# Molecular characterization and phylogeny of *Wolbachia pipientis in* mosquitoes

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**Abstract.** *Wolbachia* is an intracellular, maternally inherited bacteria that infect a wide variety of arthropods, including several mosquito such as *Aedes albopictus*, *Armigerus*, *Culex*, and *Manzonia*. However, until now there have been no reports of the existence of endosymbionts from several genera of mosquitoes from Indonesia. Therefore, we screen *Wolbachia* on several mosquito genera. The aim of the study was to determine the spreading of *Wolbachia* endosymbiont in mosquitoes from Indonesia. Mosquitoes were taken from various breeding and resting places, the captured mosquitoes were identified, the PCR product was carried out by gel electrophoresis. Visualization of amplicon bands under ultraviolet light. The result showed that the mosquitoes (*Manzonia*, *Aedes albopictus*, and *Armigerus*) tested were positive for the *Wolbachia* endosymbiont. Analysis phylogeny shows, that *Wolbachia* in three genus mosquitoes consists of two clades where the first clade consists of *Wolbachia* from *Aedes albopictus* and *Manzonia*, and the second clade consists of *Wolbachia* from *Armigerus* and other *Wolbachia* from some insects as outgroups. Subsequently, the phylogenetic trees based on the *wsp* revealed that *Wolbachia* from *Aedes* samples 1, 2, and *Manzonia* include monophyletic while *Wolbachia* from *Armigerus* is paraphyletic. *Wolbachia* from *Armigerus* close to *Wolbachia* from *Tetranychus urticae.*

**Keywords**: *Wolbachia*, Insect, Mosquitoes, PCR, Primer

## 1. Introduction

*Wolbachia pipientis* is an α-proteobacterial reproductive parasite that infects a wide variety of arthropods, including 17-75% of all insects[(1)] . Since it is vertically transmitted (passed on to offspring) exclusively in the cytoplasm of egg cells, *Wolbachia* triggers phenotypic changes in hosts to increase transmission through females. Host manipulations include parthenogenesis, feminization of males, killing of male embryos, and cytoplasmic incompatibility between uninfected females and infected males([2]). These effects skew insect populations in favor of infected females, thus enhancing the parasite's survival and reproduction[(3)]. In addition, reproductive barriers induced by *Wolbachia* infection can promote sympatric speciation (evolution of species living in the same environment) within the host population. *Wolbachia* infects an estimated 28% of mosquito species [(4)(5)(6)]. There are several potential applications of *Wolbachia* as a solution to global health issues such as lymphatic filariasis, malaria, zika, and dengue. Entomologist Steven Dobson hopes to eliminate mosquito vectors of filariasis in the South Pacific by introducing infected males to the island populations. Dobson hypothesizes that cytoplasmic incompatibility-induced sterility will effectively reduce, and even eliminate, harmful mosquito populations after many generations of reproduction. This principle is applicable to other mosquito-transmitted diseases as well. More detailed research regarding specific mosquito species and *Wolbachia* will aid in the development of these applications. Because *Wolbachia* is a widespread endosymbiont, it can be used to study the evolutionary history of arthropods [(7)]

## 2. Materials and Methods

Adults mosquito were collected from resting and breeding places of mosquitoes. From Purwokerto, Rawa Pening, and Palembang area. Several different species were identified in the laboratory using a light microscope.

### *2.1 Equipment*

The tools used are glassware (Iwaki Pyrex), a water heater (Sharp), an electric stove (Maspion), a balancer scale (Ohaus), a digital scale (Kern), a vortex (BioRad), a laminar airflow cabinet (Sanyo), micropipette (Gilson), autoclave (Sanyo), incubator (Sanyo), refrigerator (Gassio), stereo microscope (Stemi DV), microcentrifuge(Thermo scientific nanodrop spectrophotometer (Thermo Scientific), thermal cycle (BioRad), electrophoresis (Mupid 2 Plus), and gel documentation (BioRad), ABI PRISM 310 automated DNA sequencer (Applied Biosystems).

### *2.2 Chemical materials*

The chemicals used consist of 70% technical alcohol, 99% alcohol, and spirit. QIAprep-Spin Miniprep kit (Qiagen), 100 bp DNA ladder (Novagen), 6X loading buffer (Novagen), TAE buffer (BioRad), primer ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'), ITS primer 4 (5'-TCCTCCGCTTATTGATATGC-3') 1st base), agarose gel (Promega), absolut ethanol (Merk), nuclease-free water (Promega), and PCR ready mix (Bioline). The primer pairs are w81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and w691R (5'-AAAAATTAAACGCTACTCCA-3').

### *2.3 Consumables*

The consumables used are medium, chemicals, dry tissue, yellow pages, aluminum foil, plastic, rubber bands, universal pH indicator paper, micropipette tips (10L, 100 L, and 1000L) (BiologiX), microtubes, and disposable gloves (HandSeal), parafilm (American National Can), rubber bands and heat-resistant plastic (Bell), zip plastic (Ziploc), tissue Kimwipe (Kim wipes).

### *2.4 DNA Extraction*

The procedure using DNeasy tissue kit (Qiagen) was as follows: the abdomen of an adult female was cut and put in a labelled 1.5ml microcentrifuge tube. 180µl of STE (100 mM Sodium chloride, 10 mM Tris hydrochloride, and 1 mM EDTA, at pH 8.0) was added to the sample and the sample was ground using a disposable microtube pestle. 20µl of >12 mAU proteinase K and 200µl of buffer AL (lysis buffer) was added to each sample. This was then mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. 200µl of 100% ethanol was subsequently added to each sample and again the sample was vortex in order to denature the proteinase K. The mixture was then pipetted into separate DNeasy ion exchange spin columns sitting in 2ml collection tubes and centrifuged at 8000rpm for 1 minute. The flow-through and collection tubes were then discarded. The DNeasy spin columns were placed in new 2ml collection tubes and 500µl of buffer AW 1 (wash buffer 1) was added to each in order to "wash" out unwanted ethanol from the samples. Again, the flow-through and collection tubes were discarded. The DNeasy spin columns were placed, as before, in new 2ml collection tubes and 500µl of buffer AW 2 (wash buffer 2) was added. This time, the samples were centrifuged at 16,000 rpm for three minutes to dry the DNeasy membrane. The flow-through and collection tubes were again discarded. The DNeasy columns were placed in clean 1.5ml microcentrifuge tubes and 200µl of buffer AE (elution buffer) was pipetted directly onto the membrane. The samples were then centrifuged for one minute at 8000 rpm to elute the DNA. The DNeasy spin columns were then discarded and an elute was frozen (-20°C) for future use.

### *2.5 Calculation of DNA Concentration*

Calculation concentration and purification of DNA isolation products were carried out using the NanoDrop 1000 spectrophotometer user manual. Blank in the form of Milli-Q was prepared as much as 2µl and used in calibration. The extracted DNA was prepared as much 2µl. DNA concentration was

measured at a wavelength of 260. The purification of the sample was seen based on the comparison value of 260/A280 which was shown by the NanoDrop 1000 spectrophotometer software.

*2.6 Polymerase Chain Reaction*

The extracted DNA from the adult mosquitoes was analysed using the Polymerase Chain Reaction (PCR) to determine evidence of *Wolbachia* infection. The following procedure was adopted.
Manufacture instruction (Promega).
A master mix was made consisting of the following substances:

13.5µl of $H_2O$ (UHQ)
2µl of 10x polymerase buffer (Promega)
2µl of 25mM $MgCl_2$
0.5µl of (20µM) primer F
0.5µl of (20µM) primer R
0.5µl of (10mM) dNTP (deoxynucleotide triphosphate)

1µl of 1U/µl *Taq* polymerase analyzed into a 1.5ml micro-centrifuge tube. Two 0.5ml micro-centrifuge tubes were labelled. To each was added the sample (1µl) and master mix (19µl). One master mix was used to detect *Wolbachia* (using *wsp* [*Wolbachia* surface protein] gene). The forward primer is *w*81F: 5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′ and the reverse primer is *w*691R: 5′-AAA AAT TAA ACG CTA CTC CA-3′ the length is 610 bp (Braig *et al.*, 1998); as a control to detect mitochondria (using the forward primers 12SbiF: 5′-AAG AGC GAC GGG CGA TGT GT-3′ and reverse primer 12SaiR: 5′- AAA CTA GGA TTA GAT ACC CTA TTA T-3′). The micro-centrifuge tubes were then placed in a thermal cycler (Technogene). An initial denaturation at 95°C for 5 minutes was followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, followed by 72°C for 10 minutes and finally 4°C for an infinite amount of time. The cycling took approximately 3-4 hours in total. 3µl of loading buffer (15% Ficol, 0.5% bromophenol blue and 0.5% xylene cyanol) was then added to the sample and mixed thoroughly using a pipette. 17µl of the sample and 12µl of a 1 kilo base ladder (Promega) were then pipette into the wells of a prepared 1% electrophoresis gel. 200ml of gel was made as follows: 2g of agarose powder was added to 200ml of 1 X TBE (54g Tris base, 27.5ml Borax and 20ml 0.5M EDTA, pH8 for 1litre of 5X TBE, and the molarities were 0.045M Tris-borate and 0.001M EDTA. For 1X TBE: 200ml TBE 5X in 1000ml water). The powder was dissolved by heating at medium high in a microwave for 3 minutes and then allowed to cool. 7µl ethidium bromide was added to the new gel or 2µl to an old gel (Ethidium bromide was made of 1g of Ethidium bromide to 100ml of water, dissolved with a stirrer, then put in brown bottle). The gel was poured into an electrophoresis gel tray (Life Technology) and allowed to set in 1X TBE (Tris-borax EDTA). The gel was run at a voltage of 130V for 1 hour at room temperature. The gel was removed and, using ultra-violet light, a photograph of the gel was taken to determine the presence and size of the amplified DNA.

*2.7 Cycle Sequencing and Sequencing gen wsp*

The purified DNA was dissolved in 50l distilled water and used for sequencing. The PCR cycle sequencing reaction was carried out using the DNA Sequencing kit Big Dye Terminator ver.3.1.(Applied Biosystems) and two sequencing primers, namely w81F (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′ and primer w691R (5′-AAA AAT TAA ACG CTA CTC CA-3′) PCR condition of 95° C for 4 min followed by 30 cycles at 95oC for 30 s, and 50°C for 1 min, and 60°C for 4 min. The PCR products were then analyzed with the ABI PRISM 310 DNA Sequencer (Applied Biosystems).

*2.8 Sequences editing and Molecular Phylogenetic Analysis*

Sequences alignment was performed with the help of Bioedit 7.0.4.1 (Hall, 1999) software and edited manually The wsp gene sequence data was sent to the online international DNA database (GenBank) to find the percentage of homology with the Basic Local Algorithm Search Tools (BLAST) program (http://www.ncbi.nlm.nih/blast). The wsp gene sequences obtained in this study and the wsp sequences from *Wolbachia* obtained from GenBank (multiple sequences alignments) were analyzed together using the Clustal W software package program [8]. The phylogenetic tree was constructed using the Neighbor-

joining method and the distance length was calculated based on Kimura's two-parameter evolutionary model. Bootstrap testing was carried out for 1,000,000 repetitions.

## 3. Result and Discussion

### *3.1 Wolbachia in mosquitoes*

The results of the amplified DNA extraction of the PCR product obtained had the expected length, which was approximately 610 bp, and there was no difference in length between individuals. This also proves that the resulting bands are really the expected target. Our research from three different genera shows, that from adult mosquitoes of Manzonia (22 specimens) there were 13 specimens (59,09%) were positive for *Wolbachia* (Table 1), adult mosquitoes of *Aedes albopictus* (10 specimens), there were 6 (60%) were positive for *Wolbachia* (Table 2), while from adult mosquitoes of *Armigerus* 6 specimens all (100%) (Table 3) were positive for *Wolbachia*. Those results indicate that three genus mosquitoes tested were highly infected with *Wolbachia.* This finding is similar to the previous report. However, for *Aedes albopictus* test, the percentage of our result is lower than [9]. which found 98.6% for females and 95.1% for males *Aedes albopictus* in the population studied. While for Manzonia our result is higher than the previous report by [10] which found around 26-27% were infected with *Wolbachia.* While for genus *Armigerus* our result is similar to [11] from Srilangka found 100% of *Armigerus* tests were infected with *Wolbachia.* The Tables below show gel *electrophoresis* with bands that appear to indicate positive *Wolbachia.*

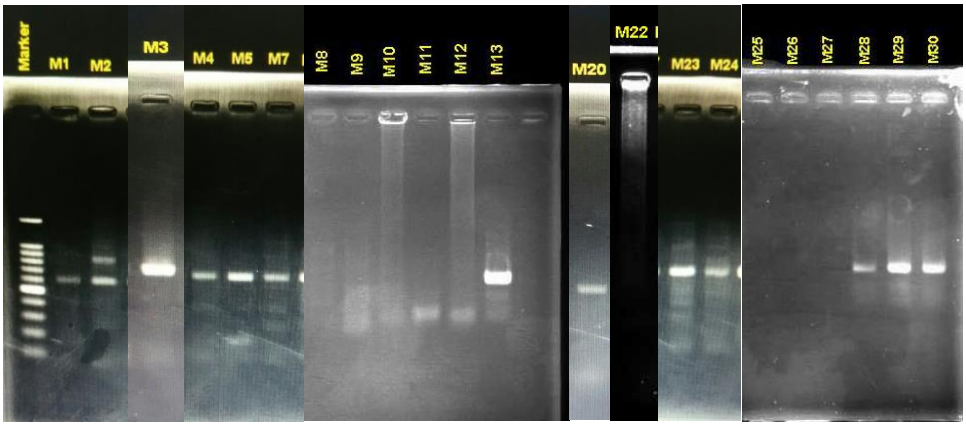


**Figure 1.** Visualization of PCR products from mosquitoes *Manzonia* sp. tested for *Wolbachia* (From 22 specimens' tests, there are 13 samples (M1, 2, 3, 4, 5, 7, 13, 20, 23, 24, 28, 29, and 30) specimens are positive for *Wolbachia*)

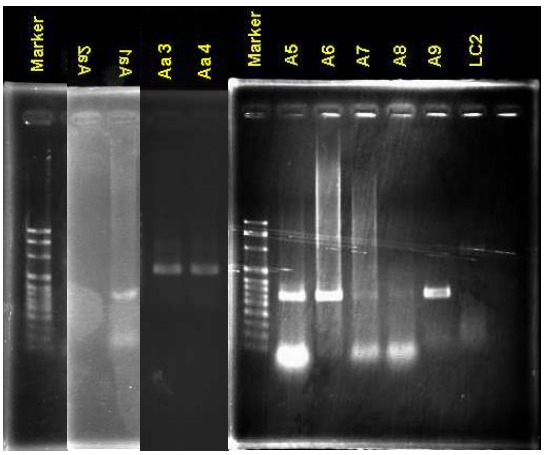


**Figure 2.** Visualization of PCR products from *Aedes albopictus* tested for *Wolbachia* (from 10 specimens tests, there are 6 samples (*Ae* 2, 3, 4, 5, 6, and 9) specimens are positive for *Wolbachia*)

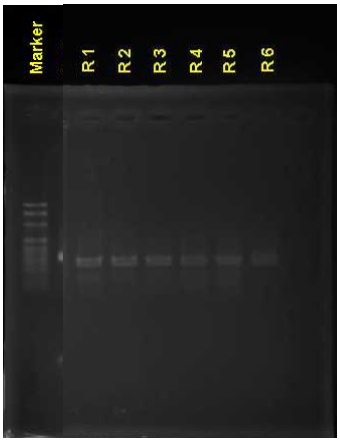


**Figure 3.** Visualization of PCR products from *Armigeres* sp. tested for *Wolbachia*
(ATotal of 6 positive samples of *Wolbachia in Armigerus* sp)

3.2 *Similarity*

Sequencing of the *wsp* fragment was successfully sequenced from three genera of mosquitoes. In BLAST, the sequences showed a different degree of sequence similarity with the wsp gene in the NCBI nucleotide database, with range values from 86,01% to 95,51%. This result indicated that sequences can be identified convincingly at the species level, according to the BLAST result, *Wolbachia* from *Ae. albopictus* sample no 1 showed sequence similarity as much as 94,80%, to *Wolbachia* endosymbiont *Aedes aegypti* isolate wAegB surface protein gene, complete cds. (accession number MN307069), while *Wolbachia* from *Ae. albopictus* sample 2 has sequence similarity as much as 86.01% to *Wolbachia* endosymbiont of *Aedes aegypti* isolate wAegB surface protein gene, complete cds. (accession number MN307069). Subsequently, *Wolbachia* from *Manzonia* has sequence similarity as much as 92,67% to *Wolbachia pipientis* strain wTos2 *Wolbachia* outer surface protein (wsp) gene, partial cds. (accession number MH886577) and *Wolbachia* from *Armigerus* has sequence similarity as much as 95.51% to *Wolbachia* endosymbiont of *Kerria lacca* outer surface protein (wsp) gene, partial cds (accession number JQ837254). The Tables below show the similarity between *Wolbachia* from three genera of mosquitoes

Table 1. Similarity of *Wolbachia* from *Aedes albopictus*

| Description | Max Score | Query cover | Identity | Total Score | Accession Number |
|---|---|---|---|---|---|
| *Wolbachia* endosymbiont of *Aedes aegypti* isolate wAegB surface protein gene, complete cds. | 804 | 98% | 94.80% | 804 | MN307069 |
| *Wolbachia* endosymbiont of *Aedes aegypti* isolate wAegB surface protein gene, complete cds. | 804 | 98% | 94.80% | 804 | MN307069 |
| *Wolbachia* endosymbiont of *Aedes aegypti* isolate wAegB surface protein gene, complete cds. | 597 | 96% | 86.01% | 597 | MN307069 |

Table 2. Similarity of *Wolbachia* from *Manzonia* and *Armigerus*

| Description | Max Score | Query cover | Identity | Total Score | Accession Number |
|---|---|---|---|---|---|
| *Wolbachia pipientis* strain wTos2 *Wolbachia* outer surface protein (wsp) gene, partial cds. | 610 | 953% | 92,67% | 953 | MH886577 |
| *Wolbachia* endosymbiont of *Kerria lacca* outer surface protein (wsp) gene, partial cds.(12) | 819 | 91% | 95.51% | 819 | JQ837254 |

*3.3 Analysis phylogeny*

Phylogenetic analysis or sometimes called cladistics which means clades or groups of descendants from a common ancestor. Phylogenetic analysis is usually represented as a branching system, such as a tree diagram known as a phylogenetic tree [(12)(13)]. Phylogenetic analysis of a nucleotide or amino acid sequence family is an analysis to determine how the family was inherited during the evolutionary process. Evolutionary relationships between sequences are described by locating sequences as outer branches of a tree. The branch relationships in the interior of the tree reflect the degree to which the different sequences are related. Two very similar sequences will be located as neighboring outside of the branches and connected in a common branch [(14)].

Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 57 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [4]

The wsp is a very useful tool for typing different *Wolbachia strains.* Our sequencing uses wsp gene. Wsp gene sequences provide many more informative characters with which to determine evolutionary relationship between strains. Figure 1 showed the phylogenetic analysis results based on the wsp gene sequences of *Wolbachia* in mosquitoes and several sequences derived from Genbank for the outgroups. The results showed that *Wolbachia* from *Aedes* samples 1,2, and *Manzonia* include monophyletic (partly descended from the same ancestor, while *Wolbachia* from *Armigerus* is paraphyletic (partly descended from the same ancestor). *Wolbachia* from *Armigerus* close to *Wolbachia* from *Tetranychus urticae.* Based on phylogeny tree, *Wolbachia* from three genera consist of two clades, first clade consist of *Wolbachia* from *Aedes albopictus* and *Manzonia* and second clade consist of *Wolbachia* from *Armigerus* and other *Wolbachia* from some insect such as *Ostrinia furnacalis, Culex quenquefasciatus, Nilaparvata muiri, Kerria lacca, Tetranychus cinnabarium,* and *Tetranychus urticae* with assession number respectively: GU166595, KJ140126, GU289811, JQ837254, AY585711, and DQ910771 sequences provide from GenBank.

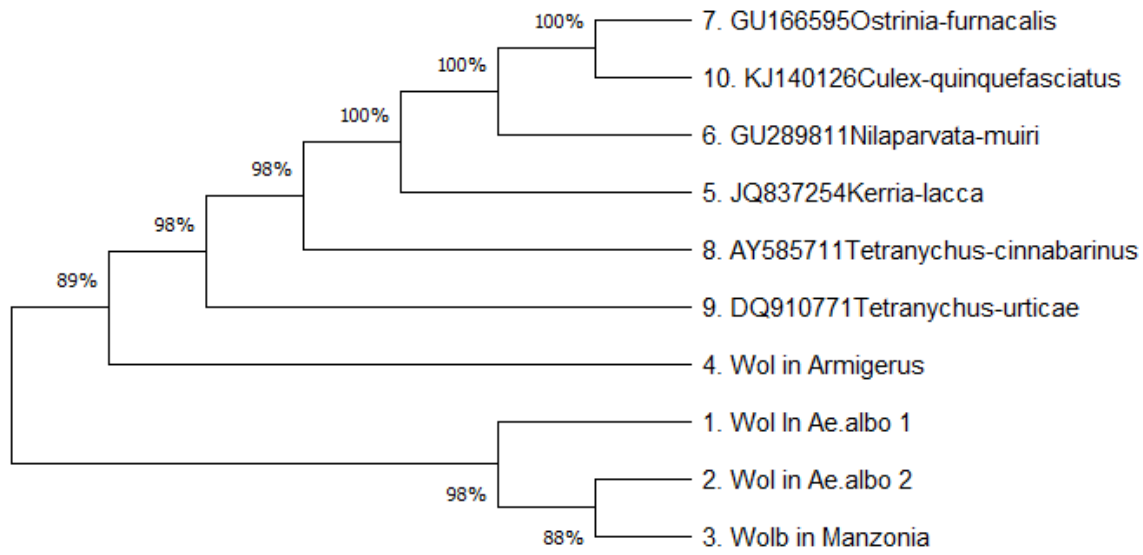


**Figure 4.** Phylogenetic tree of *Wolbachia* from *Manzonia, Aedes albopictus,* and *Armigerus* based on 610 bp of the wsp gene sequence. *Wolbachia* endosymbiont of *albopictus* and *Manzonia* were placed in the same clade and include in monophyletic, while *Wolbachia* in *Armigerus* is included in paraphyletic and close to *Wolbacia* in *Tetranychus urticae*

### *3.4 Genetic Distance Result*

Table 3. The genetic distance value within *Wolbacia* study samples and *Wolbachia* from different insects performed by pairwise distances using Mega software version 7

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1. 1. Wol In Ae.albo 1 | | | | | | | | | |
| 2. 2. Wol in Ae.albo 2 | 0.0000000000 | | | | | | | | |
| 3. 3. Wolb in Manzonia | 0.0549862207 | 0.0000000000 | | | | | | | |
| 4. 4. Wol in Armigerus | 0.8713470994 | 0.7517419246 | 0.8655008814 | | | | | | |
| 5. 5. JQ837254Kerria-lacca | 0.7071768869 | 0.6987826641 | 0.8711177038 | 0.6757023025 | | | | | |
| 6. 6. GU289811Nilaparvata-muiri | 0.7071768869 | 0.6987826641 | 0.8711177038 | 0.6757023025 | 0.0000000000 | | | | |
| 7. 7. GU166595Ostrinia-furnacalis | 0.7071768869 | 0.6987826641 | 0.8711177038 | 0.6757023025 | 0.0000000000 | 0.0000000000 | | | |
| 8. 8. AY585711Tetranychus-cinnabarinus | 0.7290653171 | 0.7231918776 | 0.9004625550 | 0.6433309352 | 0.0000000000 | 0.0000000000 | 0.0000000000 | | |
| 9. 9. DQ910771Tetranychus-urticae | 0.7290653171 | 0.7231918776 | 0.9004625550 | 0.6358719558 | 0.0181500806 | 0.0181500806 | 0.0181500806 | 0.0000000000 | |
| 10. 10. KJ140126Culex-quinquefasciatus | 0.7071768869 | 0.6987826641 | 0.8711177038 | 0.6757023025 | 0.0000000000 | 0.0000000000 | 0.0000000000 | 0.0000000000 | 0.0181500806 |

The results of genetic distance analysis showed that the shortest distance was between *Wolbachia* on *Ae. Albopictus* samples 1 and 2 (0,000). The distance between *Wolbachia* on *Ae.albopictus* is 0,055 with *Wolbachia* on *Manzonia.* While *Wolbachia* on *Ae.albopictus* is 0,871 with *Wolbachia* on *Armigerus.* Subsequently, the distance of *Wolbachia* on *Ae. Albopictus* is 0,7071 with *Wolbachia* from *Kerria lacca,* 0,7071 with *Nilaparvata muiri,* 07071 with *Ostrinia furnacalis,* 0,7290 with *Tetranychus cinnabarinus,* 0,7290 with *Tetranycus urticae,* and 0,7071 with *Culex quinquefasciatus.* Genetic distance analysis is an analysis based on calculating the matrix of the distance between base pairs between sequences that are close to evolutionary distance [15]

## 4. Conclusion

In summary, the infection status of *Wolbachia* in *three* genera of mosquitoes populations was investigated and the infection rate of *Wolbachia* was found to be 59,09% of adult mosquitoes of *Manzonia*, 60%) of adult mosquitoes of *Aedes albopictus,* while from adult mosquitoes of *Armigerus* is 100% were positive for *Wolbachia*. The phylogenetic trees based on the *wsp* revealed that *Wolbachia* from *Aedes* samples 1, 2, and *Manzonia* include monophyletic (partly descended from the same ancestor, while *Wolbachia* from *Armigerus* is paraphyletic (partly descended from the same ancestor). *Wolbachia* from Armigerus close to *Wolbachia* from *Tetranychus urticae*. Based on the phylogeny tree, *Wolbachia* from three genera consist of two clades, the first clade consist of *Wolbachia* from *Aedes albopictus* and

*Manzonia* and the second clade consists of *Wolbachia* from *Armigerus* and other *Wolbachia* from some insect as outgroups such as *Ostrinia furnacalis, Culex quenquefasciatus, Nilaparvata muiri, Kerria lacca, Tetranychus cinnabarium,* and *Tetranychus urticae* with assession number respectively: GU166595, KJ140126, GU289811, JQ837254, AY585711, and DQ910771 sequences provide from GenBank, supported by the distance figure.

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