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Existence of Two Distinct Whiteflies in Chilli-Pepper Cultivation in West Sumatra-Indonesia Based on Mitochondria Cytochrome Oxidase I Gene Sequences

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ABSTRACT

Whiteflies have attracted intensive attention from many agriculturists due to their direct feeding and plant virus transmission. The economic losses depend on certain species population existing on the field during chilli-pepper cultivation. More over, biotype characteristic will determine the effectivity of applied pest management. Based on this, detailed information on whiteflies in chilli-pepper cultivation is very important. We applied mitochondria cytochrome oxidase unit I gene sequence for detecting species and biotype of whiteflies existing in West Sumatera. Two species were successfully identified i.e., *Bemisia tabaci* and *Trialeourides vaporariorum* that are dominant in this region. Distribution of both species are separated by altitude, where *B. tabaci* exist predominantly from low altitude to medium altitude, whereas in high altitude it is not common. On the other hand, *T. vaporariorum* distribute mainly in high altitude and not in medium altitude. Furthermore biotype determination on *B. tabaci* identified the existence of B biotype in all areas studied.

Key words: *Bemisia tabaci*, *Trialeorides vaporariorum*, cytochrom oxidase I, biotype, geminivirus

INTRODUCTION

Whiteflies cover aproximately 1,500 species distributed almost all over the world (Martin, 2004). Their effect on agricultural production was reported by many authors from tropical to sub-tropical regions and amounts to billions of dollars. Economic loses caused by whitefly are not only due to crop losses by direct feeding, sucking the sap from the plant and finally disturbing plant growth. The pest also produce honeymildew, encouraging sooty mould growth and finally decreasing plant vigour (Jelinek, 2010). The most dangerous, whiteflies are responsible for transmission of about 110 plant virus species (Jones, 2003; Martin *et al*., 2000; Mugiira *et al*., 2008; Qiu *et al*., 2009).

The family of whitefly is groupped in the order of Hemiptera (Von Dohlen and Moran, 1995) and contains two subfamilies, Aleurodicinae and Aleyrodinae (Mound and Halsey, 1978). Two species from subfamily Aleyrodinae; *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) and

Trialeurodes vaporariorum (West.) (Hemiptera: Aleyrodidae) attract great attention from many agricultural practicists as well as scientists from many tropical and sub tropical continents. This is due to their role in virus transmission and spreading in a wide variety of vegetable, ornamental plants and other crops worldwide (Jones, 2003; Ma *et al*., 2007).

West Sumatera is one of the chilli pepper production centres in Indonesia. In this region chilli fruit is one of the food components playing an important role in people's diet. Based on this reason, chilli production has a significant economic role not only in the province but also on the national level. However, pepper yellow leafcurl disease (PepYLCD) which is caused by a group of begomovirus attributed as peppper yellow leaf curl virus (PepYLCV) which is transmitted by whitefly has reduced chilli production during in the last two decades (Meliansyah *et al*., 2011).

Effectivity of virus transmission is determined by its vector. Therefore, knowledge of the species is indispensable for providing basic knowledge of pest and disease interaction in terms of epidemiology studies in integrated pest management. It is also well known that compatibility plays an important role in the success of virus transmisssion via whiteflies. For this reason understanding of genetic variation between pathogens and their vectors could disclose compatibility mechanisms that could be useful for development of disease management.

So far, in Indonesia, *Bemisis tabacci* is the only species of whitefly reported as vector for PepYLCV transmision, hence existence of another whitefly species has been reported by some authors in Java Island (Fitriasari, 2010; Hartono and Wijonarko, 2007). Currently, based on morphological and bioassay analysis only two biotypes namely B-biotype and non-B biotype are currently reported (Hidayat *et al*., 2004). The difference in biotype was believed also to be associated with fast growing and spreading of viruses and also their resistence against some insecticides (Horowitz *et al*., 2011; Shadmany *et al*., 2013). Certain biotype were reported to be more resistent against organophosphates, carbamates and pyrethroids (Scott *et al*., 2007). Q-Biotype for instnace is known to be more resistant, or at least has reduced susceptibility, to a number of neonicotinoids (Pascual and Callejas, 2004; Horowitz *et al*., 2005) and some insect growth regulators. This in turn forces to adapt chemical treatment. Therefore, comprehensive information on genetic variability of whitefly including their biotype could enhance the effectivitiy of disease management in chilli pepper cultivation.

Many studies dealing with assessing genetic variation of whitefly have been carried out by using random DNA sequence for instance RAPD-PCR (Gawel and Bartlett, 1993; Perring *et al*., 1993; De Barro and Driver, 1997; Khasdan *et al*., 2005) and AFLP (Amplified Fragment Length Polymorphism) (Cervera *et al*., 2000). Currently, analysis based on specific conserved sequence region for instance ribosomal and mitochondria cytochrome oxidase unit I (mtCOI) gene sequence (De Barro *et al*., 2005; Dinsdale *et al*., 2010; Rocha *et al*., 2011) become to be more common in this field. Biotype determination is also suggested to be possible by this method.

The present study was reported on genetic variation of whitefly population in chilli cultivation in West Sumatera. Genetic variation were assessed using mtCOI gene sequence and used the data also for biotype determination among species.

MATERIALS AND METHODS

Sample collection: Fourtytwo whiteflies were collected randomly from three different altitudes representing low, medium and highland. Lowland region (0-300 m above sea level (asl)) was represented by three regencies: Padang, Pesisir Selatan and Pasaman Barat. Mediumland region (301-700 m asl) was represented by Solok and 50 Kota and highland region (700 m asl) was represented by Agam and Tanah Datar. The number of samples collected from each region is listed

Table 1: Number of whitefly samples collected from chilli pepper plant from different locations in the study*

Altitude	Locality	Number of samples	Biotype
Low (0-300 asl)	Padang	5	All "B"
	Pesisir selatan	7	All "B"
	Pasaman barat	9	All "B"
Medium (301-700 asl)	Solok	5	All "B"
	50 Kota	6	5"B", 1 *T. vaporariorum*
High (>700 asl)	Agam	5	4"B", 1 *T. vaporariorum*
	Tanah datar	5	All *T. vaporariorum*
Total number of collected sample		42	

*One sample was provided from Bogor, designated as: BGR-R and used as reference sample

in Table 1. Whitefly from Bogor was used as a reference sample, so that total number of sample is 43. Sample collection was done by capturing nymph and adult whitefly using a zipper bag with caps on the bottom side so that the imago entered into zipper bag. Each sample was labelled and in each zipper bag contained only 1 single whitefly. Samples were stored at -20°C before use.

DNA isolation: The DNA of whitefly was isolated using a protocol by Goodwin *et al.* (1994) with minor modification. Briefly, DNA isolation was done as followed: The 1 single whitefly was placed into 1.5 mL centrifuge tube and crushed into powder with a micro plastic pestel with the presence of liquid nitrogen. One hundred twenty five microliter of CTAB extraction buffer containing 2% CTAB (w/v), 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA and 1% polyvinylpyrrolidone (PVP-40) was added to the powder, vortexed and subsequently incubated at 65°C for 5 min. One hundred twenty five microliter of chloroform:isoamyl alcohol (CI) (24:1) mixture was added and further incubated at room temperature for 20 min before centrifugation at 8,000 rpm for 5 min. The supernatant was transferred into 1.5 μL sterile centrifuge tubes and after that 10 μL of 3M sodium acetate (pH 5.2) was added. Two hundred fifty microliter of cold absolute ethanol was added and incubated for 30 min at -20°C. Supernatant was discarded after centrifugation at 11,500 rpm for 15 min. The Pellet was washed with 200 μL of 70% cold ethanol and recentrifuged again at 11,500 rpm for 2 min. Ethanol was then discarded and white pellets were dried for 10 min at room temperature. The pellets were resuspended in 10 μL of sterile water and stored at -20°C before use.

DNA amplification and partial sequencing of COI-gene: The extracted DNA was amplified using a primer pair designed from the Cytochrome Oxidase-subunit I (COI) gene sequence generated from mitochondria genome (Frohlich *et al.*, 1999). The sequence of primer was as follow: C1-J-2195-FW (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and C1-J-2195-RV (5-'TCCAATGCAATAA-TCTGCCATATTA-3') (Simon *et al.*, 1994; Frohlich *et al.*, 1999). Amplification using this primer pair should produce a fragment about 880 bp in size (Rocha *et al.*, 2011). The PCR reactions were performed in a total volume of 25 μL consisting of 2 μL of DNA template, 4 μL of each forward and reverse primer mtCOI (C1-J-2195-FW/RV) (5 pmol μL^{-1}), RTG-PCR bead (GE-Healthcare, UK) and 19 μL ddH_2O. Main amplification was performed in 30 cycles using a PCR machine (Biometra-Germany), started with initial denaturation of 95°C for 3 min. Main PCR condition was set as follow: Denaturation, 95°C for 5 min, annealing temperature at 55°C for 1 min, extension for 1 min at a temperature of 72°C. One additional extensions was done at 72°C for 5 min. The PCR product was stored at 4°C before use. Controlling the success of the amplification reaction, electrophoresis was performed on 1% agarose in 0.5x TBE buffer at 100 VOLTS for 60 min (Sambrook *et al.*, 1989). Visualization of PCR product was done using UV-transiluminator (Biometra-Germany) after gel staining with Ethidium bromide. Data was

documented and stored in jpeg format. Sequencing of PCR products was performed in PT CPI (Charoen Phokphand Indonesia-Indonesia). After purification sequencing was done directly in one direction using primer C1-J-2195-FW with 20 μL of PCR products.

Sequence and bioinformatic analysis: Sequence data was edited and analyzed using BioEdit (http://www.mbio.ncsu. edu/BioEdit/bioedit.html). Homology search of the sequence product was done using BLAST programme at http://blast.ncbi.nlm.nih.gov/Blast.cgi. (Altschul *et al*., 1990) Alignment was done using ClustalW2 provided at: http://www.ebi.ac.uk/Tools/msa/clustalw2/ (Thompson *et al*., 1994) and determination of biotype was simulated *in-silico* by means clustering analysis. In this respect two mtCOI gene sequence from each B-, A-, Q and M-biotype which is available in NCBI nucleotide database were used as comparison during clustering.

RESULTS AND DISCUSSION

Agricultural practices in sampling location: Fourtytwo whiteflies in total were collected from three different geographical conditions: Low, medium and high altitude. High altitute (Agam and Tanah Datar) is a region where agricultural cultivation is dominated by vegetable (chilli pepper, bean and tomato). Those three vegetables are intensively cultivated in this region (Badan Pusat Statistik Kabupaten Tanah Datar, 2013). Furthermore, in some regions intensive polyculture system or rotating cropping systems composed of those vegetables is commonly practiced in the regions.

The whitefly problem is supported by favorable wet tropical agroclimatic condition, where temperature range between 15-25°C (Badan Pusat Statistik Kabupaten Tanah Datar, 2013). Due to the intensive vegetable cultivation, the amount of pesticide application particularly insecticides in this region is very high. Sometimes, farmers use pesticides concentration even more than recommended, thus provoking increase in insect resistencies. Medium altitude region (Solok and 50 Kota) generally is dominated by rainforest. Agricultural practices are dominated with cereal cultivation, whereas only some vegetables ie., chilli pepper, bean and tomato cultivation are sporadically practiced. Similarly in lowland region (Padang, Pesisir Selatan and Pasaman Barat), vegetable is only grown sporadically. The agricultural system practiced in the regions seemed to contributed to the different dominance of the whitefly species (Fig. 1b).

Species identification and distribution: All the 43 whitefly DNA samples were successfully amplified and produced an about 880 bp fragment (Fig. 1a). Prior to sequencing, PCR products were purified with Wizard SV-Gel and PCR Clean-Up System (Promega-USA). Since sequencing was done only from forward direction, only part of the total fragment could be identified. The sequence data was edited and trimmed for ambiguous nucleotides and the final fasta sequence was prepared for further analysis. However since not all PCR fragment could be sequenced completely, only 541 bases in average of each samples could be successfully used in BLAST search homology analysis for species determination.

BLAST homology search showed that 36 samples had significant (99% identity) with Cytochrome oxydase I gene of *Bemisia tabacci* sequence, whereas seven samples (TD-01 to TD-05, 50K-04 and AG-04) showed significant homology (99% identity) with Cytochrome oxydase I mitochondrial gene of *Trialeurodes vaporariorum* sequence. Based on this data distribution of both species is shown in Fig. 1b.

Figure 1b showed that all *B. tabaci*, could be found in both lowland (0-300 asl) (Padang, Pesisir Selatan, Pasaman Barat) and mediumland (301-700 asl) (Solok, 50 Kota) but on the other hand

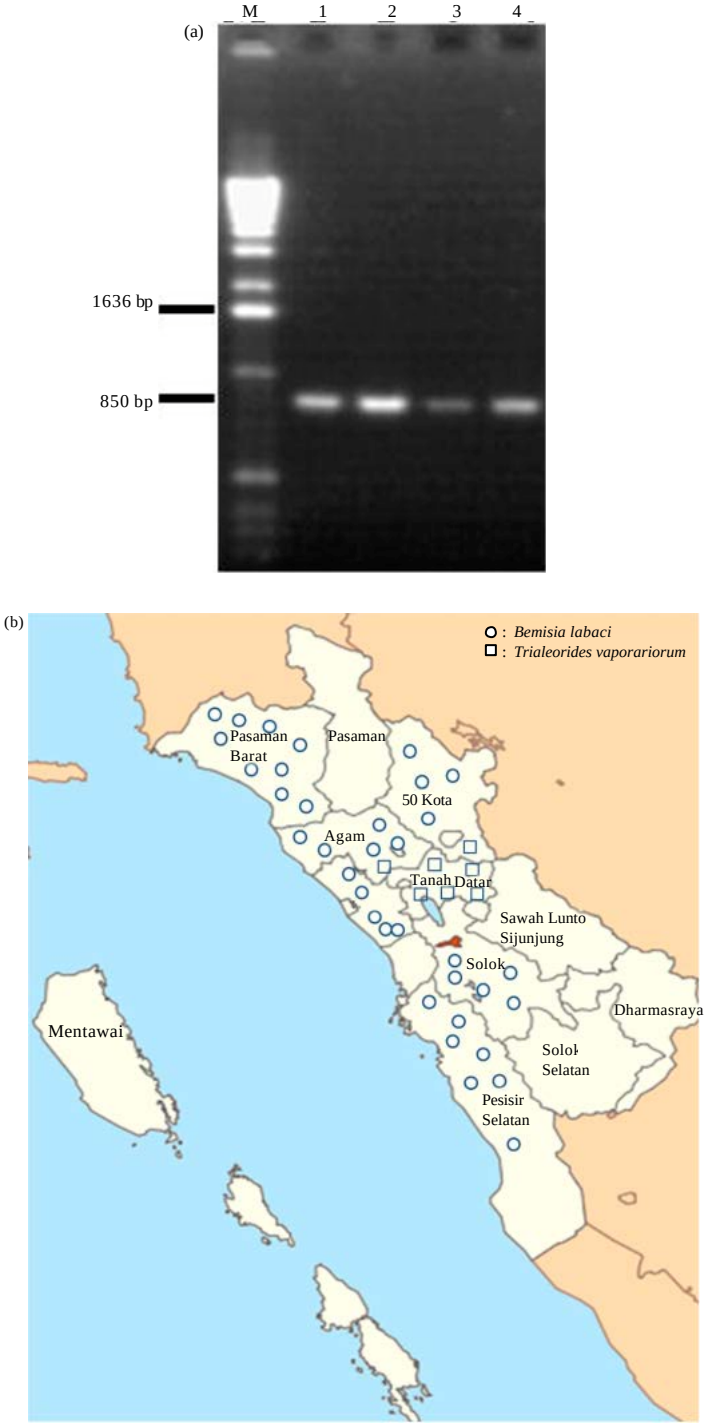

Fig. 1(a-b): Representative of PCR products from collected whitefly samples with (a) Primer combination C1-J-2195-FW/RV and (b) Whitefly species distribution in West Sumatera. Panel A, a single 880 bp fragment is expected from the amplification of mtCOI gene. Panel B, distribution of sampling location and species of whitefly based on mtCOI sequence DNA in three different altitudes

T. vaporariorum mainly (all five samples) could be found only at highland (>700 asl) (Tanah Datar) and are comparatively rare (1 from 5 in mediuml and 50 Kota and 1 from 6 at highland (Agam). Locations where *T. vaporariorum* could be found mainly in highland region (T. Datar and Agam), eventhough in mediuml and (50 Kota) *T. vaporariorum* rarely could also be found. This phenomenone is in concordance with data published by Caballero (1994) where *B. tabaci* predominantly populated in lowland region (lower than 1000 asl) whereas *T. vaporariorum* predominantly populated in highland region (above 1000 m asl). This, however, was valid for equatorial region. In other lower altitude tropical region (below 700 m asl), where temperature range from 19.9-20.3°C. *Trialeourides vaporariorum* could still also be predominant (Lourencao *et al*., 2008). In Indonesia, distribution of *T. vaporariorum* was reported predominantly in highland region (Fitriasari, 2010). This however was observed in tomatoe production centre in West Java (Hartono and Wijonarko, 2007) and no data so far reported from chilli pepper cultivation area.

Xie *et al*. (2007) reported that the highland preference of *T. vaporariorum* is supported by a broad adaptation of all growth phases of *T. vaporariorum* to low temperature compared to *B. tabaci*. Therefore it is reasonable why *T. vaporariorum* is more dominant in highland regions. Another reason why *T. vaporariorum* is more dominant in highland region compared to *B. tabaci* is probably due to its broad resistancy to diverse pesticides especially insecticides. Erdogan *et al*. (2012) reported resistance potential to chlorpyrifos ethyl-oxon and acetylcholinesterase in Turkey, whereas Gorman *et al*. (2007) reported resistance to neonicotinoid and imidacloprid.

The data indicated that favorable conditions for *B. tabaci* and *T. vaporarium* are dominantly determined by agroclimatic and agricultural system.

In order to study whether *T. vaporariorum* plays also a role in PepYLCV distribution, we amplified all four DNA from 50K-04, AG-04, TD-05 and TD-03 using CPc forward (5'-GTACAWGCCATATACAATAACAAGGC-3) and Cpv reverse primer combination (5'-ACGCCCGYCTCGAAGGTTCG-3) (Gorsane *et al*., 2004). Say that these are PepYLCV primers and possibly also where they match the genome of the virus all four samples could in fact produced an about 1,600 bp fragment as expected. Do you have a negative control for this? This indicated, that *T. vaporariorum* in fact containing PepYLCV DNA particle. However there is not yet any proof that *T. vaporariorum* is involved in geminivirus transmission, although *T. vaporariorum* can, like *B. tabaci*, ingest geminivirus particles from virus infected plants (Polston *et al*., 1990; Rosell *et al*., 1999). However, almost all publications dealing with relationship between *T. vaporariorum* and geminivirus come to the conclusion that it has no vector function (Brown and Bird, 1992; Duffus, 1987; Polston *et al*., 1990). However, the role of *T. vaporariorum* in virus transmission must not be underestimated. Recent publications showed that this whitefly plays an important role in transmission of Tomato Infectious Chlorosis Virus (TICV) (Duffus *et al*., 1996b; Wisler *et al*., 1998). They act also in *Cucumber yellows virus* (CuYV) transmission which causes a yellowing disease on cucumber (*Cucumis sativus* L.) and melon (*C. melo* L.) (Yamashita *et al*., 1979; Hartono *et al*., 2003; Lourencao *et al*., 2008). Moreover, *T. vaporariorum* also transmit some other known whitefly-transmitted yellowing viruses i.e., *Lettuce infectious yellows virus* (LIYV) (Duffus *et al*., 1986), *Tomato chlorosis virus* (ToCV) (Wisler *et al*., 1998), *Sweet potato chlorotic stunt virus* (SPCSV) (Pio-Ribeiro *et al*., 1996), *Cucurbit yellow stunting disorder virus* (CYSDV) (Celix *et al*., 1996), *Potato yellow vein virus* (PYVV) (Salazar *et al*., 2000) and *Lettuce chlorosis virus* (LCV) (Duffus *et al*., 1996a). All those viruses are classified as definitive or tentative members of the genus *Crinivirus* in the family Closteroviridae (Hartono *et al*., 2003). Because of this, cultivation of tomatoe, cucumber, lettuce, sweet potatoe and potatoe in highland altitude of West Sumatera should considered at risk.

Biotype determination of *Bemisia tabaci* based on mtCOI gene sequence data: In order to get an overview of genetic variability of our samples we aligned all 43 sequences using Clustal W software provided at http://www.ebi.ac.uk/. The result is shown in Fig. 2. Clustering analysis successfully differentiated all samples into two main clades. Main clade I was composed from

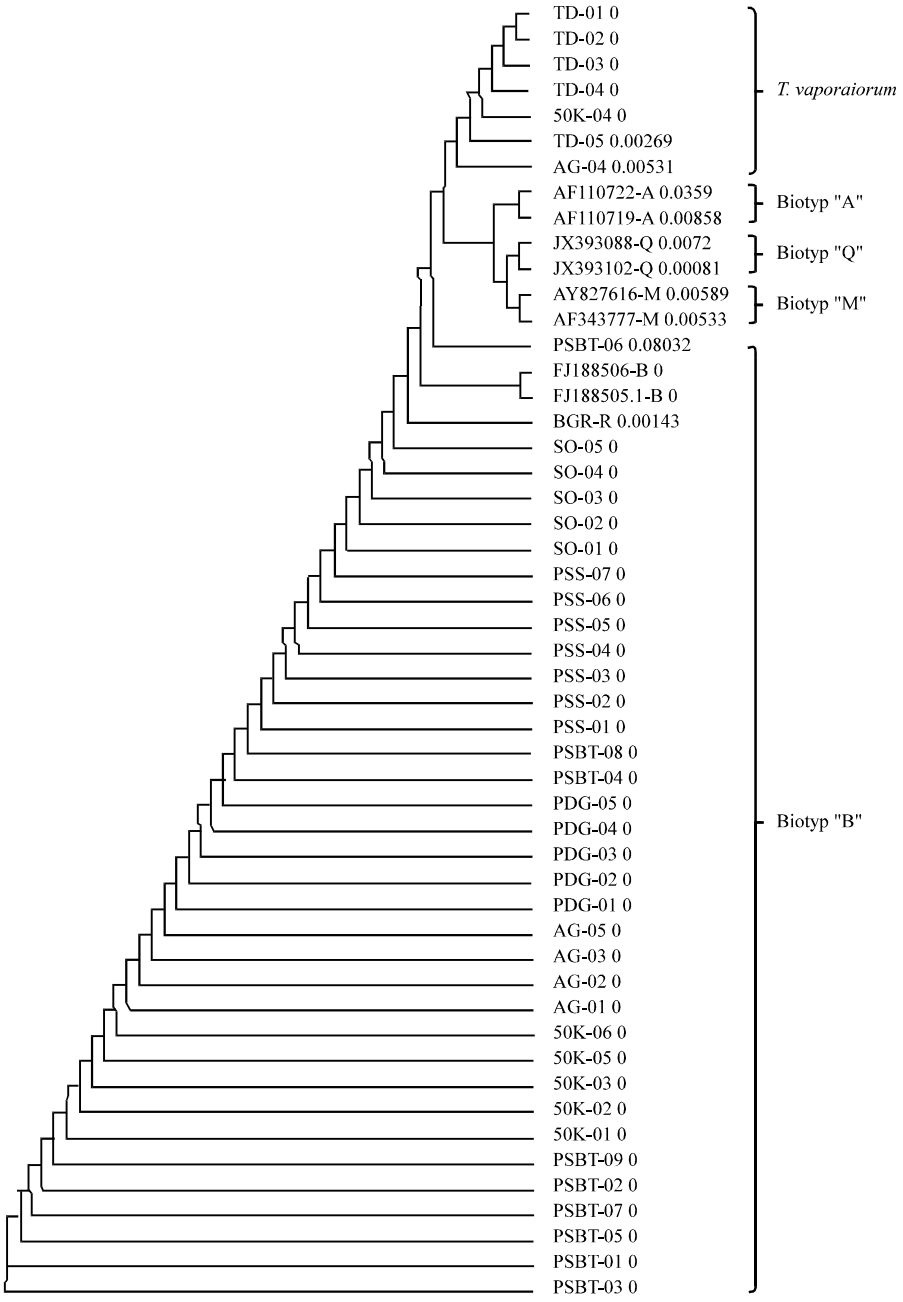

Fig. 2: Cladogram of 43 whitefly samples collected from three different geographical regions in West Sumatera. Two representative of each A-biotype (AF110722, AF110719), M (AY827616, AF343777) and Q (JX393088, JX393102) and one biotype B (FJ188505.1) were used as reference for biotype classification

36 samples mainly collected from low altitude (Padang, Pesisir Selatan and Pasaman Barat) and medium altitude (Solok and 50 Kota except 50K-04). All samples in the clade I could be definitely identified as *B. tabaci*. Almost all samples from Agam from high altitude region (except AG-04) were clustered into main clade 1. On the other hand, all samples from Tanah Datar (high altitude) were clustered into main clade II which could be identified as *T. vaporariorum*.

Another interesting aspect of *Bemisia tabacci* is their biotype determination. Biotype class determine its compatibility with plant virus pathogen and thus disease management strategy. For this purpose we used two mtCOI nucleotide sequences from each A, B, M and Q-biotype and successively run multialignment analysis by means of Clustal W2. The results are shown in Fig. 2.

All 35 samples identified as *B. tabaci* were clusterred in main clade I together with FJ186506 and FJ188505 which were identified as B-Biotype. The reference sequence from Bogor BGR-R was also clustered together with both two biotype B. Interestingly sample PSBT-06 collected from Pasaman Barat (lowland region) was more distant from almost all *B. tabaci*, eventhough it still also clustered into the B-biotype group. The results are in agreement with data published by Hidayat *et al.* (2004) from tomatoe cultivation area in West Java. However, data obtained here showed different results compared with data published by Shadmany *et al.* (2013) in Malaysia. Eventhough West Sumatera and Malaysia is not far away from each other geographically. They found Q-biotype population in vegetable and flower producing area.

Data obtained from this study suggested that pest management in both islands must be tighten to control the spreading of other biotypes of *B. tabaci*. However, attention has to be paid to the fact that B-biotype is highly polyphagous and has spread globally with the trade in ornamentals and vegetables. Moreover, B-biotype is difficult to control since it's high resistancy to most of the insecticides on the market and it has a wide host range and rapid rate of development and reproduction (Costa *et al.*, 1993; De Barro and Driver, 1997; Qiu *et al.*, 2009).

CONCLUSION

Taking all the data into account the two whiteflies namely *Bemisia tabacci* and *Trialeurodes vaporariorum* exist in the *Capscium annuum* production centre in West Sumatera. So far, this is the first report describing the occurence of *Trialeurodes vaporariorum* in addition to *Bemisia tabacci* in chilli pepper cultivation centre in West Sumatera-Indonesia. Biotype determination of *B. tabaci* based on mtCOI gene sequence showed that so far only B-biotype exists in West Sumatera.

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