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# Molecular Detection and Characterization of *Wolbachia pipientis* from *Culex quinquefasciatus* Collected from Lahore, Pakistan

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*Abstract.* The gram-negative, pleomorphic endosymbiont *Wolbachia* is known to infect a large number of insects and other arthropods naturally. This bacterium modifies the host biology, mainly causing reproductive alterations including feminization, death of male, parthenogenesis, and importantly cytoplasmic incompatibility. *Wolbachia*-induced cytoplasmic incompatibility results in nonviable offspring and vector population suppression. In addition, this bacterium rapidly spreads and propagates within the host population. This study is the first report on *Wolbachia* detection and characterization from *Culex quinquefasciatus* collected from Lahore, Pakistan. For this purpose, mosquito adults were collected from different localities of Lahore and identified at the species level. A total of 145 pairs of ovaries were individually subjected to DNA isolation, and polymerase chain reaction amplification of three (*wsp*, 16S rRNA, and *ftsZ*) genes were investigated. In all, 128 females were found positive, representing 82.3% infection rate. The phylogenetic analysis indicated that the detected endosymbiont had 100% homology with *Wolbachia pipientis w*Pip strain and supergroup B. The detection of the local strain of *Wolbachia* (*w*Pip) will be useful in investigating its potential for the control of dengue vector (*Aedes aegypti*) and reducing dengue transmission in Pakistan.

## INTRODUCTION

*Wolbachia* is a gram-negative endosymbiotic bacterium, and it belongs to the phylum α-proteobacteria, order Rickettsiales,[1] and family Anaplasmataceae.[2] *Wolbachia* was first detected as *Rickettsia*-like organisms from the common house mosquito *Culex pipiens*.[3] Since then, *Wolbachia* has been reported in a large number of arthropods and filarial nematodes. It is abundantly found in more than 65% of insects in nature.[4,5] In *Cx. pipiens*, 76–98% of total microbiota comprises *Wolbachia*.[6]

*Wolbachia* is considered as a reproductive parasite and causes various reproductive modifications in its hosts including parthenogenesis, feminization of males, death of males, and cytoplasmic incompatibility.[7] Cytoplasmic incompatibility is one of the most important characteristics of *Wolbachia* that results in reduction of the viable progeny. Cytoplasmic incompatibility can be either unidirectional in which *Wolbachia*-infected sperm fertilizes uninfected egg,[8] or bidirectional where sperm and egg both are infected but with different strains of *Wolbachia*, resulting in failure of egg hatching and leading to suppression of host population.[9]

There is a large variety of *Wolbachia* strains present in arthropods. Multiple *Wolbachia* strains and various changes in *Wolbachia* sequences within the same host have been reported.[10] A huge variety of *Wolbachia* strains have been reported in the recent decades. *Wolbachia* has incorporated a wide variety of branches in phylogenetic hierarchy, and 14 supergroups have been described so far.[11] Supergroups A and B, being the most abundant, are found in insects.[9] Molecular approach targeting different genes for detection and identification of *Wolbachia* is considered as a reliable method. The most widely used detection method is carried out by amplifying specific regions of the *Wolbachia* surface protein (*wsp*) gene by polymerase chain reaction (PCR). Other different molecular markers include the 16S rRNA gene, *dnaA* gene which is crucial for the initiation of DNA replication process, the *ftsZ* (filamenting temperature sensitive) protein-encoding gene, the *groE* operon encoding two important heat shock proteins of bacteria, *hcpA*, *gltA*, *fbpA*, *gatB*, and *coxA*.[12]

Many classified strains of *Wolbachia* (*w*MelPop and *w*MelPop-CLA) are known to inhibit transmission of parasites or pathogens through infected mosquito vectors and to decrease the rate of disease transmission.[13,14] It has been suggested that introduction of specific *Wolbachia* strains in mosquito vectors and release of these infected vectors into a local population of vectors could prevent the transmission of arboviruses and other human parasites or pathogens such as dengue viruses and *Plasmodium*.[15,16] The use of *Wolbachia* as a biological tool for mosquito vector control, detection, and identification of its strains in local insects and other organisms is of prime importance. It is being well documented that *Wolbachia* is abundantly found in reproductive tissues of various insects. Objectives of the current study are the molecular detection of *Wolbachia* in the reproductive tissues of *Culex* mosquitoes collected from various sites of Lahore and the assessment of the phylogenetic status of detected *Wolbachia* on the basis of *wsp*, 16S rRNA, and *ftsZ* genes.

## METHODS

***Culex quinquefasciatus* collection and identification.** Surveillance of *Cx. quinquefasciatus* mosquitoes was conducted for a period of 4 months from July to October 2014. Adults were collected from four different (indoor and outdoor) locations (Cantonment, Katchery Road, Model Town, and Mughalpura) of Lahore, Pakistan (Figure 1), using a backpack aspirator (John W. Hock Company, Gainesville, FL). Geographical coordinates were collected using GPS Garmin GPSMAP®; 76CSx. The collected mosquito samples were reared in the insectary, GC University Lahore, Pakistan, under standard conditions (temperature: 27 ± 2°C, relative humidity: 80% ± 5%, and light dark cycle: 12:12 hours). The identification of adult *Culex* mosquitoes was carried out up to the species level using standard morphological keys.[17,18]

**Dissections and separation of ovaries.** The females were isolated from males by examining mouth parts, antennae,

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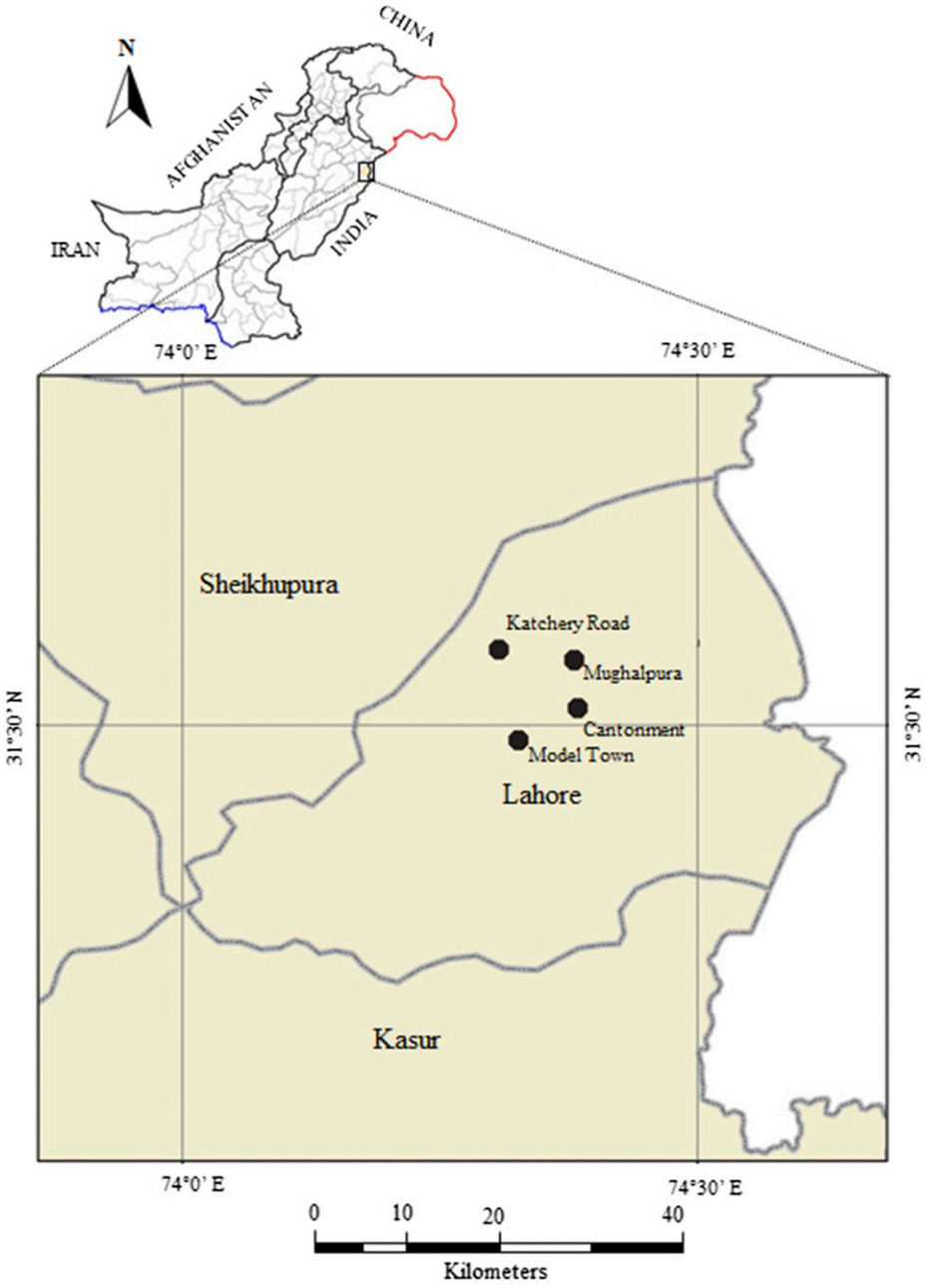


FIGURE 1. Map of Lahore, Punjab, Pakistan showing sampling sites of *Culex quinquefasciatus* for the screening of *Wolbachia*. This figure appears in color at www.ajtmh.org.

and the last portion of the abdomen. The selected females were used in the screening process and were paralyzed at 4°C for 5–10 minutes, using the freeze shock method. The mosquitoes were dipped 3× in sterile water, followed by rinsing in 70% ethanol for 5 minutes, and then transferred to successive sterile water baths 5× and once in sterilized 0.8% NaCl.[19] Each individual female was transferred to a clean glass slide containing a drop of phosphate buffer saline (PBS). The ovaries were dissected with the help of sterile dissecting needles under dissecting microscope. A single pair of ovaries was considered as one sample.

**PCR amplification of target genes.** Whole genomic DNA was extracted from each sample as described previously.[20] Exponential amplification of the extracted DNA (template) was carried out with a PCR thermal cycler (Techne Progene, Cambridge, United Kingdom) in a final volume of 50 μL containing 1× *Taq* buffer (without $MgCl_2$), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 mM each primer, 1 U *Taq* polymerase, and 2 μL whole genomic DNA. Genomic DNA from *Aedes aegypti* and *Aedes albopictus* was used as negative and positive control, respectively. The reagents were mixed in 0.5-mL PCR tubes placed on ice in a safety cabinet. The primer sequences targeting three genes (*wsp*, 16S rRNA, and *ftsZ*) along with the respective annealing temperature used in PCR are mentioned in Table 1. The following thermal profiles were used: initial denaturation at 94°C for 2 minutes followed by 35 cycles (denaturation at 94°C for 1 minute, annealing optimized at 51°C for 16S rRNA and *wsp*, and

TABLE 1
The details of gene primers used for the detection and characterization of *Wolbachia* from *Cx. quinquefasciatus* collected from Lahore Pakistan

| Primers pair 5′-3′ | Annealing | Target gene | Estimated product size | GenBank accession number |
|---|---|---|---|---|
| *wsp*_F AAGGAACCGAAGTTCATG *wsp*_R AAAAATTAAACGCTACTCCA | 55°C | *wsp* | 510 bp | KX650071 |
| 16S rRNA_F TTGTAGCCTGCTATGGTATAACT 16S rRNA _R GAATAGGTATGATTTTCATGT | 51°C | 16S rRNA | 890 bp | KX611381 |
| *ftsZ*_F TACTGACTGTTGGAGTTGTAACTAAGCCGT *ftsZ*_R TGCCAGTTGCAAGAACAGAAACTCTAACTC | 55°C | *ftsZ* | 570 bp | KY753917 |

55°C for *ftsZ* for 1 minute, and extension at 72°C for 1 minute), and final extension at 72°C for 10 minutes.

**Agarose gel electrophoresis.** The PCR products were subjected to 2% agarose gel electrophoresis using 0.5 µg/mL ethidium bromide and 1× TAE buffer. Four microliters of PCR products along with 2 µL of 3× loading dye (bromophenol blue) was loaded with 1-kb ladder as a standard to determine the size of the PCR products. The amplicons were visualized and photographed by using a UV transilluminator and BioDoc-It® TS imaging system (Ultra-Violet Products Limited, Cambridge, United Kingdom). The DNA samples were stored at –20°C until DNA sequencing.

**Sequencing of amplicons and data analysis.** The selected amplified PCR products (two samples from each locality) were purified using gel extraction method followed by DNA sequencing from First BASE Laboratories Sdn Bhd, Kembangan, Malaysia. Briefly, the PCR products underwent Sanger sequencing in both directions using their respective (forward and reverse) primers using a DNA sequencer (96-capillary 3730xl DNA Analyzer; Applied Biosystems, Foster City, CA) and an ABI BigDye Terminator v3.1 cycle sequencing kit.

The quality of the obtained results (chromatogram) was observed using Chromas Lite software (version 2.5.1), and then FASTA format of the forward and reverse partial gene sequences was aligned using the MEGA6 software. Ambiguous sequences were omitted from the results. The partial DNA sequences of selected gene were subjected to BLAST search against the National Center for Biotechnology Information (NCBI) public sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences generated in the present study were deposited in GenBank, and accession numbers were obtained. The phylogenetic tree of each strain was constructed with other taxa of related *Wolbachia* strains using the Maximum Likelihood method rooted on the Tamura-Nei model with the help of MEGA6 software. Maximum composite likelihood approach was used, and all possible codon frames were considered in the analysis after eliminating gaps.

## RESULTS

The present study involves detection and characterization of *Wolbachia* from local *Cx. quinquefasciatus* adults collected from the selected localities of Lahore, Pakistan, using the PCR method. *Wolbachia* infection was calculated as 82.3% (128/145) in collected *Cx. quinquefasciatus* females using *Wolbachia*-specific *wsp* gene primers in PCR. Partially amplified DNA fragments of *wsp*, 16S rRNA, and *ftsZ* genes revealed 510, 890, and 570 bp bands of selected four samples on agarose gel (Figure 2). DNA sequencing results of all genes revealed the presence of a single strain of *Wolbachia* in *Culex* mosquitoes collected from selected localities.

*Wsp* gene sequence was submitted to GenBank with Accession no. KX650071 and its NCBI BLAST results (Table 2) showed 100% homology (Max score 926) with *Wolbachia* endosymbiont of *Cx. quinquefasciatus* (Accession no. KJ140126), *Culex modestus* from China (Accession no. KU723567), and *Cx. pipiens* from Turkey (Accession no. KT964225). The *wsp*-gene based phylogenetic tree with the highest log likelihood (–4,762.2634) is given in Figure 3. The final dataset involved 13 nucleotide sequences with 501 positions. The evolutionary tree inferred closeness with *Cx. modestus* (Accession no. KJ140126) *Cx. quinquefasciatus*, followed by EruWolCpip2 of *Cx. pipiens* (Accession no. KT964225).

The 16S rRNA gene sequence of *Wolbachia pipientis* was submitted to GenBank as Accession no. KX611381. NCBI BLAST results of the obtained 16S rRNA sequence showed 99% homology with the *w*Pip strain of *Wolbachia* endosymbiont found in *Cx. quinquefasciatus* Pel, indicating that the strain obtained in this study belongs to subgroup *w*Pip. The evolutionary tree was traced on 13 nucleotide sequences with 895 positions in the final dataset including the highest log likelihood (–7,883.4825). The phylogenetic tree shows that the query sequence is closely related to *Wolbachia* of *Ectropis grisescens* (Lepidoptera, Geometridae) (Accession no. KT862198) and *w*Pip strain of *Cx. quinquefasciatus* (Accession no. AM999887) (Figure 4).

DNA sequence of the *ftsZ* gene was submitted to NCBI GenBank under Accession no. KY753917. NCBI BLAST results indicated 98% homology with *Wolbachia* of *Cx. pipiens* from Argentina (Accession no. GU901160) and *w*Pip *Wolbachia* of *Cx. quinquefasciatus* (Accession no. AM99887) reported from Australia. The tree was constructed with 13 nucleotide sequences with the highest log likelihood (–6,983.9439) including 570 positions. The phylogenetic tree indicated a close relationship of the obtained *ftsZ* gene sequence to the supergroup B *w*Pip isolate of *Cx. quinquefasciatus* (Accession no. AM99887) (Figure 5).

## DISCUSSION

*Wolbachia* has received increased attention in recent years because of its high rates of distribution in several insects and its unique effects on host physiology. They are the most widely

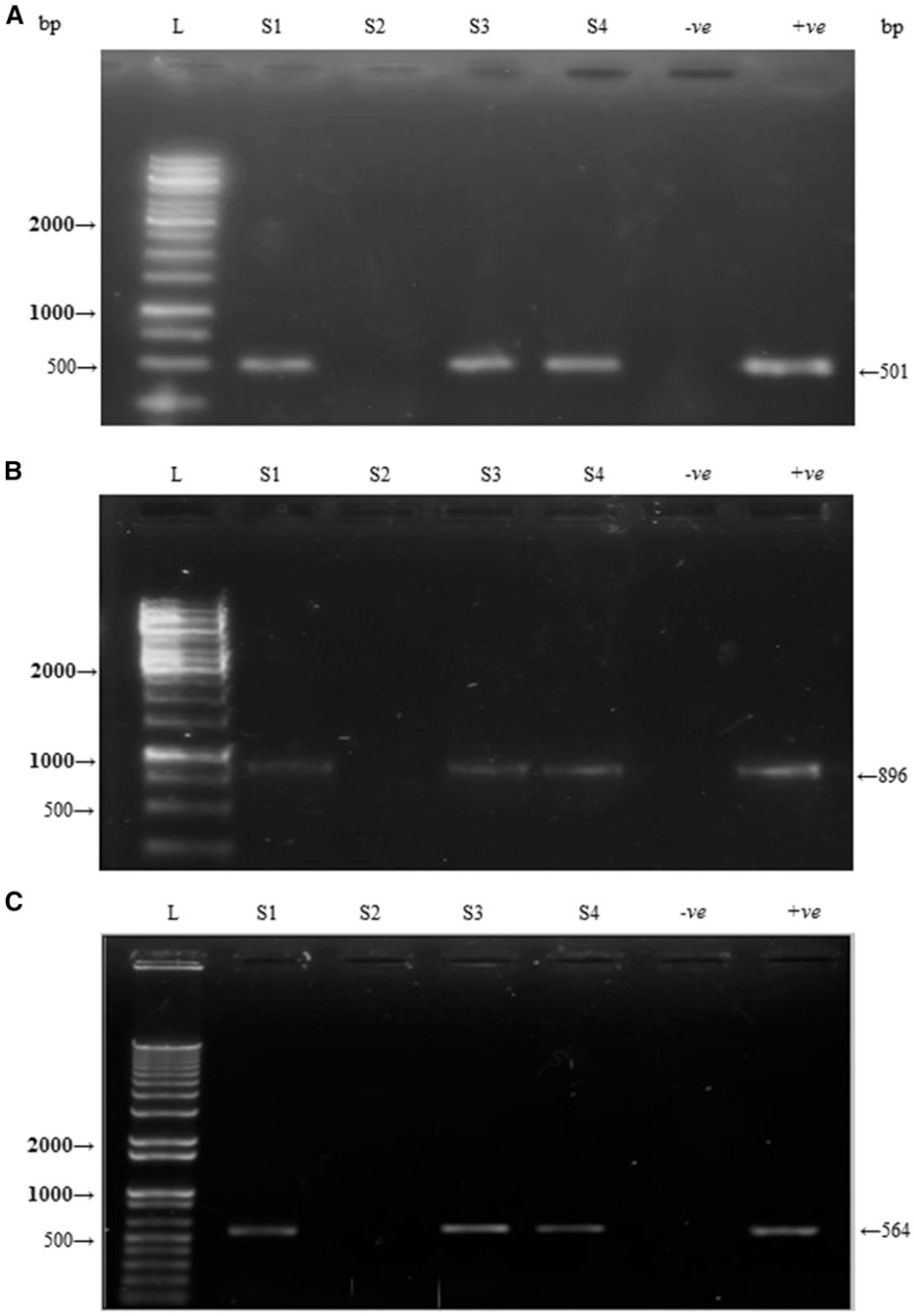


FIGURE 2. Gel electrophoresis analysis of polymerase chain reaction products using *wsp* (**A**), 16S rRNA (**B**), and *ftsZ* (**C**) gene primers targeting genomic DNA of *Culex quinquefasciatus* collected from different areas of Punjab, Pakistan.

occurring intracellular bacteria in arthropods found in major insect orders, including Orthoptera, Diptera, Coleoptera, Hemiptera, Lepidoptera, and Hymenoptera.[21] These obligate parasites are also found naturally in some of the medically important insects including mosquitoes such as *Culex* and *Ae. albopictus*, which are important vectors for the transmission of viruses such as West Nile Virus and Dengue viruses etc. respectively. The detection of *Wolbachia* strain in any given organism is done either by using staining methods[3,22] or by amplification of *Wolbachia*-specific genes including 16S rRNA, *wsp* and *ftsZ* genes, etc.[23] The molecular approach, including gene amplification and characterization, is more significant to find out the phylogenetic status of *Wolbachia*.[7] The current study deals with the use of 16S rRNA and *wsp* and *ftsZ* genes for the detection and molecular characterization of *Wolbachia* isolates from *Cx. quinquefasciatus* collected from the different regions of Lahore, Pakistan.

In this study, 82.3% *Wolbachia* infection was found in *Cx. quinquefasciatus* female mosquitoes. Various *Wolbachia* infection levels have been reported in different regions of the world. In a study from Iran,[24] all of the *Cx. quinquefasciatus* tested were found to be positive whereas other species including *Culex tritaeniorynchus* and *Culex theileri* were found to

TABLE 2

Details of arthropods having identical gene sequences with *wsp* partial cds (Accession KX650071) of *w*Pip *Wolbachia* in *Cx. quinquefasciatus* collected from Lahore Pakistan

| Sr. No. | Strain/isolate | Host | Order, family | Country/origin | Total score | Query coverage (%) | E value | Ident (%) | Accession |
|---|---|---|---|---|---|---|---|---|---|
| 1 | GD13098 | *Culex quinquefasciatus* | Diptera, Culicidae | China | 926 | 100 | 0.0 | 100 | KJ140126 |
| 2 | AISK-w035 | *Culex modestus* | Diptera, Culicidae | China | 926 | 100 | 0.0 | 100 | KU723567 |
| 3 | EruWolCpip3 | *Culex pipiens* | Diptera, Culicidae | Turkey | 926 | 100 | 0.0 | 100 | KT964225 |
| 4 | wTde-HEB | *Trichogramma dendrolimi* | Hymenoptera, Trichogrammatidae | China | 926 | 100 | 0.0 | 100 | JX027991 |
| 5 | EW 13-7 | *Polygonia calbum* | Lepidoptera, Nymphalidae | Not Available | 926 | 100 | 0.0 | 100 | JN093149 |
| 6 | Not available | *Hercinothrips femoralis* | Thysanoptera, Thripidae | Japan | 926 | 100 | 0.0 | 100 | AB245521 |
| 7 | Wbra2 | *Trichogramma brassicae* | Hymenoptera, Trichogrammatidae | China | 926 | 100 | 0.0 | 100 | AF452646 |
| 8 | WDen Qiqihae | *T. dendrolimi* | Hymenoptera, Trichogrammatidae | China | 926 | 100 | 0.0 | 100 | DQ017751 |
| 9 | WHecCI1 | *Eurema hecabe* | Lepidoptera, Pieridae | Japan | 926 | 100 | 0.0 | 100 | AB094393 |
| 10 | WBra | *Mamestra brassicae* | Lepidoptera, Noctuidae | Japan | 926 | 100 | 0.0 | 100 | AB094375 |
| 11 | Wds | *Cx. pipiens pallens* | Diptera, Culicidae | China | 926 | 100 | 0.0 | 100 | AF216860 |
| 12 | EruWolCpip2 | *Cx. pipiens* | Diptera, Culicidae | Turkey | 924 | 99 | 0.0 | 100 | KT964224 |
| 13 | wPip | *Cx. quinquefasciatus* | Diptera, Culicidae | Not available | 922 | 99 | 0.0 | 100 | AM999887 |
| 14 | EruWolCpip6 | *Cx. pipiens* | Diptera, Culicidae | Turkey | 920 | 100 | 0.0 | 99 | KT964228 |
| 15 | wtauFJ1 | *Bactrocera tau* | Diptera, Tephritidae | China | 920 | 100 | 0.0 | 99 | DQ450892 |
| 16 | WDen | *E. hecabe* | Lepidoptera, Pieridae | China | 920 | 100 | 0.0 | 99 | DQ017750 |

be negative for *Wolbachia*. It has also been reported previously that *Wolbachia* infection frequency in *Cx. quinquefasciatus* from three villages of South India was 91.2%.[25] In addition, Kittayapong et al.[26] conducted a survey of *Wolbachia* infection in mosquitoes from Thailand and found 42.1% *Wolbachia* infection in 29 species of the genus *Culex* by targeting *ftsZ* and *wsp* genes. The authors did not find any *Wolbachia* infection in *Anopheles* mosquitoes.

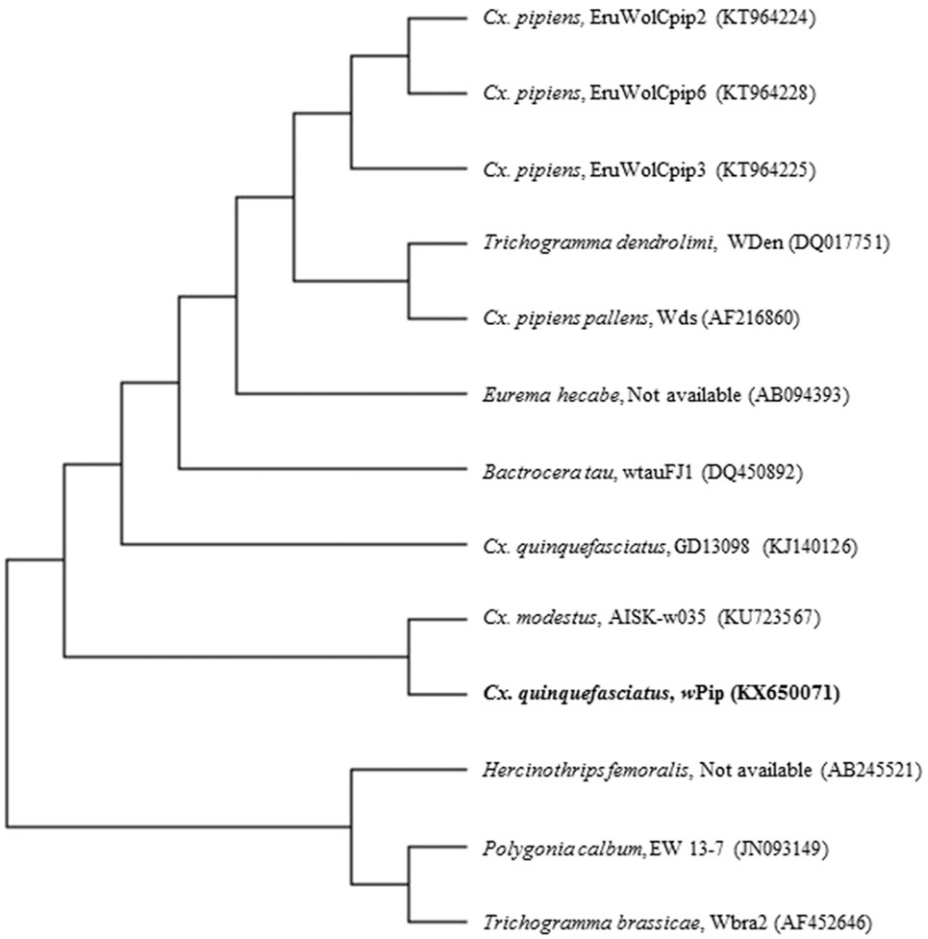


FIGURE 3. *wsp* gene-based phylogenetic tree of *Wolbachia pipientis w*Pip strain found in *Culex quinquefasciatus* using maximum likelihood method (Accession no. KX650071).

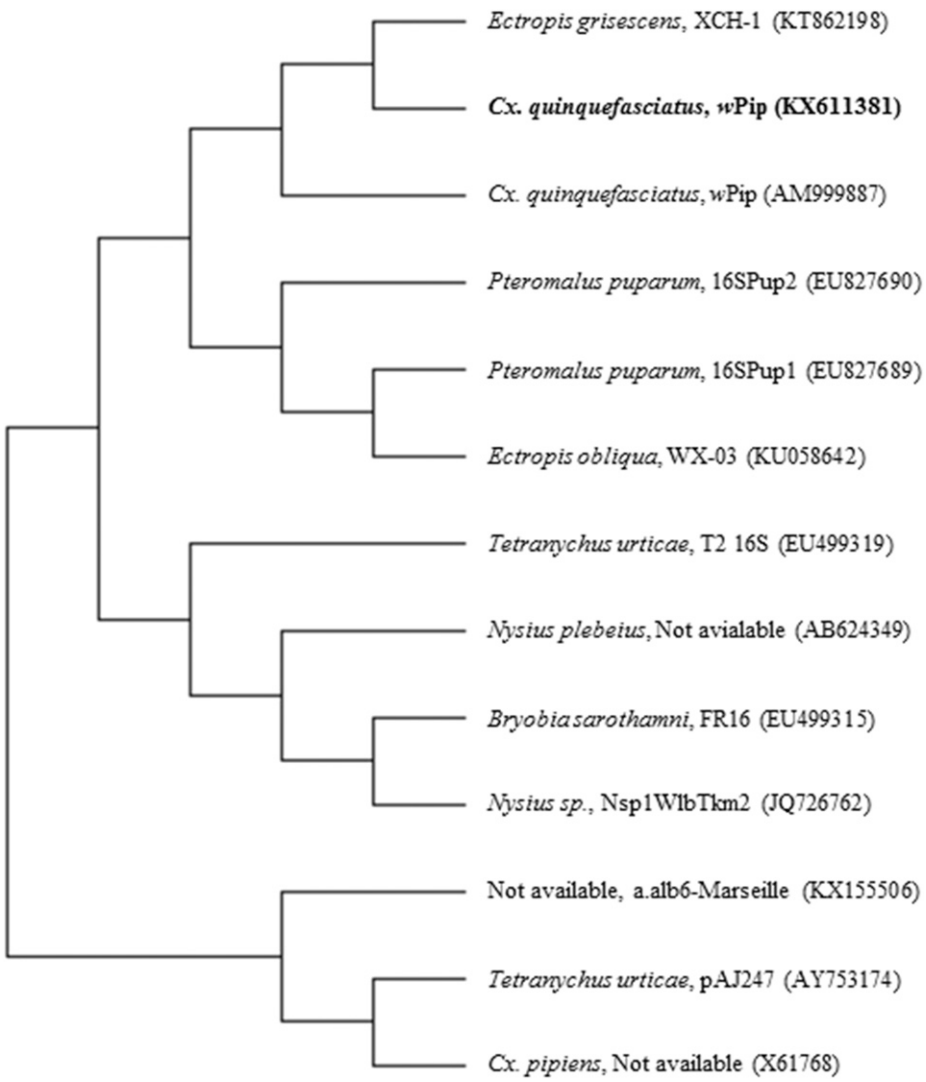


FIGURE 4. 16Sr RNA gene-based phylogenetic tree of *Wolbachia pipientis w*Pip strain found in *Culex quinquefasciatus* using maximum likelihood method (Accession no. KX611381).

In another report, 14 mosquito species related to five genera (*Aedes*, *Culex*, *Anopheles*, *Ochlerotatus*, and *Culiesta*) from North America were tested for the presence of *Wolbachia*. Only species of the *Cx. pipiens* complex were found to be positive.[27] Ricci et al.[28] investigated 26 mosquito species out of which five (three *Culex* and two *Aedes* species) carried *Wolbachia* infection. Karami et al.[29] screened *Wolbachia* in *Cx. pipiens* collected from three provinces of Iran and found 87.3% infection. In a report of *Wolbachia* infection in the *Cx. pipiens* complex of mosquitoes from Germany and the Philippines, *Wolbachia* infection was confirmed in all of the *Culex* mosquitoes tested based on the of 16S rRNA gene of *Wolbachia*.[30]

A number of valuable molecular methods have provided the basis for phylogenetic studies. General *Wolbachia* primers and *Wolbachia* supergroup A- and B-specific primers may be used for the *wsp* gene.

Characterization of the detected strain of *Wolbachia* in the current study showed that it is related to supergroup B and subgroup (strain) *w*Pip. The current results are consistent with some previously reported studies in different regions of the world. In India, Patole and Shouche[12] found *Wolbachia* in *Cx. quinquefasciatus* which was related to the *w*Pip strain and supergroup B. In another study, it was also reported that three tested *Culex* species (*Culex torrentium*, *Cx. pipiens*, and *Cx. modestus*) fall in supergroup B.[28] Recently Karami et al.[29] also placed a type strain from *Cx. pipiens* within supergroup B. Furthermore, Kittayapong et al.[26] reported *Wolbachia* in three *Culex* species (*Cx. quinquefasciatus*, *Culex fuscocephala*, and *Culex sitiens*) collected from the Southeast Asian region belonging to supergroup B and *w*Pip strain. Similar findings were also reported by Yildirim et al.[5] who studied *Cx. pipiens*.

The current study reports the detection and characterization of *Wolbachia* in *Cx. quinquefasciatus* for the first time in Pakistan. The information obtained so far about *Wolbachia* abundance in different arthropods found in Pakistan is inadequate. Therefore, there is dire need to determine the distribution and type of *Wolbachia* infections in insects. Moreover, the potential of *Wolbachia* strain may be evaluated as biological control agent to combat vector-borne diseases (dengue and malaria) in endemic areas of Pakistan.

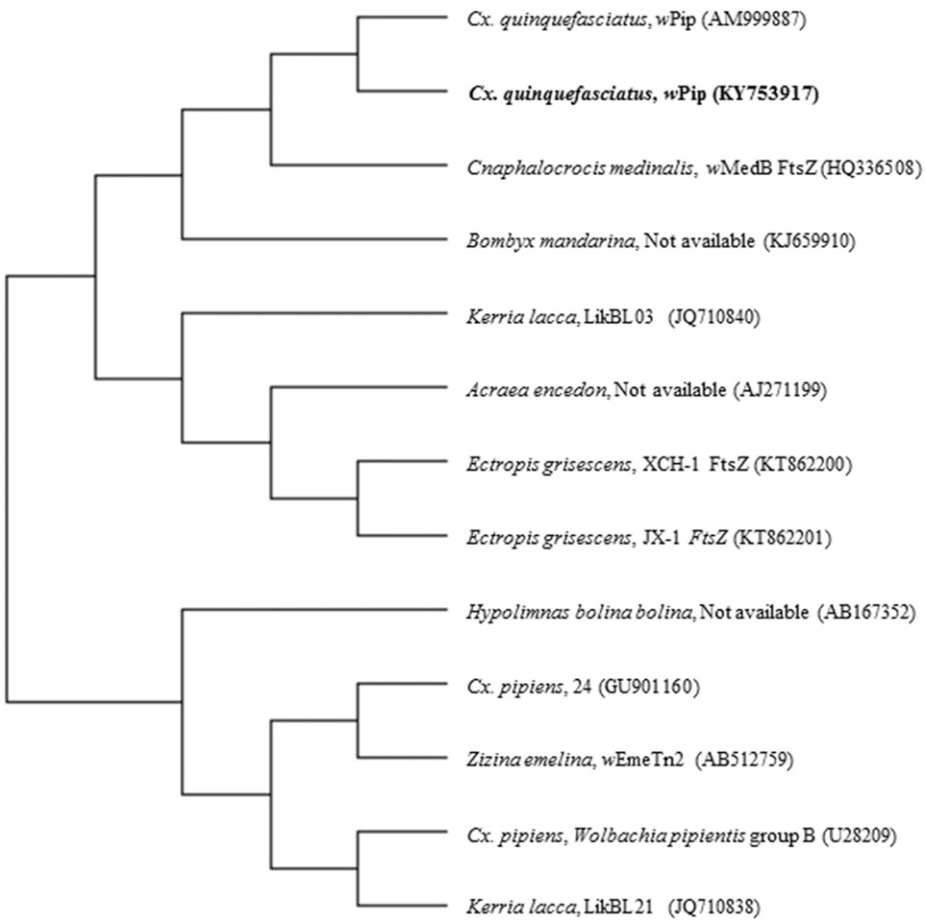


FIGURE 5. *ftsZ* gene-based phylogenetic tree of *Wolbachia pipientis* *w*Pip strain found in *Culex quinquefasciatus* using maximum likelihood method (Accession no. KY753917).

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