BIODIVERSITAS
Volume 23, Number 5, May 2022
Pages: 2321-2328

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230510

Wolbachia genetic similarity in different insect host species: *Drosophila melanogaster* and Yogyakarta's (Indonesia) *Aedes aegypti* as a novel host

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Manuscript received: 19 February 2022. Revision accepted: 20 April 2022.

Abstract. *Rovik A, Daniwijaya EW, Supriyati E, Rahayu A, Kumalawati DA, Saraswati U, Handayaningsih AE, Rachman MP, Oktriani R, Kurniasari I, Candrasari DS, Nurhayati I, Sholeh R, Arianto B, Tantowijoyo W, Ahmad RA, Utarini A, Arguni E. 2022.* Wolbachia *genetic similarity in different insect host species:* Drosophila melanogaster *and Yogyakarta's (Indonesia)* Aedes aegypti *as a novel host. Biodiversitas 23: 2321-2328. Wolbachia* naturally presents in a large number of insects and other arthropod species. The *Wolbachia* strain *w*Mel from *Drosophila melanogaster* has been stably transinfected into *Aedes aegypti* where it stops the mosquito host from being infected with medically important arbovirus like dengue. Consequently, *Ae. aegypti* infected with *w*Mel have been released in Indonesia as a public health intervention against dengue. This study genetically compared *w*Mel from Yogya field-caught *D. melanogaster* and the *w*Mel in stably transfected *Ae. aegypti* used for field releases in Yogyakarta, Indonesia. The genetic similarity between *w*Mel *Wolbachia* was evaluated by sequencing of *Wolbachia* surface protein (*wsp*) gene and some polymorphic genomic regions of insertion sites (IS) and variable number tandem repeats (VNTR) loci. The sequence of the *Wolbachia* surface protein (*wsp*) gene was 100% identical between hosts. There is no insertion sequence among specimens. The insertion sequence IS-WD1310 was identical between *w*Mel from both hosts and among other strains, as well as the IS-WD516/7. The VNTR-141 period was identical within *w*Mel from both hosts and among other strains, the VNTR-105 as well. *Wolbachia* Yogya field-caught *D. melanogaster* and *Wolbachia* strain *w*Mel present in *Ae. aegypti* used for bio-control of dengue were genetically identical. These findings provide beneficial understanding to answer the public attention on safety issues, especially on the genetic similarity between *Wolbachia* strain in the natural and transfected hosts of this novel technology for dengue control.

Keywords: *Aedes aegypti*, *Drosophila melanogaster*, genetic similarity, novel host, *Wolbachia*, *w*Mel

INTRODUCTION

Wolbachia is obligate intracellular endosymbiotic bacteria (Calvitti et al. 2010). It maternally transmitted and naturally presents in many insect species (Werren et al. 2008). *Wolbachia* can manipulate the tissues and reproductive cycles of the host to increase its spread through insect populations (Stevens et al. 2001; Tram et al. 2003). Therefore, *Wolbachia* infection is estimated to occur naturally in 40-65% of insects, as well as other arthropods and some nematode species (Hilgenboecker et al. 2008; Kumalawati et al. 2020; Werren et al. 2008; Zug and Hammerstein 2012). In its early discovery, *Wolbachia* has classified as a strain of one species, *Wolbachia pipientis* (Calvitti et al. 2010). Currently, *Wolbachia* is named commonly by their hosts, such as the native *w*Ri strain of *Drosophila simulans* (isolated in Riverside, California), the native *w*AlbB strain of *Aedes albopictus (Ae. albopictus)*, the original *w*Pip strain of *Culex pipiens (Cx. pipiens)*, and the *w*Cau strain native to *Cadrautella* sp.

Wolbachia has varied effects on the host, including both pathogenic and mutualistic effects. In the host, *Wolbachia* interferes with its host reproduction resulting in male parthenogenesis, homicide or feminization, sex ratio distortion (Dyson et al. 2002; Hurst et al. 2002), and cytoplasmic incompatibility (Ilinsky and Zakharov 2011). In contrast, *Wolbachia* also allows mutualistic effects for insect hosts, such as in wasps, *Drosophila*, and bedbugs (Dedeine et al. 2005; Hosokawa et al. 2010; Starr and Cline 2002). *Aedes aegypti* (*Ae. aegypti*) mosquitos, the most notable vector for arboviruses transmission, are not naturally infected with *Wolbachia*. Meanwhile, independent studies have reported that *Wolbachia* infection is naturally present in other mosquito species, such as *Cx pipiens* and *Ae. albopictus* (Afizah et al. 2015; Rasgon and Scott 2004; Tsai et al. 2004). Several *Wolbachia* strains have been successfully artificially transferred into the *Aedes* mosquito, such as *w*MelPop (McMeniman et al. 2009), *w*Mel (Walker et al. 2011), *w*AlbB (Flores et al. 2020; Xi et al. 2005), *w*Ri, *w*MelCS, *w*Pip (Fraser et al. 2017), *w*AlbBA, and *w*Au (Ant et al. 2018).

Wolbachia has applied in various fields, including biotechnology, agriculture, and public health. *Wolbachia* strain *w*MelPop in *D. melanogaster* has been shown to shorten the life of the host. This effect is also seen when *w*MelPop has been transferred to its novel host, *Ae. aegypti* (McMeniman et al. 2009). The *w*MelPop and *w*Mel strains have been shown to reduce dengue (Bian et al. 2010; Blagrove et al. 2012; Ferguson et al. 2015; Flores et al. 2020; Frentiu et al. 2014; Moreira et al. 2009; Walker et al. 2011), zika (Aliota et al. 2016; Dutra et al. 2016), and chikungunya virus transmission potential of mosquitoes (Blagrove et al. 2013; Moreira et al. 2009; Van den Hurk et al. 2012), as well as the malaria parasite *Plasmodium gallinaceum* (Moreira et al. 2009) and *Plasmodium berghei* (Kambris et al. 2010). In some studies, *Wolbachia* strains that have been transferred from an original host to the new host are very stable. This condition generally occurs when *Wolbachia* is transferred to a new host, within or between related species in the same genus or family (Dobson et al. 2002; Xi et al. 2005). The success of *Wolbachia* transfer may depend on the bacteria's ability to adapt to the new intracellular environment. In other cases, *Wolbachia* strains exhibit fluctuating infection densities and varying degrees of transovarial transmission. This condition is encountered when the transfer occurs between phylogenetically distant hosts. As a result, the infection is often lost in several generations of the host (Dobson et al. 2002) and has the potential to undergo phenotypic changes (Chrostek and Teixeira 2015).

The application of *Wolbachia* in eliminating dengue has been performed in several countries which are involved in the World Mosquito Program (WMP) initiative, such as Australia, Vietnam, Brazil, Columbia, Vanuatu, Sri Lanka, Fiji, Kiribati, New Caledonia, and Indonesia. The WMP Yogyakarta Indonesia released the *w*Mel-infected *Ae. aegypti* in 2015 in a small area of Sleman and Bantul Regency (Tantowijoyo et al. 2020), and the City of Yogyakarta in 2016 (Indriani et al. 2020; Utarini et al. 2021). The current study compared the genetic similarity of *Wolbachia* (Riegler et al. 2005; 2012) from two different hosts: *D. melanogaster* and Yogya *Ae. aegypti* as a novel host.

MATERIALS AND METHODS

Backcrossing of *w*Mel-infected Yogya *Aedes aegypti*

The *w*Mel-infected *Ae. aegypti* was sourced from the laboratory of WMP Australia (Center for Research and Development of Biomedical and Basic Health Technology, Ministry of Health, Indonesia issued an import recommendation No. YF.01.11/III/4554/2012). The *w*Mel strain was artificially transferred from *D. melanogaster* to *Ae. aegypti* by embryonic microinjection (Walker et al. 2011). A *w*Mel-infected Yogya *Ae. aegypti* was generated by performing backcrossing for a few generations to introgress the local genetic profile into the *w*Mel-infected *Ae. aegypti* (details provided in Tantowijoyo et al. 2020).

Rearing of wMel-infected Yogya *Aedes aegypti*

The *w*Mel-infected Yogya *Ae. aegypti* mosquitoes were reared in a controlled laboratory (details provided in Tantowijoyo et al. 2020).

Rearing of *Drosophila melanogaster*

The wild-type *Drosophila* sp. was sourced from the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. The morphologically identified *D. melanogaster* was reared and maintained in a rearing cage and fed with banana pulp regularly. A total of 50 *Drosophila* specimens were sampled and preserved with ethanol 80%.

DNA extraction

The samples were individually rinsed with ethanol 96% and aquadest. Then, the DNA samples were extracted following the DNA extraction procedure of the Genejet Genomic DNA Purification Kit (Thermo Scientific). A total of 1 µL of extracted DNA was put on a NanoDrop machine (NanoVue) to quantify its concentration and purification. (Kumalawati et al. 2020)

DNA amplification

Wolbachia genetic loci were amplified from genomic DNA by using specific primers. The *Wolbachia* surface protein (*wsp*) gene permitted an initial screening of the collected sample. The *wsp* detection was also used as a quality control for DNA extraction which was amplified using the primers *wsp*-81F and *wsp*-691R. The 81F-691R primer set detects the *Wolbachia* surface protein of length 590-632bp depending on the individual *Wolbachia* strain (Zhou et al. 1998). Polymerase chain reaction (PCR) tests were run using thermal-cycler machines (BioRad C-1000 Touch). PCR conditions were as follows 95˚C, 4min; 34 cycles [95˚C, 30s; 50˚C, 30s; 72˚C, 60s]; 72˚C, 10min; 12˚C, 5 min (for 81F-691R and *IS-WD1310*); 95˚C, 4min; 34 cycles [95˚C, 30s; 50˚C, 30s; 72˚C, 90s]; 72˚C, 10min; 12˚C, 5 min (for *VNTR-141*, *VNTR-105*, INV [*WD0394-WD0541*]); 95˚C, 4min; 34 cycles [95˚C, 30s; 50˚C, 30s; 72˚C, 120s]; 72˚C, 10min; and 12˚C, 5 min (for *IS-WD516/7*) (details provided in Riegler et al. 2005; 2012; Zhou et al. 1998).

Visualization of PCR product

PCR products were verified using 1% (w/v) agarose gel electrophoresis (Mupid® eXu submarine electrophoresis system). The electrophoresis was performed at 135 volts for 60 minutes. The resulting bands were visualized using Gel Doc Documentation System (Protein Simple) and compared with DNA Ladder (100bp or 1kb Ladder, Thermo Scientific) to know the estimation size and their possibilities of polymorphism. Sanger sequencing was performed by the 1st Base Pte. Ltd. Company, Singapore.

Phylogenetic analysis of *wsp* gene

Sequence analysis was performed by using BioEdit ver. 7.2 software. The phylogenetic tree was constructed by using a neighbor-joining (NJ) method within the MEGA ver. X software. A 1,000 bootstrap replication was used to construct the evolution distance (Afizah et al. 2015). The *wsp* sequence was compared to the *Wolbachia* endosymbiont among different species and hosts, including *Wolbachia* endosymbiont of *Drosophila melanogaster* isolate *w*Mel (KX650072), *Wolbachia pipientis* strain *w*Mel (DQ235407), *Wolbachia* endosymbiont of *Drosophila simulans* strain Riverside 1988 (EF423761), *Wolbachia* endosymbiont of *Bemisia tabaci* isolate 67.3 (KM404298), *Wolbachia* endosymbiont of *Eurema hecabe* isolated from Indonesia (AB278218) and Malaysia (AB278206), *Wolbachia* endosymbiont of *Culex quinquefasciatus* isolate Pers6 (MN893364), *Wolbachia pipientis* strain *w*MelPop (AF338346), *Wolbachia* endosymbiont of *Drosophila melanogaster* isolate Beijing (KU870673), *Wolbachia* sp. *w*Mel isolate Wuhan (FJ403330), *Wolbachia* sp. *w*Mel isolate Yunnan (FJ403332), *Wolbachia* endosymbiont of *Drosophila* sp. isolate D3G (MN900914), and *Wolbachia* endosymbiont of *Drosophila* sp. isolate D3E (MN900913).

Sequence analysis of insertion sites and tandem repeat loci

The sequence from different hosts was aligned within MEGA ver. X software to evaluate the presence or absence of insertion sites, the tandem repeat loci as well. The periodicity or copy number of the tandem repeat sequence was analyzed by using Tandem Repeat Finder ver. 3.1 which is available at (http://tandem.bu.edu/trf/trf.html) (details provided in Riegler et al. 2012). The periodicity of *VNTR-141* and *VNTR-105* loci were compared to reference sequences including *Wolbachia* strain *w*Mel (JF797613) and *w*Mel (JF797619).

Ethics approval

This present study was part of the World Mosquito Program (WMP) Yogyakarta (previously known as the Eliminate Dengue Program) at Phase 1. The research protocol has been approved (No. KE/FK/01/EC/2012) by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

RESULTS AND DISCUSSION

Results

In this study, some polymorphic genomic markers were sequenced to explore the extent of similarity between a *w*Mel strain of Yogyakarta-sourced *D. melanogaster* and the *w*Mel strain of origin that has been stably transinfected in *Ae. aegypti* mosquitoes by targeting *Wolbachia* surface protein (*wsp*) gene, insertion sites (IS5 family), and variable number tandem repeat (VNTR) loci.

All 50 *D. melanogaster* and all 50 *w*Mel-infected Yogya *Ae. aegypti* were infected by *Wolbachia* as determined by detection of the *wsp* gene (Figure 1). Figure 2 showed that the *wsp* gene was 100% identical among tested hosts and reference *wsp* gene of *Wolbachia* endosymbiont of *Drosophila melanogaster* isolate *w*Mel. Agarose gel analysis of insertion sites (IS5 family) amplification products presented identical sizes of expected bands (Figure 3.A). It showed that the IS-WD1310 insertion site was absent from all specimens, the IS-WD516/7 insertion site as well (Figure 3.B). The agarose gel analysis of tandem repeat loci presented identical sizes of expected bands for the VNTR-105 period and showed a slight difference of the VNTR1-141 period between *Wolbachia* endosymbiont of *D. melanogaster* and *w*Mel-infected Yogya *Ae. aegypti* (Figure 3.C-D).

In this study, the analysis results of insertion sites (IS-WD1310) amplification showed that the sequence was identical among tested hosts and reference genome (KX650072). Therefore, all samples lack the IS-WD1310 element based on the size of the sequenced fragment (Table 1). Like IS-WD1310 analysis, the sequenced element of IS-516/7 insertion showed no consistent difference between hosts, *D. melanogaster* and *w*Mel-infected *Ae. aegypti*. The amplicon size (including the IS-WD516/7 element) is predicted to be 2,488bp. Primer set targeting the IS-WD516/7 element might be difficult to amplify those sequences. It suggests using additional internal sequencing primers to allow for enough overlapping sequence and properly address the locus.

The analysis results of tandem repeat locus (VNTR-141) amplification showed a gap of 140bp for the sequence of *Wolbachia* endosymbiont of *D. melanogaster*. Meanwhile, all sequences of *w*Mel-infected *Ae. aegypti* match the reference sequence (JF797613) in terms of length. Site with tandem repeats having period sizes from 18bp to 264bp and internal match percentage from 90% to 99%. The sequence periodicity of the VNTR-141 period of *Wolbachia* sequence was different among host i.e., 6.3 for *D. melanogaster* and 7.3 for *Ae. aegypti* specimens (Table 2).

In this study, the analysis results of tandem repeat locus (VNTR-105) amplification showed no difference in the number of repeats across all samples, *Wolbachia* endosymbiont of *D. melanogaster* and *w*Mel-infected *Ae. aegypti*. There is no change in repeat number based on the reference sequence (JF797619). Site with tandem repeats having period sizes from 80bp to 291bp and internal match percentage from 97% to 98%. The sequence periodicity of the VNTR-105 period of *Wolbachia* sequence from both hosts was identical i.e., 4x1 + 2x0.5 for *Ae. aegypti* and *D.*

melanogaster specimens (Table 2). It showed that the structure of the VNTR-105 period is less conserved than the VNTR-141 period.

Discussion

Wolbachia infection has been studied widely in the context of evolution, biology, and ecology, and its application in various fields, such as health, agriculture, and vector control. Several studies (e.g Bing et al. 2014; McMeniman et al. 2009; Morrow et al. 2014) have transferred *Wolbachia* across species and genera within and between insect orders, both single and multiple hosts; for example, Dobson et al. (2002) found that infection of *Wolbachia* strain *w*Ri was maintained stably in the host cells of *Drosophila*, *Spodoptera*, and *Aedes* mosquitoes. Stable transfection has been successfully achieved for several species of mosquitoes including *Ae. aegypti*, *Ae. albopictus*, *Ae. polynesiensis*, *Cx. pipiens*, *An. stephensi*, and *An. gambiae* (Bian et al. 2013; McMeniman et al. 2009; Hoffmann et al. 2011; Jin et al. 2009; Walker et al. 2011; Xi et al. 2005). Those studies aimed to determine the ability of *Wolbachia* to increase host immunity against various pathogenic infections in a different host.

Wolbachia have been reported to suppress viruses from a range of RNA virus families. In laboratory research, *Wolbachia*-infected *Ae. aegypti* can inhibit the replication of the dengue virus in the mosquito's body (Bian et al. 2010; Ferguson et al. 2015; Flores et al. 2020; Moreira et al. 2009). In some field studies, *Wolbachia*-infected *Aedes* mosquitoes are shown to reduce the dengue cases e.g., Nazni et al. (2019), Ryan et al. (2019), Tantowijoyo et al. (2020), Indriani et al. (2020), and Utarini et al. (2021). During field-application of *Wolbachia*-infected *Ae. aegypti*, various safety issues might be raised by the public or communities, including the possibilities of genetic changes of *Ae. aegypti* in their natural habitat.

Several studies reported the possibilities of genotypic and phenotypic changes of *Wolbachia* in a transfected host e.g., McMeniman et al. (2008), Schneider et al. (2013), and Woolfit et al. (2013). The genetic similarity of *Wolbachia* between original species and local infected *Ae. aegypti* mosquito was important to increase self-evidence to convey this novel technology to local people residing in the release area in Yogyakarta, and hopefully when implemented to other areas throughout Indonesia. Therefore, monitoring of genetic changes is important to be conducted.

The *Wolbachia* surface protein (*wsp)* has been one of the most widely used for *Wolbachia* identification and systematics. It is well-known that there are 11 supergroups of *Wolbachia* which have been designated based on their *ftsZ*, *wsp*, and 16S rRNA genes (Riegler et al. 2012; Zhao et al. 2021). Zhou et al. (1998) proposed that the *wsp* sequence similarity among *Wolbachia* strains should be greater than 97.5% identical. In this study, the analysis of sequence similarity showed that *Wolbachia* was 100% identical among tested hosts and reference *wsp* gene of *Wolbachia* endosymbiont of *D. melanogaster* isolate *w*Mel (KX650072) (Figure 2). It showed that *Wolbachia* native to Yogya *D. melanogaster* has no difference with *w*Mel strain in a novel host, Yogya *Ae. aegypti*.

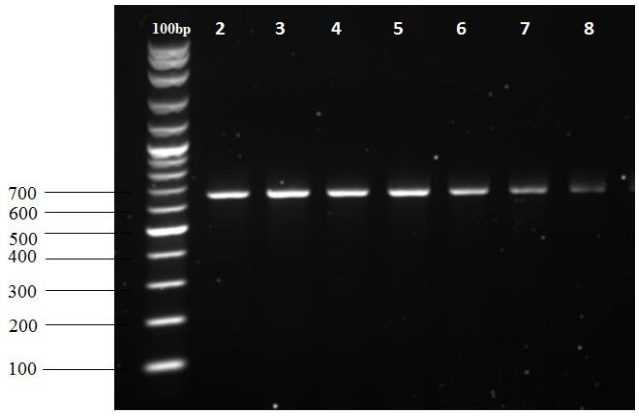

Figure 1. Initial screening of collected samples for DNA quality assessment. Visualization of *wsp* gene amplicon from *Drosophila melanogaster* (column 2-4), *w*Mel-infected Yogya *Aedes aegypti* (column 5-7); and *w*MelAlbB (column 8), A 100bp DNA marker (1).

Table 1. Sequence analysis of insertion sites in different *Wolbachia* hosts using MEGA ver. X software

Host	*Wolbachia* strain	Marker	Presence/ Absence
Drosophila melanogaster	*w*Mel	IS-WD1310	Absent
	*w*Mel	IS-WD516/7	Absent
Yogya *Ae. aegypti*	*w*Mel	IS-WD1310	Absent
	*w*Mel	IS-WD516/7	Absent

Table 2. Sequence analysis of tandem repeat loci in different *Wolbachia* hosts using a Tandem Repeat Finder ver. 3.1.

Host	Periodicity	
	VNTR-141 period	VNTR-105 period
*w*Mel (JF797613)*	7.3	n/a
*w*Mel (JF797619)*	n/a	4x1 + 2x0.5
Drosophila melanogaster isolate 1	6.3	4x1 + 2x0.5
Drosophila melanogaster isolate 2	6.3	4x1 + 2x0.5
Drosophila melanogaster isolate 3	6.3	4x1 + 2x0.5
Drosophila melanogaster isolate 4	6.3	4x1 + 2x0.5
Drosophila melanogaster isolate 5	6.3	4x1 + 2x0.5
Aedes aegypti isolate 1	7.2	4x1 + 2x0.5
Aedes aegypti isolate 2	7.2	4x1 + 2x0.5
Aedes aegypti isolate 3	n/a	4x1 + 2x0.5
Aedes aegypti isolate 4	7.3	4x1 + 2x0.5
Aedes aegypti isolate 5	7.3	4x1 + 2x0.5

Note: *wMel reference sequence (containing tandem repeat loci). n/a: not applicable

The current study evaluated some polymorphic markers as published by Riegler et al. (2005; 2012), even if the inversion period was not evaluated in this study. Wu et al. (2004) has submitted a complete genome map of *Wolbachia* strain *w*Mel. The *Wolbachia* genome shows a very high proportion of insertion sites of the transposable element, the VNTR loci, and genes encoding ankyrin return domain. The results of this study showed that insertion sites (IS-WD516/7 and WD1310) were absent in the sequenced fragment in both *Wolbachia* hosts i.e., *D. melanogaster* and *Ae. aegypti*. The proportion of tandem repeat periods seems to vary among isolates. The analysis of VNTR-141 loci matches the reference, even if a gap of 140bp sequence was found for *Wolbachia* endosymbiont of *D. melanogaster*. Riegler et al. (2012) reported the periodicity of 141bp element in *w*Mel genome is 7.3 (consisting of the internal 15bp direct repeat A, a 23bp hairpin with a 9bp palindromic stem, an 18bp insertion, and the internal 15bp direct repeat B). Meanwhile, the periodicity of 105bp element in *w*Mel full genome is 4x1 + 2x0.5 (containing four complete 105bp periods and two periods with 25bp internal deletions). The analysis of the VNTR-105 loci matches the reference in terms of length.

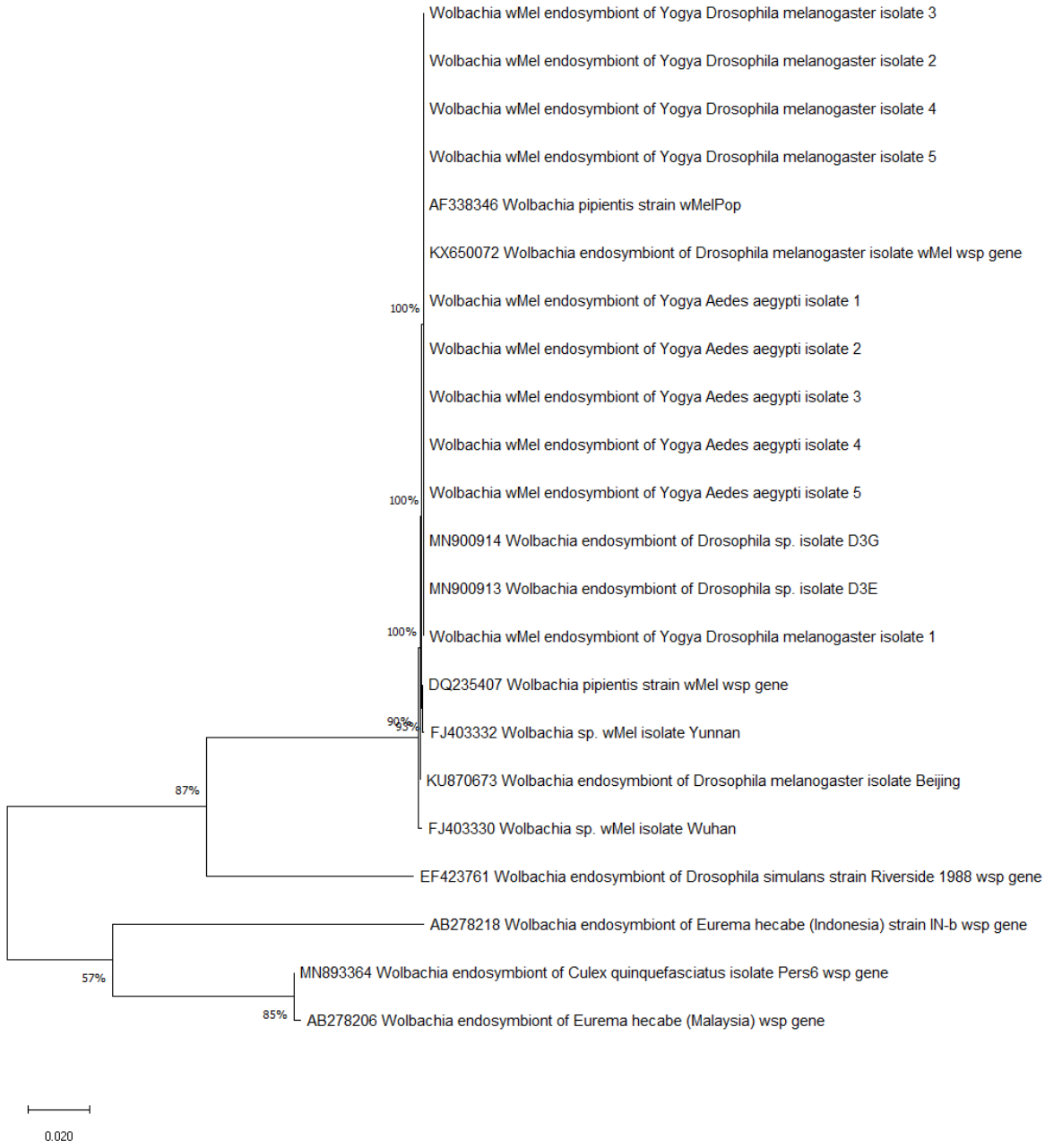

Figure 2. Phylogenetic tree of *Wolbachia* endosymbiont of *Drosophila melanogaster* and *w*Mel-infected Yogya *Aedes aegypti* based on the *wsp* gene sequence. *Wolbachia* endosymbiont of *Drosophila melanogaster* and *w*Mel-infected Yogya *Aedes aegypti* were placed in the same clade with *w*Mel reference sequence (KX650072), as well as *Wolbachia* strain *w*MelPop and *w*Mel.

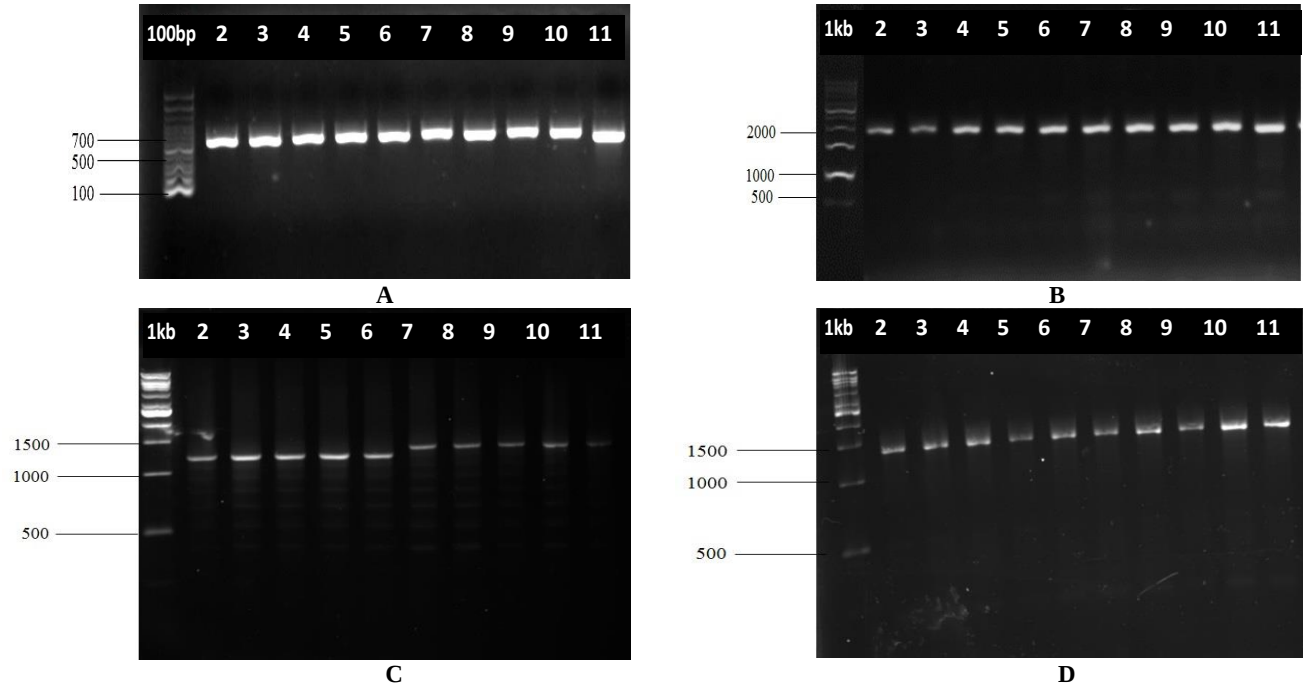

Figure 3. Visualization of PCR products from *Drosophila melanogaster* (n= 10) and *w*Mel-infected Yogya *Aedes aegypti* (n= 10) based on the insertion sites IS-WD1310 (A), IS-WD516/7 (B), tandem repeat loci VNTR-141 (C), and VNTR-105 (D) from *Drosophila melanogaster* (column 2-6) and *w*Mel-infected Yogya *Aedes aegypti* (column 7-11).

In conclusion, this study showed no difference in the genetic of *Wolbachia* among tested hosts and *w*Mel reference genome (NC002978). It means that *Wolbachia* from Yogya *D. melanogaster* and *Wolbachia* strain *w*Mel present in *Ae. aegypti* used for bio-control of dengue were genetically identic. This result supported previous studies which found that there are no or only slight genetic differences among *Wolbachia* strains in the natural host, transfected or novel host, and field-caught specimens. The key attribute of *Wolbachia* is that the World Mosquito Program is basing its intervention on its demonstrated ability to interfere with the replication of dengue in *Wolbachia*-infected *Aedes* mosquitoes. Thus this information of genetic similarity will be a positive response for community safety concerns, not only for the concern of capability of reducing dengue transmission but more importantly, that *Wolbachia* strain that being transfected into mosquito is similar to those in the natural organism. Questions such as the persistence of the virus blocking capacity of *Wolbachia* after generations in the natural population are essential to be answered in the future.

ACKNOWLEDGEMENTS

The authors thank all of World Mosquito Program (WMP) Yogyakarta team and Tahija Foundation for the financial support for the activities of WMP Yogyakarta, Indonesia.

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