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**RESEARCH ARTICLE**

# Phylogenetic Analysis of *Culex tritaeniorhynchus* and *Culex vishnui* Vector of Japanese Encephalitis Virus

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**Abstract**

*Culex tritaeniorhynchus* and *Culex vishnui* are medically essential mosquitoes that transmit the Japanese encephalitis (JE) virus. There is less information about the recording data and research due to genetic character differences among them. The objective of this study was to examine the genetic variation of *Cx. tritaeniorhynchus* and *Cx. vishnui* in 3 sites of Central Java using polymerase chain reaction randomly amplified polymorphic DNA (PCR-RAPD). The study was done in January to November 2017 in Pekalongan city, Pekalongan regency, and Semarang regency. Adult female mosquitoes collected by human bite method. DNA of ten *Cx. tritaeniorhynchus* samples and fifteen samples of *Cx. vishnui* purified using DNA extraction kit. Furthermore, PCR amplification was conducted with 5 RAPD primers (OPA 11, 12, 15, 16, and 20) and would run into 2% gel electrophoresis for 45 minutes. Cluster analysis was using MVSPTM software (version 3.1). The results showed 213 genetic characters of *Cx. vishnui*, while 142 characters shown by *Cx. tritaeniorhynchus*. The dendrograms showed three distinct groups of *Cx. vishnui* from 2 sites of Pekalongan and one site of Semarang, while *Cx. tritaeniorhynchus* showed two distinct groups, which were 1 group from Pekalongan and 1 group from Semarang. Low genetic similarity (<10%) shown *Cx. vishnui* from Pekalongan city and Pekalongan district, and there was no genetic similarity in *Cx. tritaeniorhynchus* from Pekalongan and Semarang. It concluded that the polymorphism of *Cx. tritaeniorhynchus* and *Cx. vishnui* reached 100%.

**Key words:** *Culex tritaeniorhynchus*, *Culex vishnui*, JE-vector, PCR-RAPD, phylogenetic analysis

# Analisis Filogenetik *Culex tritaeniorhynchus* dan *Culex vishnui* Vektor Virus *Japanese Encephalitis*

**Abstrak**

Nyamuk *Culex tritaeniorhynchus* dan *Culex vishnui* memiliki peran penting di bidang medis terutama dalam penularan virus *Japanese encephalitis* (JE). Sampai saat ini data dan riset tentang karakter genetik vektor JE masih sangat terbatas. Penelitian ini bertujuan menjelaskan variasi genetik *Cx. tritaeniorhynchus* dan *Cx. vishnui* di 3 lokasi di Jawa Tengah berdasar *polymerase chain reaction randomly amplified polymorphic DNA* (PCR-RAPD). Studi ini dilakukan dari bulan Januari sampai November 2017 di Kota Pekalongan, Kabupaten Pekalongan, dan Kabupaten Semarang. Metode *human bite* digunakan untuk koleksi nyamuk. Ekstraksi DNA nyamuk dilakukan pada 10 ekor *Cx. tritaeniorhynchus* dan 15 ekor *Cx. vishnui* menggunakan kit ekstraksi DNA. Selanjutnya, diamplifikasi dengan 5 macam primer RAPD (OPA 11, 12, 15, 16, dan 20), serta dielektroforesis pada 2% agar selama 45 menit. Analisis klaster dilakukan menggunakan program MVSPTM (versi 3.1). Ditemukan 213 dan 142 karakter genetik masing-masing pada *Cx. vishnui* dan *Cx. tritaeniorhynchus*. Analisis dendogram menunjukkan 3 grup yang berbeda untuk *Cx. vishnui*, sedangkan untuk *Cx. tritaeniorhynchus* terdapat 2 grup yang berbeda, yaitu 1 grup dari Pekalongan dan 1 grup dari Semarang. Similaritas genetik yang rendah (<10%) ditunjukkan *Cx. vishnui* dari Kota Pekalongan dan Kabupaten Pekalongan, bahkan tidak ada persamaan genetik pada *Cx. tritaeniorhynchus* dari Pekalongan dengan Semarang. Disimpulkan bahwa polimorfisme *Cx. tritaeniorhynchus* dan *Cx. vishnui* mencapai 100%.

**Kata kunci:** Analisis filogenetik, *Culex tritaeniorhynchus*, *Culex vishnui*, PCR-RAPD, vektor JE

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## Introduction

Japanese encephalitis (JE) is a zoonotic viral disease and a health problem in Asia, including Indonesia. In Asia, there were about 68,000 cases annually, with 30% of the case-fatality rate for encephalitis.[1] In Indonesia, the first JE case reported in 1962[2] and the number rises since then. In 2001–2004, there were 163 of JE cases, and 94 cases were serologically JE infection.[3] In 2016, there were 326 cases of JE in 11 provinces mostly in Bali (69.3%).[4] JE virus infection also reported in West Sumatera, West Kalimantan, Yogyakarta, Central Java, East Java, West and East Nusa Tenggara, and Papua.[5]

JE is a severe disease that may cause death and spread by the mosquito bite. In India, there is a success story in preventing this disease by intensive vector surveillance and immunization.[6,7] The significant vectors for JEV transmission belong to *Culex vishnui* subgroup, which comprises of *Cx. pseudovishnui* Colles. Though JEV isolated from 16 species of mosquitoes, the majority of the isolations are from *Cx. vishnui* complex, which breeds extensively in the rice ecosystem.[6] In Hongkong, there were 30 species of mosquitoes identified positively infected with the JE virus, which belonged to 5 genera: *Anopheles*, *Culex*, *Aedes*, *Armigeres*, and *Mansonia*, species of *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Cx. pseudovishnui*, *Cx. vishnui*, and *Cx. fuscocephala* becomes the vector.[8] In Indonesia, *Cx. tritaeniohynchus* is the primary vector of the JE and *Cx. vishnui* as a secondary or alternative vector of JE.[4]

The virus infects mainly in animals, pigs and wild birds. The viral agent of disease transmits to humans by the infected mosquito bite, from the genera of *Culex* mainly, *Cx. tritaeniorhynchus* that breeds in a rice field and also *Cx. vishnui* subgroup.[6] In Indonesia, there was limited information regarding recording data and research in terms of vector surveillance, and even less in the molecular study. The purpose of the study was to determine the genetic variation of *Cx. tritaeniorhynchus* and *Cx. vishnui* from Pekalongan city, Pekalongan regency, and Semarang regency using polymerase chain reaction rapid analysis polymorphism DNA (PCR-RAPD).

## Methods

The method was a descriptive study conducted from January to November 2017 in the areas of Pekalongan city, Pekalongan regency, and Semarang regency in Central Java, Indonesia. Adult mosquitoes collected by human bite methods using an aspirator and identified using a manual book from the Ministry of Health.[9] Five mosquito from each site extracted for the DNA using the DNA extraction kit,[10] "gSYNCTM" (Geneaid, Cat. No. GS 100, PT. Genetika Science Indonesia). Nine of 10-mer RAPD primers (1st BASE; OPA 1, OPA 2, OPA 8, OPA 9, OPA 11, OPA 12, OPA 15, OPA 16, OPA 20) has selected for the subset of mosquito DNAs, and five of them (OPA 11, OPA 12, OPA 15, OPA 16, and OPA 20) produced clear bands, and it applied to samples.[11] The five primers of RAPD used for the DNA amplification shown in Table 1.

There were 35 cycles for the PCR (Thermal Cycler, Boeco), steps, and annealing modified from the previous works.[10,12] The samples were then electrophoresis (Mini Run Gel Electrophoresis System GE-100), run in 2% of gel agarose for 45 minutes. The marker was 100 bp DNA ladder (Geneaid). Samples finally observed by UV transilluminator (Biorad).

The data from each electrophoresis were manually counted and scored for the real baseband value compared with the marker. If there was a band, the score is 1 (one); however, if there was no band, the score was 0 (zero). Cluster analysis using Multi-Variate Statistical Package (MVSP) software (version 3.1) used for data analysis. Dendrogram of each mosquito from each site analyzed by synthesis-descriptive analysis as percent similarity. This study approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Gadjah Mada University-Dr. Sardjito General Hospital with number Ref: KE/FK/0612/EC/2017.

## Results

*Culex tritaeniorhynchus* adult female was dominant and abundant in Semarang regency, while in Pekalongan city and Pekalongan regency *Cx. vishnui* was dominant and abundant. The optimum amplification DNA fragments obtained using five of nine OPA primers that produced numbers of *Cx. vishnui* and *Cx. tritaenirhynchus* DNA fragments.

There were, in total, 142 genetic characters of *Cx. Tritaeniorhynchus* from 2 sites of mosquito collection, while there were 213 genetic characters of *Cx. vishnui* from the three sites of mosquito

**Table 1 Primers for DNA Amplification**

| Primers[12] | Sequences (5'–3') | Annealing[9] (t°C) |
|---|---|---|
| OPA-11 | CAA TCG CCGT | 35°C |
| OPA-12 | TCG GCG ATAG | 35°C |
| OPA-15 | TTC CGA ACCC | 35°C |
| OPA-16 | AGC CAG CGAA | 35°C |
| OPA-20 | GTT GCG ATCC | 35°C |

collection (Table 2). The phylogenetic analysis was performed in percent of similarities and was showed in Figure 1–Figure 4.

Figure 2A showed two distinct groups of *Cx. vishnui* samples from Pekalongan city and Pekalongan regency, which show low similarities, less than 36%. The compilation of *Cx. vishnui* samples from Pekalongan city and regency (B2) separated in different group with the samples from Semarang regency (B1).

In Figure 4, *Cx. tritaeniorhynchus* from the two sites of collection, whereas 1 was Semarang regency and 2 was Pekalongan regency, both showed in two distinctive groups. The groups revealed that there was no (nul, 0%) similarities, it meant that *Cx. tritaeniorhynchus* from the two sites of collection had high genetic variation.

## Discussion

The use of molecular techniques, particularly the PCR-based DNA techniques, significantly improves our knowledge and understanding of the mosquito population and dynamics.[13] The study of phylogenetic was essential to understand the relationship between transmission and epidemiology. The technic can be used as a control of disease also the genetic structure of mosquito vector-based in the genetic characterization,[14] especially for the field mosquito species of *Cx. tritaeniorhynchus* and *Cx. vishnui*.

As the above results, in Table 2, the genetic character differences between mosquitoes may be due to the geographical and ecological character differences between the three sites of mosquitoes collection. The sites of mosquitoes collection in Pekalongan regency were a combination of rural-urban area type, with rice fields in some areas and crowded housing and a lot of avian and other domestic animals. However, in Pekalongan city, the sites were urban type housing, where there was no rice field. Because of the lack of breeding sites, *Cx. tritaeniorhynchus* might not collect from this site. The ecological character in Semarang regency is the rural type with rice field and domestic animal, avian, and mammal (cow).

Individual genetic variation influenced by the reproduction pattern of the species in the population. The variation due to the randomly individual selection as its couple, and it will produce random mating in the population.[15] Besides, natural selection and the ability of many habitat-exploitation of mosquito may cause this fauna to become a cosmopolitan insect, and the environmental changing will support the gene flow and develop the high genetic variation.[16]

There were 104 characters between *Cx. vishnui* from Pekalongan regency (Figure 1A), while there were 96 characters from Pekalongan city (Figure 1B) and 48 characters of samples from Semarang regency (Figure 1C). Figure 1 showed that there were two different groups from each sample site. *Culex vishnui* from Pekalongan city showed the highest genetic similarities, 20–35% compared with the two other sites, and there was about less than 20–30% and 5–35% for Pekalongan regency and Semarang regency respectively. These genetic similarities showed the closer of kinship, so mosquitoes from Pekalongan city revealed to be closer in kinship if compared with mosquitoes from Pekalongan regency and Semarang regency. There were 165 characters in the compilation

**Table 2 Genetic Characters of *Cx. tritaeniorhynchus* and *Cx. vishnui* from Pekalongan City, Pekalongan Regency, and Semarang Regency based on PCR-RAPD**

| Species | Number of Genetic Character from Sites of Collection | | | Total Character |
|---|---|---|---|---|
| | Pekalongan City | Pekalongan Regency | Semarang Regency | |
| *Culex tritaeniorhynchus* | – | 80 | 63 | 142 |
| *Culex vishnui* | 96 | 104 | 48 | 213 |

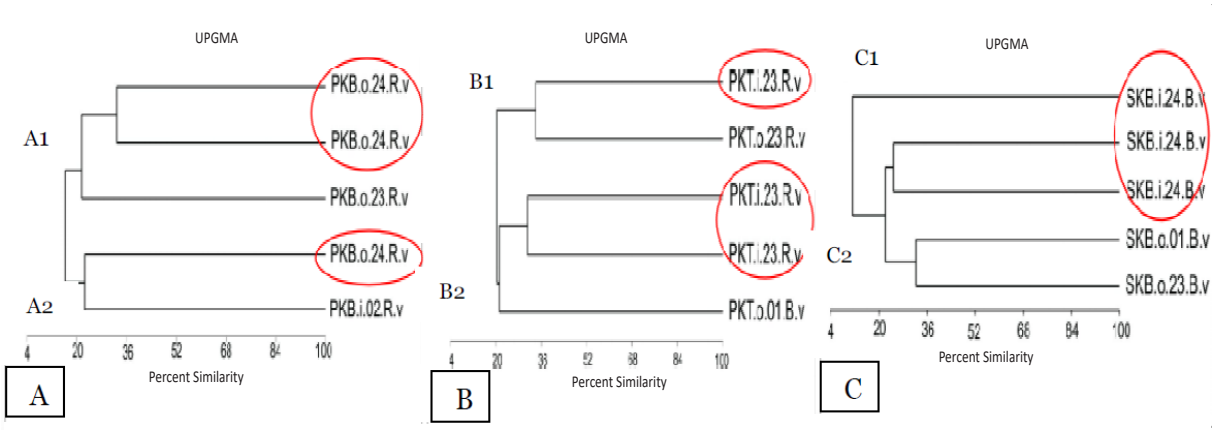


**Figure 1 Dendrogram Percent Similarities of *Culex vishnui* Genetic Character from Pekalongan Regency (A), Pekalongan City (B), and Semarang Regency (C)**

Axis refers to the distance (%) of genetic similarities of mosquito samples, PKB=Pekalongan regency, PKT=Pekalongan city, SKB=Semarang regency, o=outdoor, i=indoor, 24=24 hours of collection time, 23=23 hours of collection time, 02=2 hours of collection time, 01=1 hours of collection time, R=resting, B=biting, v=*Cx. vishnui*; Red circle in A=same code of mosquitoes (PKB.o.24.Rv) collected from Pekalongan regency in A1 and A2 groups, outdoor, at the 24 hours time, and catched at rest; Red circle in B=same code of mosquitoes (PKT.i.23.Rv) collected from Pekalongan city in B1 and B2 groups, indoor, at the 23 hours time, and at rest; Red circle in C=same code of mosquitoes (SKB.i.24.Bv) that collected from Semarang regency in C1 and C2 groups, indoor, at the 24 hours time, and cathed at bite

between *Cx. vishnui* samples from the two sites (Figure 2A), while there were 213 characters in compile data from the three collection sites (Figure 2B).

Three samples of *Cx. vishnui* from the same site and time of collection (code: PKBo.24.R.v) in Pekalongan regency also showed a different group of similarities. It could be understood that the mosquito was originally from different breeding sites.[17,18] *Culex vishnui* samples also showed a

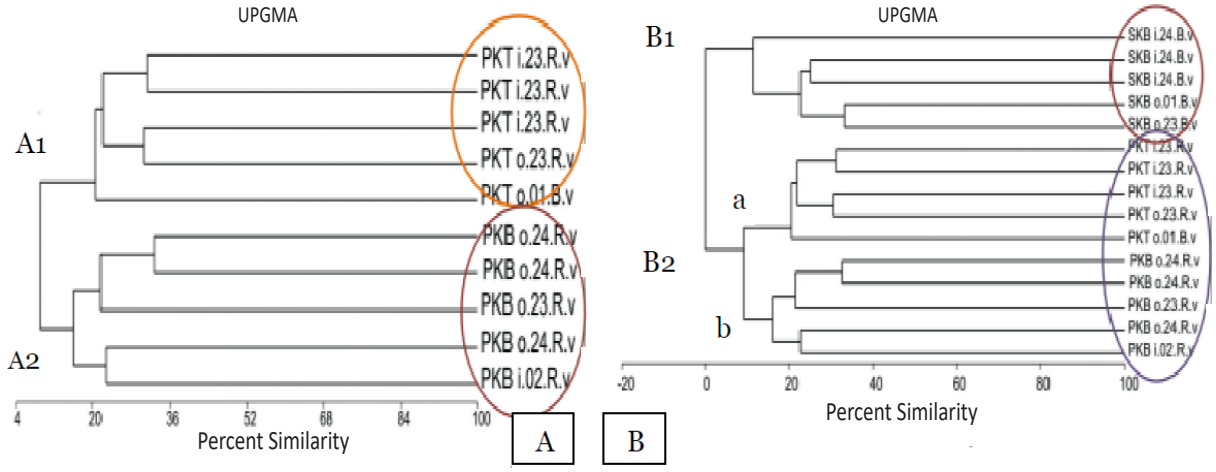


**Figure 2 Dendrogram Percent Similarities Compilation of *Culex vishnui* Genetic Character from Two (A) and Three (B) Sites of Collection**

Axis refers to the distance (%) of genetic similarities of mosquito samples, PKT=Pekalongan city, PKB=Pekalongan regency, SKB=Semarang regency, i=indoor, o=outdoor, 23=23 hours of collection time, 01=1 hours of collection time, 24=24 hours of collection time, 02=2 hours of collection time, R=resting, B=biting, v=*Cx. vishnui*; Red circle in A=same code of mosquitoes collected from Pekalongan city (A1, PKT.i.23.Rv) and Pekalongan regency (A2, PKB.o.24.Rv) groups, i=indoor, o=outdoor, at the 23 and 24 hours of collection time respectively and catched at rest; Brown circle in B=same code of mosquitoes collected from Semarang regency (B1, SKB.i.24.Bv); Blue circle in B=same code of mosquitoes collected from Pekalongan city (B2a, PKT.i.23.Rv), indoor, at 23 hours of collection time, at rest; and same code mosquitoes from Pekalongan regency (B2b, PKB.o.24.Rv), o=outdoor, 24=24 hours of collection time, R=resting, v=*Cx. vishnui*

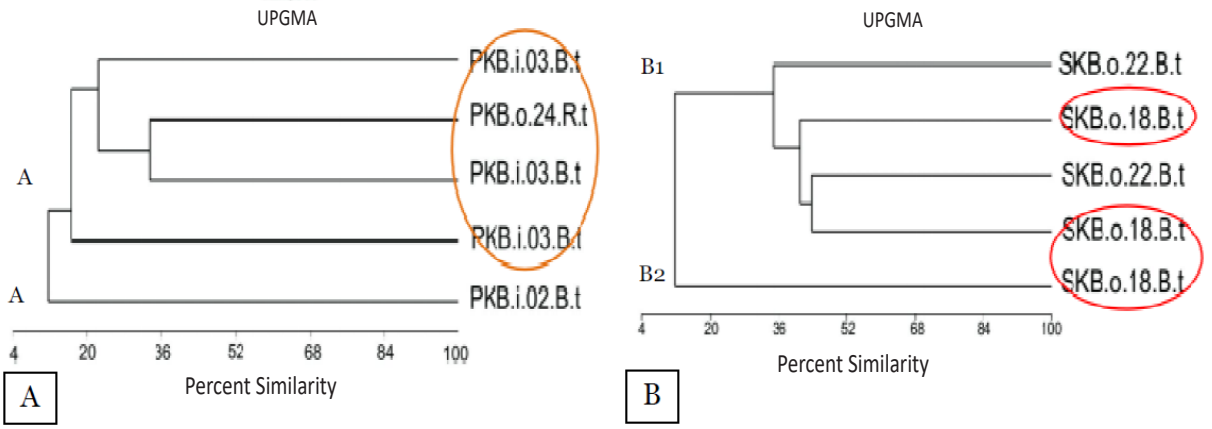


**Figure 3 Dendrogram Percent Similarities of *Culex tritaeniorhynchus* Genetic Character from Pekalongan (A) and Semarang Regency (B)**

Axis was the distance (%) of genetic similarities of mosquito samples; PKB=Pekalongan regency, SKB=Semarang regency, i=indoor, o=outdoor, 03=3 hours of collection time, 24=24 hours of collection time, 02=2 hours of collection time, 22=22 hours of collection time, 18=18 hours of collection time, B=biting, R=resting, t=*Cx. trytaeniorhynchus*; Red circle in A=same code of mosquitoes collected from Pekalongan regency (group A1 and A2), indoor, at the 03 hours of collection time and catched at bite; Red circle in B=same code of mosquitoes collected from Semarang regency (B1 and B2 groups), outdoor, at the 18 hours of collection time, and cathed at bite; It showed two distinct groups and had about 10% in similarity

significant distinct group and low similarities from Pekalongan city (PKT.i.23.R.v), two samples in one group, and 1 sample in the other group of similarities. Figure 2B showed that there were no character similarities at all of *Cx. vishnui* from Semarang and Pekalongan. This study revealed that there was up to 100% polymorphism of the genetic character of *Cx. vishnui* from the three sites of collection.

These findings showed there were significant genetic differences among the *Cx. vishnui* population. Kiliç et al.[18] said that the genetic

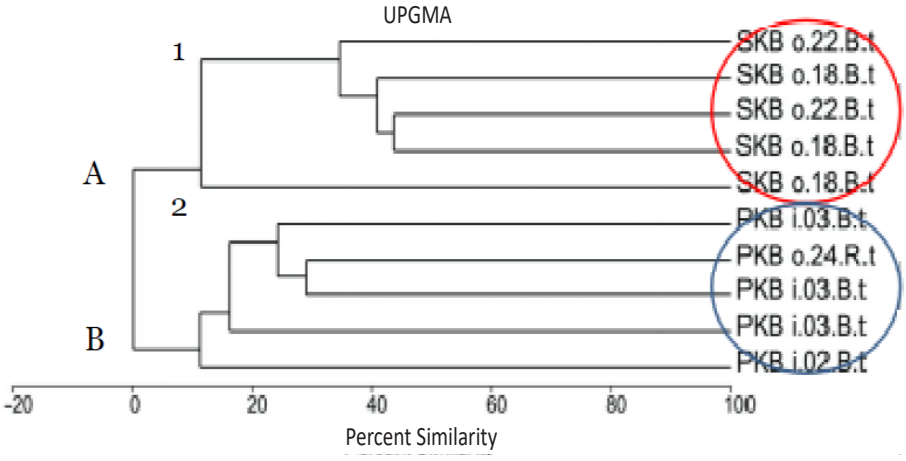


**Figure 4 Dendrogram Percent Similarities Compilation of *Culex tritaeniorhynchus* Genetic Character from Pekalongan and Semarang Regency**

Axis was the distance (%) of genetic similarities of mosquito samples; SKB=Semarang regency, PKB=Pekalongan regency, o=outdoor, i=indoor, 22=22 hours of collection time, 18=18 hours of collection time, 03=3 hours of collection time, 24=24 hours of collection time, 02=2 hours of collection time, B=biting, R=resting, t=*Cx. trytaeniorhynchus*; Red circle in group A (1 and 2)=same code of mosquitoes (SKB.o.18.Bt and SKB.o.22.Bt) collected from Semarang regency, outdoor, at the 18 and 22 hours of collection time and catched at bite; Blue circle=same code of mosquitoes collected from Pekalongan city in B (PKB.i.03.Bt), indoor, at the 03 hours of collection time, and at rest; It showed two distinct groups; The red and blue circle showed two distinct groups and there was no similarity (0%)

differentiation among *Cx. pipiens* in Aegean, Turkey indicated the high rate of gene flow among the population. Those findings suggest that *Cx. pipiens* are freely moving around the Aegean region in diverse habitat. Joice et al.[17] said that there was a significant finding of genetic divergence of *Cx. pipiens* population from five habitats in Merced in Central valley in California.

The genetic character of *Cx. tritaeniorhynchus* from Pekalongan regency were 80 characters and also showed high variation with two different groups. As shown in Figure 3A, there were three mosquito samples (PKBo.03.B) from the same time, behavior, and place that showed three different lines (group) of similarities. One of them separated and showed less than 20% of character similarities than the other two. These mosquitoes from Semarang regency (Figure 3B) showed 63 genetic characters and separated into two groups. Three samples of *Cx. triteniorhynchus* from the same collection time, behavior, and site had separated into three different lines in the dendrogram, with character similarity less than 10% (Figure 4). This condition may be due to the low genetic flow in Semarang regency rather than in Pekalongan regency. The low genetic flow might be due to the ecological character in Semarang regency that showed a close area with surrounding hardwood and rubber plantation. However, Pekalongan regency was an open area, rice field area. The *Cx. tritaeniorhynchus* samples from Pekalongan and Semarang regency showed almost dissimilarity of the genetic character, and it revealed that there was genetically 100% polymorphism.

In a significant population, naturally, random mating happens. The parental genetic combination will support to produce high genetic variation. The offspring individually may be the same in genotype but different in the phenotype. Otherwise, the offspring individually has the same phenotype but is genetically different.[16] This condition explains the genetic variation in the *Cx. vishnui* and *Cx. tritaeniorhynchus* population from the sites of collection.

There was minimal information regarding the surveillance and control of the JE vector in Indonesia; this might correlate with the variation of the geographic sites (topography, and annual rainfall), ecotype, and habitat of the vector in the areas of the city and regency. All this time, the vector surveillance, especially for *Cx. tritaeniorhynchus* was done in sporadic works if there were JE outbreaks. This obstacle might also correlate with supporting funding, as a researcher in Hongkong stated that the control program does not work because of time-consuming and expensive. It is challenging to cover all mosquito habitats, and it may cause environmental pollution. The JE vector control program may work by joining together with other mosquitoes controls.[19] In the future, the systematics of JE surveillance and standardized diagnosis should be established for better assessment and control program.[20]

In Indonesia, JE cases firstly reported from Lombok in 1960, and the virus was isolated from *Cx. trytaeniorhynchus* mosquito in 1972.[20] In 2018, there were 29 of 34 provinces reported as endemic areas for JE. The JE virus isolated from 10 species of mosquito: *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Cx. vishnui*, *Cx. fuscocephala*, *Cx. bitaeniorhynchus*, *Cx. quinquefascaitus*, *An. vagus*, *An. kochi*, *An. annularis*, and *Armigeres subalbatus*.[20] In Jambi province they found the first evidence of the JE genotype 1 was in *Cx. gelidus*.[21]

In Cambodia, a study in peri-urban and a rural pig farming showed that there were seventeen of mosquito species was founded, and *Cx. gelidus* was to be the most abundant, followed by *Cx. vishnui* group and *Cx. trytaeniorhynchus*.[22]

It reported that there were 3 billion people within 24 countries in Southeast Asia and Western Pacific have transmission risk of the JE disease.[23] WHO announced that JE vaccination should be extended in endemic areas if JE became a public health problem. Comprehensive JE immunization program is done in Japan, South Korea, Taiwan, and Thailand. Furthermore, the development of immunization programs will continue in China, India, Nepal, Sri Lanka, Vietnam, and also in Indonesia.[24]

There was declined of JE incident in Taiwan, South Korea and Japan, this because of the change of pig farming management and declined the land used management.[25] The good manage of land for rice field and pig farming would be reduce the breeding sites of mosquitoes and followed by reducing the risk factor of JE infection.[25,26] Besides that, it was also applied of vaccination, data showed that vaccination program have significant impact to reduce JE cases.[23,25] In Indonesia, there has announced that the immunization program to reduce and prevent the JE cases will start from March 2018.[4]

As a result, that immunization itself gives cost-effective, prevent and reduce for new JE cases, and it will be followed by an appropriate control measured.[6,20] Also, the results will give benefits for the success of the JE control program, as in India.[6] The used of mosquito repellent, long-sleaved cloths, coil or vaporizers were good for personal preventive from mosquitoes biting that was infected with virus.[23] In recent year, the PCR-RAPD technique is abandoned, because of the time consuming, and the bias of the data. There were other molecular techniques proposed and give better results and analysis, such as RT-PCR.

## Conclusions

There were low genetic similarities (less than 10%) of *Cx. vishnui* from Pekalongan city, Pekalongan regency, and Semarang regency. There were no genetic similarities of *Cx. tritaeniorhynchus* from Pekalongan and Semarang regency. This study revealed that there was up to 100% polymorphism of *Cx. tritaeniorhynchus* and also *Cx. vishnui* from Pekalongan city, Pekalongan regency, and Semarang regency.

## Conflict of Interest

There is no conflict of interest among the authors.

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