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Molecular identification of coffee (*Coffea arabica*) pollinator insects in North Sumatra, Indonesia based on designed COI primers

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Abstract. *Sitompul FA, Siregar EH, Roesma DI, Dahelmi, Prasetya E. 2018. Molecular identification of coffee (*Coffea arabica*) pollinator insects in North Sumatra, Indonesia based on designed COI primers. Biodiversitas 19: 1877-1883.* Coffee (*Coffea arabica* L.) is one of the most important economic commodities in the province of North Sumatra, Indonesia. Insects associated with pollination of *C. arabica* are one of the key factors for successful cultivation of *C. arabica*, but, the research regarding of these was still limited. The population of coffee plant is scattered across the highlands of Indonesia and the pollination of *C. arabica* is strongly believed linked to a diverse group of pollinating insects. However, lack of taxonomic identification of insects pollinating these plants has become one of constraints to succeed the cultivation of *C. Arabica*. This study aimed to analyze types and variations of pollinating insects of *C. arabica* in the province of North Sumatra, Indonesia, using DNA barcoding. DNA barcoding is now considered an alternative method of molecular identification. Sixteen of *C. arabica* flower visitors were captured in different planting location in North Sumatra province. Using *mtDNA* markers, the *cytochrome oxidase subunit sequence I* (COI), about 12 pollinator insect species were identified based on the COI sequence i.e *Amegilla cingulata, Apis dorsata, Apis cerana, Trigona chanchamayoensis, Idiella divisa, Dolichopodidae* sp., *Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* Rhiniidae sp., *Melipona bicolor,* and Hymenoptera sp.

Keywords: *Coffea arabica, cytochrome oxidase subunit I,* mtDNA, North Sumatra, pollinator insects

INTRODUCTION

Indonesia is the fourth largest producer of coffee in the world from 2016 to 2017 with the production output reaching 11.49 million/kg (ICO 2017). The production of coffee in Indonesia are located in several islands with suitable appropriate climatic conditions (Schroth et al. 2015). of which Sumatra Island produced coffee by 74.2% of all national production of which provinces of South Sumatra contributes 21.4%, Lampung contributes 12.6%, Nanggroe Aceh Darusallam is 8.7%, Bengkulu is around 7.4%, and East Java is around 7.2% (Wahyudi et al. 2012). North Sumatra Province is one of the central coffee plantations in Sumatra Island.

Coffee is a member of Rubiaceae family, Gentianales order and *Coffea* genus with more than 124 member species (Davis et al. 2011). Commercial coffee production is dominated by two main species i.e., *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehener (Tran et al. 2017) with 66% and 34% of the coffee market's production (Camargo 2009).

Coffea arabica is the most well-known coffee in the market which is widely produced (DaMatta et al. 2007). *Coffea arabica* has a high quality in terms of flavor and aroma (Flament 2002). *Coffea arabica* comes from the rainforest in the Ethiopian highlands (Monaco 1968), growing at the altitude of 1000-2800 m above sea level (Schmitt 2006), with 1200-1800 mm of rainfall and temperatures ranging between 18-20ºC (Alégre 1959). Flowering period of *C. arabica* varies in different regions but it generally occurs between January to April (Ngo et al. 2011). The flowers wilt within 1-2 days after pollination (Aga 2005) and continue with fruit development in 7-10 months (Eira et al. 2006). Most literature state that the percentage of flowers can develop into fruits ranging from 20 to 40% (Free 1993).

Almost 90% of flowering plants heavily rely on pollinator insects (Klein et al. 2007). The productivity of majority of cross-pollinated crops can be increased by improving the pollination process with the help of pollinator insects (Klein et al. 2007; Abrol 2012). The diversity and abundance of pollinator insects can increase the number and quality of fruits (Garibaldi et al. 2011).

The success of pollination processes is strongly influenced by the diversity of pollinating insects (Mayer et al. 2011). Lack of information about identification of pollinating insects is an obstacle in conducting this research (FAO 2009). DNA barcoding using mtDNA as a marker can be used to identify species diversity (Hebert and Gregory 2005; Floyd et al. 2009). This becomes a solution for the identification of pollinator insects in agriculture (FAO 2009). This study aimed to identify pollinator insects of *C. arabica* in the province of North Sumatra, Indonesia, using DNA barcoding.

MATERIALS AND METHODS

Study area

Insect pollinator samples were obtained from three districts in the province of North Sumatra, Indonesia i.e North Tapanuli, South Tapanuli, and Dairi. All three districts are the center of coffee cultivation in North Sumatra (Figure 1, Table 1). The used samples were fresh samples of pollinating insects obtained from coffee planting in North Sumatra. Sampling of pollinator insects on coffee flower planting was done on sunny days with the scan sampling method (Bookhout 1996).

Procedure

Genomic DNA extraction

DNA was extracted using a Purelink ™ kit Genomic DNA Mini Kit. A total of 25 mg of insect tissue was taken into the micro centrifuge tube by adding 180 µL purelink genomic digestion buffer and 20 µL K proteinase. It was then incubated for 2 hours at 55 ºC temperature and centrifuged for 3 minutes at 10,000 x g speed. After the supernatant was transferred to a new microcentrifuge tube, 20 µL of RNAse A was added and incubated at 27ºC for 2 minutes. A total of 200 µL of purelink genomic lysis and 200 µL of ethanol 96% were added to the microcentrifuge tube and then homogenized. The solution sample was transferred into a spin column and centrifuged for 1 minute at a rate of 10,000 x g. DNA was washed using wash buffer I and wash wafer II, followed by centrifugation for 1 minute at 10,000 x g. DNA was eluted using 100 µL of purelink genomic elution buffer.

Amplification of mtDNA COI sequence

COI (*cytochrome oxidase subunit I*) sequences were amplified using a pair of forward primers LCO1490 5'-GGT-CAA-CAA-ATC-ATA-AAG-ATA-TTG-G-3' and reverse primer HCO2198 5'- TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-CA-3' (Folmer et al. 1994). Total volume of PCR (polymerase chain reaction) reaction was 25 µL (2.5 µL DNA template; 2.5 µL primer forward; 2.5 µL primer reverse; 5 µL distilled water; 12.5 µL PCR master mix (MyTaq™ DNA Polymerases). The reaction was run in PCR with PCR condition as follows: pre-denaturation at temperature of 97ºC for 5 minutes followed by 40 cycles with denaturation reaction conditions at temperature of 94ºC for 1 minute 30 seconds, annealing at temperature of 52ºC for 1 minute, and extension at 72ºC

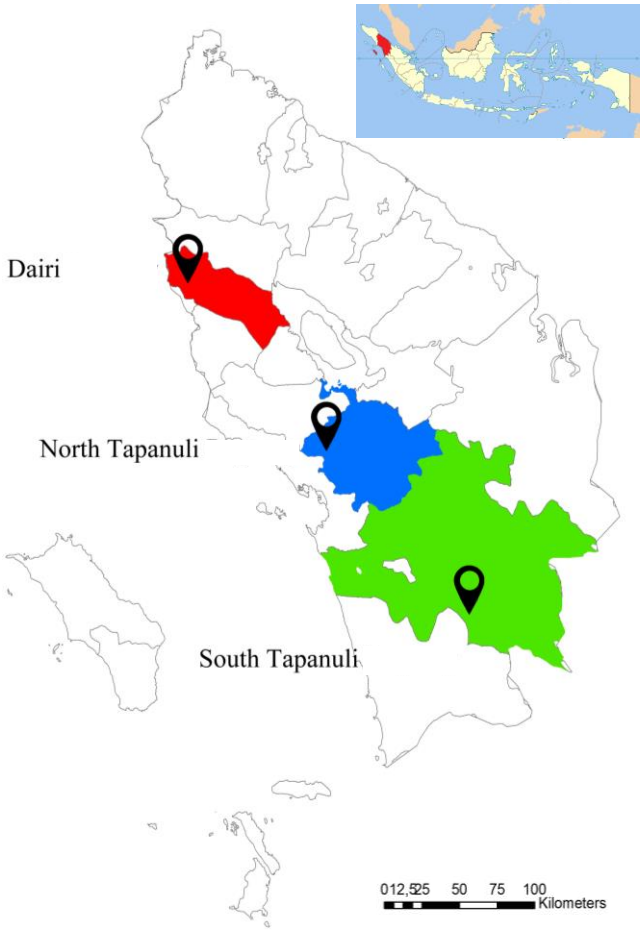

Figure 1. Sampling location in North Sumatera Province, Indonesia

Table 1. Samples of pollinator insects used for analysis

Sample	District	Sub-district	Village	Elevation (m asl.)	Latitude	Longitude
Sample 4	Dairi	Parbuluan	Lae Hole II	1321	N 02^0 41' 27.9"	E 098^0 24' 2.06"
Sample 53	Dairi	Parbuluan	Lae Hole II	1321	N 02^0 41' 27.9"	E 098^0 24' 2.06"
Sample 85	Dairi	Siempat Nempu Hulu	Lae Nuaha	1056	N 02^0 46' 1.04"	E 098^0 18' 25.06"
Sample 95	Dairi	Siempat Nempu Hulu	Lae Nuaha	1056	N 02^0 46' 1.04"	E 098^0 18' 25.06"
Sample 314	North Tapanuli	Pangaribuan	Rahut Bosi Onan	1280	N 01^0 58' 9.2"	E 099^0 11' 25.7"
Sample 335	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 336	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 337	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 338	North Tapanuli	Pangaribuan	Batu Nadua	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 340	North Tapanuli	Pangaribuan	Rahut Bosi Onan	1280	N 01^0 58' 09.2"	E 099^0 11' 25.7"
Sample 341	North Tapanuli	Pangaribuan	Sidori	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 348	North Tapanuli	Pangaribuan	Sidori	1180	N 01^0 58' 49.7"	E 099^0 11' 8.7"
Sample 399	South Tapanuli	Mamancar	Aek Sabaon Julu	981	N 01^0 30' 31'0"	E 099^0 13' 35.3"
Sample 445	South Tapanuli	Dolok	Siranap	903	N 01^0 31' 32.8"	E 099^0 12' 22.5"
Sample 454	South Tapanuli	Dolok	Siranap	903	N 01^0 31' 32.8"	E 099^0 12' 22.5"
Sample 461	South Tapanuli	Dolok	Siranap	903	N 01^0 31' 32.8"	E 099^0 12' 22.5"

temperature for 1 minute, then PCR process ended with post extension at 72°C temperature for 5 minutes. The PCR product was visualized using 1% agarose gel added with 4 µL SYBR® Safe DNA Gel Stain. A total of 6 µL of PCR products were added with 1 µL of loading dye in running with a 100 bp DNA ladder marker using an electrophoresis engine with a mobile phase of 1X TBE buffer at 45 volts for 30 minutes. Visualization of ribbon emerging by gel documentation (Biostep). The PCR product with the next visible band was sent to 1st Base DNA Sequencing Service for sequencing.

Data analysis

Sequence data that has been obtained was edited using BioEdit ver program. 7.0.1 to combine the results of the forward and reverse primary sequences. The combination of mtDNA sequence of COI data was analyzed by sequencing homology using mega BLAST program which can be accessed at the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences homology analysis was performed by comparing COI sequence of pollinator insects samples with NCBI GenBank Database. Phenogram analysis was performed using the MEGA 7 program.

RESULTS AND DISCUSSION

Sample collection

Samples were obtained from 3 coffee planting centers in North Sumatra province. A total of 60 pollinator insect samples were used in the analysis and only 16 samples were successfully amplified using COI primers. Four samples were obtained from Dairi District, 8 samples were obtained from North Tapanuli District, and 4 samples were obtained from South Tapanuli District. A total of 16 samples were used in the present study.

Alignment of sequences

A total of 799 bp of the alignment result of the sequencing product was observed successfully. From these data, there were 155 conservative characters, 619 character variable sites, and 496 characters of informative parsimony. This suggests that the mtDNA COI sequence on insects is less conservative and has a high degree of variability. The mean frequency of nucleotides in the COI sequence in the sample was 39.0% (T), 16.3% (C), 30.4% (A), and 14.3% (G) (Table 2). In Table 3, each entry showed the probability of substitution from one line to another. Different transitional substitution levels were shown in bold and transverse substitutions were shown in italics. This sequence was rich in A/T (69.4%), while G/C was 30.6%. The approximate transition/transversion bias (R) was 0.88. The substitution pattern and approximate rate were based on the 2-parameter model kimura (Kimura 1980).

Sequencing analysis of PCR amplified region

The sequencing results of the PCR amplification product of 16 pollinating insects were presented in Table 4. The nucleic acid sequence of samples was analyzed using the BLASTN program to determine the identity of pollinator insects. The BLASTN analysis showed that sequences of 20 isolate insect insects had 79% -99% similarity with 12 insect species, *Amegilla cingulata, Apis dorsata, Apis cerana, Trigona chanchamayoensis, Idiella divisa,* Dolichopodidae sp., *Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* Rhiniidae sp., *Melipona bicolor,* and Hymenoptera sp. Pearson (2003) stated that two genes or homologous DNA fragments having similarity with range of 80% or 25% of amino acid sequences between two genes or DNA fragments mostly having identical species performances.

Phenogram construction analysis

The entire sequence of individual sequenced results was analyzed and phenogram was made by Neighbor-Joining (NJ) method to see differences in genetic distance and analyze similarities between samples. Phenogram analysis was performed to compare the results of alignment of nucleotide sequences of pollinator insect samples with some homologous sequences (selected from *GenBank*) as reference using MEGA 7 program. The alignment results showed COI sequence in the sample and the references used has a high level of variety. This was indicated by the least conservation sequence (306/1050) and the high value of the site variable (688/1050). Each sample clumped together with a homologous reference sequence (Figure 2).

Table 2. Variation in length, AT content and GC content on COI sequences on Insect

Sample	T	C	A	G	Total	%AT	%GC
Sample 4	43.2	15.0	27.6	14.3	526.0	70.7	29.3
Sample 53	40.8	15.0	32.7	11.5	642.0	73.5	26.5
Sample 85	41.3	14.5	33.8	10.4	671.0	75.1	24.9
Sample 95	47.1	11.6	31.7	9.6	622.0	78.8	21.2
Sample 314	36.6	17.2	29.3	16.9	656.0	65.9	34.1
Sample 335	31.6	19.6	30.4	18.4	648.0	62.0	38.0
Sample 336	34.5	18.2	29.2	18.1	565.0	63.7	36.3
Sample 337	35.1	18.1	29.4	17.4	609.0	64.5	35.5
Sample 338	39.2	14.1	30.6	16.1	633.0	69.8	30.2
Sample 399	40.7	14.9	32.6	11.8	610.0	73.3	26.7
Sample 340	38.8	14.7	29.7	16.8	585.0	68.5	31.5
Sample 341	38.3	14.7	30.2	16.8	572.0	68.5	31.5
Sample 348	40.4	13.7	30.3	15.5	611.0	70.7	29.3
Sample 445	41.4	15.2	32.8	10.6	592.0	74.2	25.8
Sample 454	40.7	18.3	27.6	13.5	624.0	68.3	31.7
Sample 461	41.8	14.6	32.6	11.0	610.0	74.4	25.6
Average	39.0	16.3	30.4	14.3	603.3	69.4	30.6

Table 3. Maximum composite probability estimate of the pattern of nucleotide substitution

	A	T	C	G
A	-	*9.24*	*3.90*	**6.06**
T	*7.31*	-	**9.84**	*3.36*
G	*7.31*	**23.29**	-	*3.36*
C	**13.19**	*9.24*	*3.90*	-

Table 4. Results of sequencing and BLASTN analysis on 16 pollinator insects on coffee

Sample number	Location (District)	Fragmen size (bp)	Identification result	Query cover	Identical
4	Dairi	526	*Amegilla cingulata* (KY842920.1)	100%	96%
53	Dairi	642	*Apis cerana* (KU963187.1)	100%	99%
85	Dairi	674	*Apis dorsata* (KU752355.1)	99%	98%
95	Dairi	623	*Trigona chanchamayoensis* (KC853315.1)	100%	99%
314	North Tapanuli	656	*Idiella divisa* (KY031806)	94%	99%
335	North Tapanuli	648	Dolichopodidae sp. (KY833537.1)	98%	86%
336	North Tapanuli	565	Dolichopodidae sp. (KY833537.1)	97%	97%
337	North Tapanuli	609	Dolichopodidae sp. (KY833537.1)	99%	97%
338	North Tapanuli	633	*Allactoneura* sp. (KT175590.1)	98%	99%
340	North Tapanuli	585	*Stomorhina discolor* (KY031819.1)	98%	98%
341	North Tapanuli	572	*Phytomia erratica* (KR831181.1)	99%	95%
348	North Tapanuli	611	Rhiniidae sp. (KX054613.1)	100%	99%
399	South Tapanuli	610	Hymenoptera sp. (KF200070.1)	100%	98%
445	South Tapanuli	593	Hymenoptera sp. (KF200070.1)	98%	99%
454	South Tapanuli	626	*Melipona bicolor* (KC853346.1)	93%	79%
461	South Tapanuli	610	Hymenoptera sp. (KF200070.1)	99%	98%

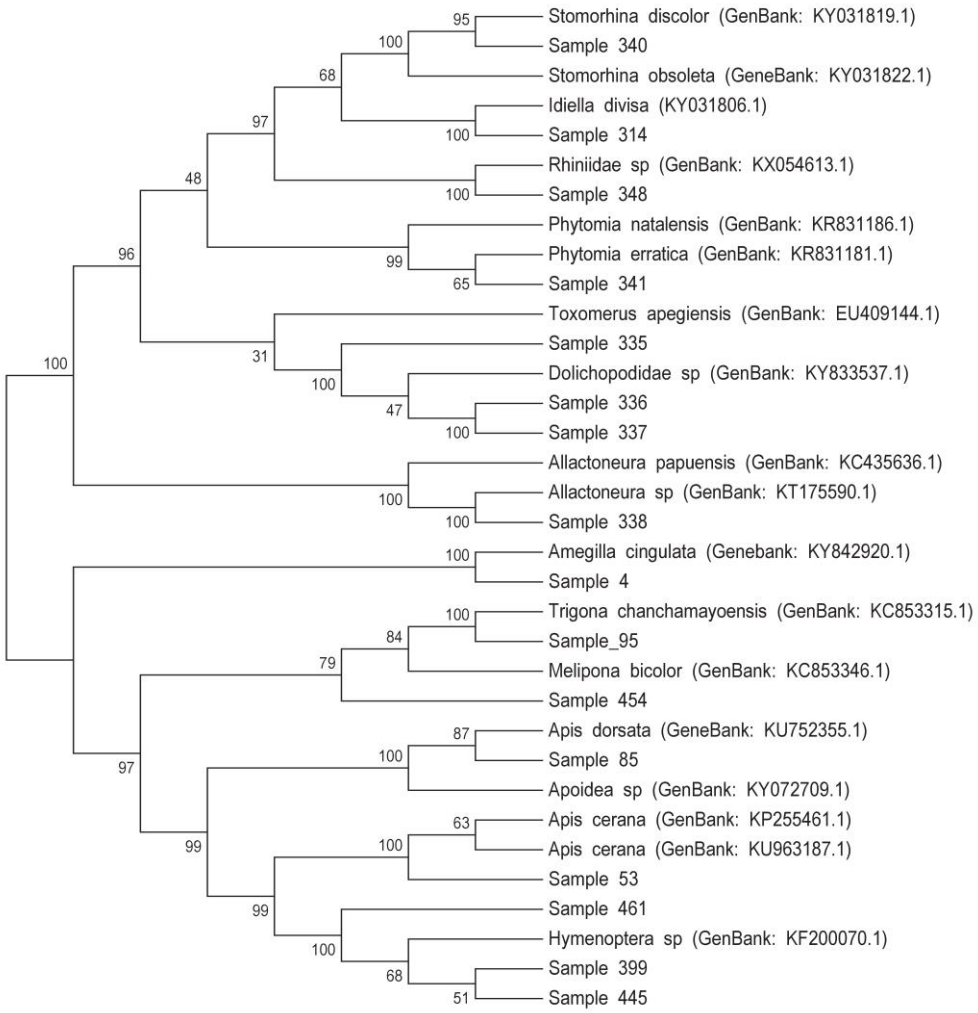

Figure 2. Phylogenetic sequences of insect pollinators in coffee plants. Twelve species were identified using COI sequences. Identified insects include *Amegilla cingulata* (4), *Apis cerana* (53), *Apis dorsata* (85), *Trigona chanchamayoensis* (95), *Idiella divisa* (314), Dolichopodidae sp. (335, 336, 337), *Allactoneura* sp. (338), *Stomorhina discolor* (340), *Phytomia erratica* (341), Rhiniidae sp. (348), Hymenoptera sp. (399, 445, 461), and *Melipona bicolor* (454). The numbers on the branches show the confidence level of the branch separation

Discussion

Wind is an important source of pollination process for 18% of species in the Angiosperm family (Culley et al. 2002). Le Pelley (1973) noted that based on the grain structure of pollen on *C. arabica*, winds cannot carry pollen moving away and cross-fertilization is mostly associated with insects. Bees have long been known for its important role in pollination of *C. arabica* (Ricketts 2004). Klein et al. (2003) stated that social bees more often visit *C. arabica* in Indonesia with the most frequent visitors are *Apis nigrocincta, Apis dorsata,* and *Apis cerana*. Flowering of single coffee flower outside flowering events is still accompanied by the existence of solitary insects (*Megachile frontalis* and *Amegilla* sp.) (Ngo et al. 2011). Solitary bees are considered to be more efficient as pollinators than social bees although the rate of social bees' visit to coffee flowers is higher (Willmer and Stone 1989; Klein et al. 2003). Amaral (1972) observed that the honeybee is the main visitor of the coffee flower on *C. arabica* compared to *Trigona* and *Partamona* bees and wood bees (*Xylocopa* sp.), which are the least frequent visitors. Roubik (2002) reported that besides *Apis mellifera* which visits flowers of *C. Arabica, Trigona* spp., *Melipona* spp., *Bombus* spp., and *Centris* sp. were also visitors.

The application of a long-term agroforestry system has created a specific habitat for coffee cultivation, including Berytidae, Calliphoridae, Dolichopodidae, Dytiscidae, and Histeridae, which were only found in coffee plantations (Kinasih et al. 2016). This study also identified several species that do not have references as pollinator insects in coffee plants such as *Idiella divisa, Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* and Rhiniidae sp. *Idiella divisa,* which were found across Indonesia (Sulawesi), Vietnam (The Catalogue of Life Partnership 2017), India, and Taiwan (Yang et al. 2014). *Stomorhina discolor* are spread across Bangladesh, Australia, Indonesia, Pakistan, China, and Malaysia (Ratnasingham and Hebert 2007). *Rhiniidae* is known as pollinator on bitter ground, *Momordica charantia* Linn (Subhakar et al. 2013). *Allactonoura* is known to only be found in Asia, northern Australia New Guinea, a series of islands in the Indian Ocean, and East Africa (Zaitzev 1981).

Pollinator Insects of coffee plants have been shown to increase crop productivity (Klein et al. 2003). The pollination process by insects benefits the major crops by up to 75%, representing 35% of the world's crop production (Klein et al. 2007). Flowering coffee plants that are pollinated by insects have 15.85% higher fruit set than their own self-pollinated flowers (Ngo et al. 2011).

Earlier works on using COI sequences to identify the pollinator insects are scanty. Our present study confirms that DNA barcoding based on COI sequences can be applied for taxonomic identification of pollinating insect species in coffee plants. The aim of our study was to ensure that molecular identification methods could be applied to pollinating insects of coffee plants in the province of North Sumatra, Indonesia. Sometimes, the morphological markers used in insect identification showed similarities so that it confused to determine the species of insects. Molecular markers can facilitate the identification of insects in shorter period of time, making this method more efficient. DNA barcode is able to facilitate integrative approaches in species identification but it still involves a classical taxonomic approach (Imtiaz et al. 2017).

Subunit of *cytochrome oxidase subunit I* (COI) has been used extensively by molecular biologists around the world to identify insect species (Jalali et al. 2015). COI barcoding sequences can be used to identify insect species at all stages of development (Armstrong and Ball 2005; Ball and Armstrong 2006). COI sequences are not only widely used in Diptera (Alessandrini et al. 2008), but also in Coleoptera identification (Paul et al. 2009; Fang 2009). In East Asia, mtDNA including COI (Kim et al. 2000) and COII (Suzuki et al. 2004) has been used in identifying evolutionary relationships and biogeography in some Coleoptera families. COI barcoding sequences have been used to identify species in a number of studies (Harvey et al. 2003).

DNA barcoding has been applied to identify the Coleoptera order (Greenstone et al. 2005), Hymenoptera (Fisher and Smith 2008), Lepidoptera (Hajibabaei et al. 2006), Orthoptera (Trewick 2007), and Diptera (Burns et al. 2008). DNA barcoding of COI sequences could be used to identify six orders of insects (Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera) with significant variation among orders on Neighbor-Joining Tree analysis with the highest identification proportions in Hymenoptera and Orthoptera and the lowest one in Diptera (Virgilio et al. 2010). In the present study, two insect orders had been identified, namely Diptera order (*Idiella divisa, Dolichopodidae* sp., *Allactoneura* sp, *Stomorhina discolor, Phytomia erratica,* and *Rhiniidae* sp.) and Hymenoptera (*Amegilla cingulata, Apis cerana, Apis dorsata, Trigona chanchamayoensis, Hymenoptera* sp., and *Melipona bicolor*).

In this study, COI sequence analysis showed that there were various types of pollinating insects in coffee. Pollinator insects of coffee plants are identified using *mtDNA* markers, the COI sequence, i.e., *Amegilla cingulata, Apis dorsata, Apis cerana, Trigona chanchamayoensis, Idiella divisa, Dolichopodidae* sp., *Allactoneura* sp., *Stomorhina discolor, Phytomia erratica,* Rhiniidae sp., *Melipona bicolor,* and Hymenoptera sp. DNA barcoding using COI sequences could be an effective screening method for pollinating insects. Research on the identification of pollinating insects in coffee plants can improve the yield productivity by providing information about insects that are efficient for coffee pollination. The coffee conservation and management strategy focus on conservation of important pollinating insect communities. This research can be used to know the identification of the pollinating insect communities, which are beneficial to coffee plants.

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