Artikel 7. Molecular identification, phylogeography, and genetic .....ATMPH_2017_10(2)_341-347

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Submission date: 27-Apr-2020 01:13PM (UTC+0700)
Submission ID: 1308860604
File name: ,_phylogeography,_and_genetic.....ATMPH_2017_10_2__341-347.docx (98.62K)
Word count: 2480
Character count: 14367

Annals of Tropical Medicine and Public Health

ORIGINAL ARTICLE
Year : 2017 | Volume : 10 | Issue : 2 | Page : 341--347

Molecular identification, phylogeography, and genetic diversity of Culex quinquefasciatus in Central Java province, Indonesia

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Abstract

Species identification of Culex quinquefasciatus mosquitoes is crucial for planning vector control program. The progress of molecular entomology tool supports a better understanding of the species identification. In the molecular study, it has grown ITS2 sequences which are used as a potential marker for species identification and phylogenetic analysis. The genetic diversity of Culex quinquefasciatus mosquitoes has been reported worldwide, but until now there has been no study of diversity of Cx. quinquefasciatus mosquitoes in Indonesia. The purpose of this study is to determine the genetic diversity of Culex quinquefasciatus as a filariasis vector in Central Java on the basis of ITS2 genes from ribosomal DNA. This study is done descriptively by collecting samples from filariasis endemic areas in Central Java. Results of the morphological and molecular analysis showed a difference of identification. ITS2 sequence alignment results of Cx. pipien complex from Central Java isolates have similarities with some isolates in many worlds. Phylogenetic analysis showed that ITS2 sequence of mosquitoes in this study is not monophyletic. The further results indicate the fact of introgression history ITS2 from Cx. pipien toCx. quinquefasciatus in Central Java. The introgression may occur before Cx. quinquefasciatus proliferate and spread in the Central Java region.

How to cite this article:
Chakim I, Tyasningrum WS, Wardani HC, Sayono S. Molecular identification, phylogeography, and genetic diversity of Culex quinquefasciatus in Central Java province, Indonesia.Ann Trop Med Public Health 2017;10:341-347

How to cite this URL:
Chakim I, Tyasningrum WS, Wardani HC, Sayono S. Molecular identification, phylogeography, and genetic diversity of Culex quinquefasciatus in Central Java province, Indonesia. Ann Trop Med Public Health [serial online] 2017 [cited 2020 Apr 24];10:341-347
Available from: http://www.atmph.org/text.asp?2017/10/2/341/208718

Full Text

Introduction

Culex mosquitoes, especially C. pipiens and C. quinquefasciatus, are the main vectors of filariasis in many regions of the world[1] including Middle East and Eastern Mediterranean countries.[2],[3] Culex is also responsible for the transmission of some viral diseases such as Rift Valley Fever,[4],[5],[6],[7] the West Nile virus,[5],[6],[7],[8] Saint Louis Encephalitis (SLE)[9] and Eastern Equine Encephalitis (EEE).[10] Their ability to transmit numerous disease agents greatly increases the potential risk of a sudden outbreak of any one of the diseases.[11] The distribution of Culex mosquitoes in various regions of the world, including Indonesia and its relationship with the incidence of filariasis has been reported.[1],[12],[13] The existence of Culex quinquefasciatus mosquito habitat was associated with the incidence of Filariasis.[14]

Species identification is very important to determine which species that become the main vector in various different geographical region, so understand the role of these species in the vector-borne disease can be understood and can be used for planning a vector-control program. The survey of vector density showed that Culex sp. is the most dominant species, both in Malaysia and Indonesia.[15],[16],[17] The density of Culex quinquefasciatus mosquitoes reaches 5.91 mosquitoes/person/hour in the house and 4.75 mosquitoes/person/hour in the outdoors.[18] The studies report in Indonesia informed that the Culex quinquefasciatus mosquito is the predominant species morphologically. However the variability in the morphological characteristics of interspecies and the emergence of sibling species reduces the effectiveness of morphological identification techniques. Sibling species is a species that is difficult or impossible to distinguish on the basis of morphological characteristics.[19] So it requires molecular confirmation. The progress of molecular entomology tool can support a better understanding of the species identification and genetic structure of the mosquitoes' population.[20]

The research managed to distinguish between Cx. pipien and Cx. quinquefasciatus based on ITS2 barcode was done in 1996.[21] In the same year, a research conducted by Miller et al. (1996) is the first study reveals the variability of interspecies on the basis of geographical region in the Cx. pipien complex.[22] After the 1990s, in the 21st century, research and publications related to Cx. pipien complex increasingly widespread. In 2006, a study explained the genetic distribution worldwide including America, Asia, and Africa region.[23] This study, which includes isolates from 28 countries, informed that Cx. quinquefasciatus divided into two different groups; Pacific group and New World group.[24] The molestus form of Cx. quinquefasciatus has similar characteristics with Cx. pipien. The same lineage of Cx. quinquefasciatus isolates from Malaysia and the isolates from East Africa and Asia is confirmed by Low et al. (2014) based on COI and COII genes. The low genetic diversity pattern in the COI and COII genes is increasingly giving confidence regarding to their uniformity at the genetic level.[25] This information is reinforced by Deghan et al. (2013) which showed a clear separation between Cx. quinquefasciatus and Cx. pipien.[26]

The research proves a strong correlation and at the same time also illustrates the variability of Cx. pipien complex. In Indonesia, especially in Central Java, the genetic diversity of Cx. quinquefasciatus mosquito as a main vector of filariasis has not been reported yet. On the other hand, the importance of species identification for planning a vector control program is very needed.

This study will identify the Cx. quinquefasciatus mosquitoes which are located in the Central Java province, Indonesia, and characterize their genetic diversity using ribosomal DNA Internal transcribed spacer II (ITS2). The ITS2 sequences from rDNA have been found and used for taxonomic classification.[27] The use of ITS2 region as a DNA barcode is because it is a potential phylogenetic marker and used widely for phylogenetic analysis at the genus or species level.[28],[29] So this study will provide important information related to Cx.

quinquefasciatus which became the main vector of filariasis in Indonesia.

Materials and Methods

Mosquito specimens

The specimen is Cx. quinquefasciatus mosquitoes which identified morphologically in the endemic areas of filariasis, Central Java, that are Semarang City, Demak District, Grobogan District, Jepara District and Pekalongan District [Figure 1]. Laboratory testing has done in Eijkman laboratory of molecular biology.{Figure 1}

DNA extraction and amplification

The DNA extracted from the specimens using Chelex-100 ion exchanger metho ITS2 region from rDNA for molecular identification is amplified using forward (Cx_ _F) and reverse (Cx_ITS2_R) primers.[21] In the ITS2 amplification, the PCR reaction contains 2.5 μL of PCR buffer, 0.5 μL of MgCl2 25 mM, 0.5 μl of dNTP 10 mM, 0.25 μL of forward and reverse primers, 0.2 μL Taq polymerase (KAPA), 2.5 μL of DNA template for each samples and 18.3 μL of ddH20. Thermocycling conditions for ITS2 amplification, that is, predenaturation with 95°C for 5', 40 cycles of denaturation at 95°C for 30", annealing at 50°C for 1', and extension at 72°C for 1' and postextension at 72°C for 5'. The ch ogram from the sequence is edited manually using Finch TV program. The comparison with the sequences data which is available on the GenBank using BLAST searching tool from ncbi.nlm.nih.gov/.

Sequences alignment and phylogenetic tree construction

ITS2 sequences of the samples is aligned with Cx. pipien and Cx. quinquefasciatus sequences from various areas worldwide which is obtained from GenBank using BioEdit 7.1.9 program. The phylogenetic correlation from the samples and GenBank sequences analyzed using maximum likelihood (ML) method based on neighbor-joining model with bootstrap test (1000 bootstrap) using MEGA 6 program. GenBank sequences which are used for comparison are Cx. quinquefasciatus and Cx. pipien with accession number Z48468.1 and X75817.1. However, the average number of polymorphic sites is counted using DnaSp 5.10.1.

Results

Morphological analysis res on mosquito samples collected from five districts/cities in the endemic areas of filariasis, Central Java, showed that all of the samples are identified as Cx. quinquefasciatus species. On the other hand, the molecular analysis showed the existence of species identification variation, that is, Cx. quinquefasciatus (66.6%) and Cx. pipien (33.5%) from all of the sample sites [Figure 2]. The ITS2 fragment of these complex Cx. pipien ranges 400–476 base pairs. The variation in the length of the base is because there is an indel in the sequences. Average of the GC content is 57.68%.{Figure 2}

The reference sequences that used are Cx. quinquefasciatus and Cx. pipien isolates with accession number Z48468.1 and X75817.1 along 512 base pairs. After they are aligned, the result of 486 base pairs of the sequences found 70 alignment gaps and 268 monomorphic sites [Figure 3]. Furthermore, this study has detected the average value of p-distance is 0.9% from isolates in this study compared with Cx. quinquefasciatus and Cx. pipien isolates from various region worldwide (USA, China, Brazil and Bangladesh). P-distance values among Cx. pipien and Cx. quinquefasciatus originating from Jawa Tengah are 0.4% and 0.7% respectively. Meanwhile, according to the geographical region, that is, Semarang, Grobogan, Jepara, Pekalongan and Demak has a range between 0.8-2.5%. If compared with outgroup taxa, the p-distance value becomes 7%.{Figure 3}

Phylogenetic analysis has done with neighbor joining method and 1000 boostrap [Figure 4]. The sequences that become reference in the filogenetic analysis were Cx. quinquefasciatus (Z48468.1) and Cx. pipien (X75817.1). The other sequences from various regions (USA, China, Brazil, and Bangladesh) become comparator in the analysis. Cx. tritaeniorhynchus, Cx. pseudovishnui, Aedes sylvaticum, and Aedes vittatus become the outgroup taxa. Phylogenetic analysis showed that the Cx. pipien dan Cx. quinquefasciatus in this study are not monophyletic. Phylogenetic tree showed that Cx. quinquefasciatus and Cx. pipien in this study are in the same branching with the isolates from Bangladesh, China, Japan, Iran, USA and also Cx. quinquefasciatus referral (Z48468.1). However the isolates from Semarang (Semarang contig 1) broke away from the clades, along with the isolates from Brazil, Bangladesh and Cx. pipien referral (X75817.1).{Figure 4}

Discussion

The result showed that all of the morphological identification of the sample identified as Cx. quinquefasciatus, but the molecular analysis provided more varied identification result, that there is a part of the samples which is Cx. pipien species. This is possible because the determination keys for Cx. pipien complex species is still not fully able to distinguish the morphological variation in the complex.[30],[31] The other study suggests latitude on the wings as well as genital organ of female mosquitoes can be used for determination of Cx. pipien complex species.[23],[32]

P-distance value in this study based on the original region in Central Java Province, that is, Semarang City, Demak District, Pekalongan District, Grobogan District, and Jepara District ranges 0.8–2.5%. It demonstrates the high level of variation in Cx. pipien complex in Central Java. For comparison, the ITS2 variation in Cx. quinquefasciatus in Bangladesh shows the p-distance ranges 0.3–1.1%.[33] P-distance average in Cx. quinquefasciatus from Brazil is 0.9%.[34] These facts indicate that Cx. pipien complex isolates from Central Java have higher variation compared with other regions worldwide. It may be caused by the distance variation and geographical location, so it has the impact on genetic variation of the species.[19]

The ITS2 sequence alignment result of Cx. pipien complex from Central Java showed the similarity with Vinogradova et al. that there is a difference in the number of ACG at the base 243.[24] An insertion of ACG has present at the base 244-246. In all of the sequences, CGT was repeated twice in the nucleotide region of 344 and once in 360. In addition, there are four indel bases at position 109-114which is TACC, it is in line with the Cx. pipien quinquefasciatus from China (AF305553.1).[35] Furthermore, at the base 223-225 was also found an indel of GTC. These indel was also found in Cx. quinquefasciatus from Bangladesh (FJ416058.1), but with different formation of the base that is GCC. The sequence similarity of several loci and indel with other region showed an offspring variation to Cx. pipien complex from Central Java. It indicates a high recombination activity in the ITS2 region, especially from Central Java.

Phylogenetic analysis showed that the sequence from this study was not monophiletic. This phenomenon may occur as a result of changes in multiple pseudogene sequences that have undergone many substitution bases relative to the functional alleles.[36] Phylogenetic analysis of Cx. pipien complex from Central Java also indicates that contingents are located in different clusters with several sequences from various regions. This ambiguity shows the evolutionary forces that vary in ITS2 region.

Cases of noncoding regions, such as ITS2, integrated evolution often have a permanent impact on interspecies differences and maintain homogeneity of intragenomic. Ribosomal DNA has high homogeneity efficiency.[37] However, Cx. pipien complex from Central Java showed a high intragenomic variation with p-distance value ranges 0.8-2.5%. If compared with interspecies diversity in some other areas, it becomes 0.1-0.9%. These results indicate that the mutation rate of ITS region was much higher than the level of homogenization.[37],[38] On the basis of the result, different chromosomes in each individual genome contain various types of ITS2 sequences which are not homogenous. The low homogenization level eventually has an impact to the integrated evolution effect within a species. Intragenomic wide variation to ITS2 segments illustrates that the region has not been evolved to do with consistency than other chromosomal region.[22],[23]

Phylogenetic analysis also showed that Cx. pipien and Cx. quinquefasciatus both isolates from various regions worldwide and Central Java are not separated properly. In South Africa and United states, most of Cx. quinquefasciatus are simpatric with Cx. pipien. Research has shown that in South Africa, both Cx. quinquefasciatus and Cx. pipien is still a separate species,[39] parental polymorphisms which is happened in an integrated manner is the most plausible explanation to the simpatric phenomenon.[40] On the other hand, the rate of interspecific gene widespread in United States and with introgression to some parts of ITS2 sequence from Cx. pipien and Cx. quinquefasciatus population may be responsible to the simpatric phenomenon that has been observed.[23],[39],[40] In Indonesia, the possibility of interspecific introgression is because Cx. pipien species had never been found before.[16],[17],[41] Most of the sequence of Cx. quinquefasciatus and Cx. pipien from this research has a different genetic structure with other region, it showed a distinct evolutionary history in the contingent. This finding has similarity with Hasan et al. in the analysis of ITS2 to Cx. pipien complex from Bangladesh.[33] A possible explanation to the phenomenon is the existence of parental introgression to the offspring in some parts of ITS2 sequence from Cx. pipien that occurred before the separation of Cx. quiquefasciatus in the Central Java region[33].

Acknowledgments

The authors wish to thank people who have consented to take a part in this study, to Eijkman Institute for Molecular Biology in Jakarta; Health Office of Semarang City, Grobogan District, Jepara District, Demak District, and Pekalongan District, and those who have helped.

Financial support and sponsorship

This research was funded by Kementrian riset, teknologi dan pendidikan tinggi.

Conflicts of interest

There are no conflicts of interest.[42]

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