | India*       |
| 7.    | LR736014      | *Anopheles stephensi*  | AE6              | COI gene, partial cds; mitochondrial | 2017     | India*       |
| 8.    | NC_028223     | *Anopheles stephensi*  | ASTEP20150811V3  | Mitochondrion, complete genome | 2015     | USA          |
| 9.    | MH538704      | *Anopheles stephensi*  | voucher BUZOOT   | COI gene, partial cds; mitochondrial | 2018     | India        |
| 10.   | KX467337      | *Anopheles stephensi*  | voucher MOSQ02-16 | COX1 gene, partial cds; mitochondrial | 2018     | India        |
| 11.   | KF406680      | *Anopheles stephensi*  | voucher NIBGE DIP-00281 | COI gene, partial cds; mitochondrial | 2007     | Pakistan     |
| 12.   | NC_028216     | *Anopheles culicifacies* | voucher ACUL20150811V4 | Mitochondrion, complete genome | 2015     | USA          |
| 13.   | KR732656      | *Anopheles culicifacies* | isolate B        | Mitochondrion, complete genome | 2015     | China        |
| 14.   | KJ010898      | *Anopheles culicifacies* | voucher UNB-04   | COI gene, partial cds; mitochondrial | 2013     | UK           |
| 15.   | DQ424962      | *Anopheles culicifacies* | -                | COI gene, partial cds; mitochondrial | 2006     | India        |
| 16.   | KR817729      | *Anopheles culicifacies* | voucher BUZOO-M-Ac | COI gene, partial cds; mitochondrial | 2015     | India        |
| 17.   | KJ010896      | *Anopheles culicifacies* | voucher F04      | COI gene, partial cds; mitochondrial | 2013     | Srilanka     |
| 18.   | KJ010892      | *Anopheles culicifacies* | voucher H06      | COI gene, partial cds; mitochondrial | 2013     | Srilanka     |
| 19.   | NC_035159     | *Aedes aegypti*       | strain LVP_AGWG  | Mitochondrion, complete genome | 2017     | USA          |

*this study
