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Surveillance of European honeybee subspecies distribution in Chiang Mai, Thailand

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Abstract
Nowadays the most common subspecies of western honeybee or European honey bee (*Apis mellifera*) have 28 subspecies around the world. The subspecies are divided into four major branches were confirmed by mitochondrial DNA analysis. African subspecies belong to branch A, north western European subspecies branch M, south western European subspecies branch C and Middle Eastern subspecies branch O. The different sub species of European honeybees have different characterization factors such as tolerances of environmental temperature changes, specific diseases resistant, defence behaviour and other economically important Traits. In this studied we collected the samples from 50 colonies of *Apis mellifera* from January to March 2017 at Chiang Mai, Thailand and used PCR-RFLP to identify mitochondrial lineages and subspecies of European Honeybees. There have three different subspecies of *Apis mellifera* such as Thai A1 Thai A2 and Thai B in Chiang Mai. Province, Thailand. Thai A group are similar match to C or east European lineage *A.m. ligustica* and *A.m. carnica* and Thai B group are similar to the match of O or Middle Eastern lineage, *A.m. syriaca* and *A.m. lamarckii*. The Haplotype group of ThaiA1 (38% of colonies) and group ThaiA2 (52%) Haplotype group ThaiB (10%).

Keywords: *Apis mellifera*, PCR-RFLP

1. Introduction
In Thailand, there are four native species of honey bees: *Apis andreniformis*, Apis cerana, Apis dorsata and Apis florea (Oldroyd and Wongsiri, 2006) [17]. Thailand is a tropical country it has various plantation and several kinds of flora are blooming in all seasons. Especially in the north of Thailand, have plenty of nectar resources and suitable climate for beekeeping (Oldroyd and Wongsiri, 2006, Chantawannakul, de Guzman, Li, and Williams, 2015) [17, 4]. There are four native species of honey bees: *Apis andreniformis*, *Apis cerana*, *Apis dorsata* and *Apis florea* (Oldroyd and Wongsiri, 2006) [17]. Today beekeeping in Thailand have more than 500,000 of European honey bee colonies and 1,000 beekeepers all over the country (Suwannapong, Benbow, and Nieh, 2011) [25]. *Apis mellifera* L. was introduced to Chulalongkorn University, Bangkok Thailand in the early 1940s and 1953 Introduced to Kasetsart University (Seanbualuang, P., 2012) [26]. At 1970s there were imported large numbers of *A. mellifera* from Taiwan to Lampoon and Chiang Mai in northern Thailand for commercial purposes (Wongsiri *et al*., 1995) [27]. Beekeeping for *Apis mellifera* were spread out in northern Thailand, especially Chiang Mai province, Thailand. Beekeepers distributions in northern Thailand 7 provinces such as Chiang Mai, Chiang Rai, Phra, Lamphun, Lampang, Phayao, and Nan. In the Northern Thailand have 66% of total honey bee colonies in Thailand and 52% of total beekeepers (Suwannapong, G., *et al*. 2011) [25].
There are 28 subspecies have been described from *A. mellifera*'s native range in Europe, Africa and the Middle East, based primarily on morphometric variation (Ruttner, 1988; Engel, 1999) [20]. The different sub species of European honey bees have different characterization factors such as tolerances of environmental temperature changes, specific diseases resistant, defence behaviour and other economically important Traits (Ruttner, 1988) [20]. The Studies of *A. mellifera* mitochondrial DNA have at least 4 major mitochondrial lineages such as M or West European, C or Eastern Mediterranean, A or African, (Hall and Muralidharan, 1989;

Arias and Sheppard, 1996) [15, 2] and O or Middle Eastern lineage (Franck *et al*., 2000) [12]. A possible fifth lineage, Y, has been described from Ethiopia (Franck *et al*., 2001) [13]. Until 2006 the ancestry of *A. mellifera* in Thailand is unknown, then the matrilineal origins of *Apis mellifera* were recorded as 5 rear and 3 major subspecies of *Apis mellifera* by PCR-RFLP and direct DNA sequencing (Suppasat. T., *et al*., 2007) [24].

The RFLP (Restriction Fragment Length Polymorphisms) is the revealed by the digesting of the whole entire mitochondrial genome with restriction endonucleases. PCR-RFLP is more convenient than RFLP because it is revealed within a specific PCR-amplified region. The most popular PCR amplification of the non-coding region located between the tRNAleu and COII genes by digestion with the *Dra* I restriction enzyme, commonly known as *Dra* I test. It is widely used in Honey bees maternal identification Clarke *et al*., 2002; Pinto *et al*., 2004) [5, 18]. In this research to clarify the maternal ancestry of *A. mellifera* in Chiang Mai Provence, Thailand used PCR-RFLP analysis of four regions of mtDNA restricted with the 6- base restriction enzymes *Dra I* and a 4-base restriction enzyme, *Hinf I*.

2. Materials and Methods

2.1 Collections

Apis mellifera samples were collected between January to March 2017 from 50 colonies from 5 different distinct in Chiang Mai Thailand. All samples were kept in 95% ethanol and stored at -20 ◦C until extraction.

2.2 PCR-RFLP analysis

Total DNA was extracted from one honey bee thorax per colony by RBC Real genomic DNA extraction kit extraction methods. To amplify the intergenic region with the Primers E2 (5' GGCAGAATAAGTGCATTG 3') located 5' end of the gene tRNAleu and H2 (5' CAATATCATTGATGACC 3') located close to the 5' end of the gene COII. PCR thermal reaction were used the denaturation at 94◦C for 60 s and follows by 30 cycles of 94 ◦C for 30 s, 55 ◦C for 30 s, 72 ◦C for 60 s and final elongation step of 10 min at 72 ◦C. And enzyme digestion with *Dra I* and *Hinf I* methods. *Dra I* test has widely used for honey bee maternal identification. Because it is helpful in phylogeographical studies, easy to understand in natural hybrid zone, detecting of new species from foreign queen's introduction and tracking on temporal changed of honey bee maternal compositions (Cánovas *et al*., 2008, Jensen *et al*., 2005, Clarke *et al.*, 2002. Franck *et al*., 2001; Franck *et al*., 2000 De la Rúa *et al.*, 1998; Franck *et al*., 1998) [3, 16, 5, 13, 12, 7, 11].

PCR-based assays, colony identification using the *DraI* test can be performed in a small-sized laboratory equipped with basic equipment. The *DraI* test was adopted as the standard for honey bee maternal identification, for several reasons. Because it has proven to be the most powerful and informative. And COI-COII/DraI variation has been widely documented across natural and introduced honey bee ranges. The combination of length and restriction-site polymorphisms produced by the *DraI* test has resolved over 100 haplotypes (Franck *et al.*, 2001; Collet *et al.*, 2006; Shaibi *et al*., 2009; Alburaki *et al.*, 2011; Rortais *et al*., 2011; Pinto *et al.*, 2012) [13, 6, 23, 1, 22, 19], which have been correctly assigned to evolutionary lineages. *DraI* test compared to other assays, it can be identified as *A. m. carnica* or *A. m. ligustica*, because this kind of haplotype is present in both populations, although at different frequencies. The same applies to an A1 haplotype which could be carried by an *A.m. iberiensis, A.m. adansonii* or other African subspecies (Franck *et al*., 2001; Cánovas *et al*., 2008) [13, 3]. In this research to determine the RFLP pattern, the digested products of each samples are electrophoresed in 1.5% Agarose gel at 110V for 90 min.

3. Result

Restriction enzyme digestion of PCR products are summarized in amplification of tRNAleu-COII revealed fragments of two sizes. Digestion of this fragment with *Dra I* produced a single pattern and *Hinf I* have two restriction patterns found. According to recent research got the amplification of tRNAleu-COII revealed fragments of two sizes such as 573 bp and 837p. The smaller fragment was defined as "ThaiA", which is the typical of the C lineage. The digestion of this fragment with *Hinf I* revealed two restriction patterns which defined as "ThaiA2" and the larger fragment were defined as "ThaiB". When the compared of recent research the result of the European Honey Bee Sub species distribution in Chiang Mai Province have three different subspecies such as "Thai A1", "Thai A2" and "Thai B". Thai a group are similar match to C or east European lineage *A.m. ligustica and A.m. carnica* and Thai B group are the match similar with O or Middle Eastern lineage, *A.m. syriaca* and *A.m. lamarckii*. The Haplotype group of ThaiA1 (38% of colonies) and group ThaiA2 (52%) Haplotype group ThaiB (10%).

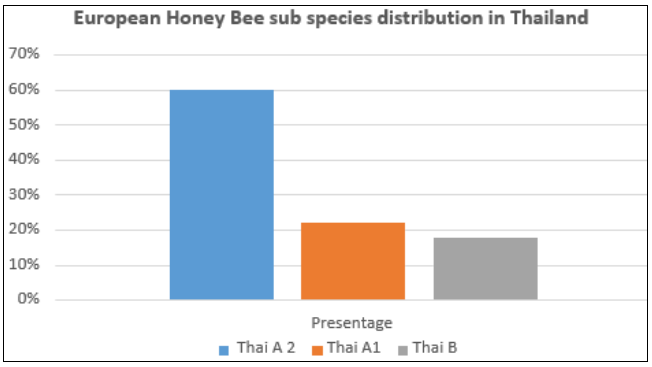

Fig 1: Percentage of European Honey Bees subspecies distribution in Thailand (Suppasat. T., *et al.* 2007) [24]

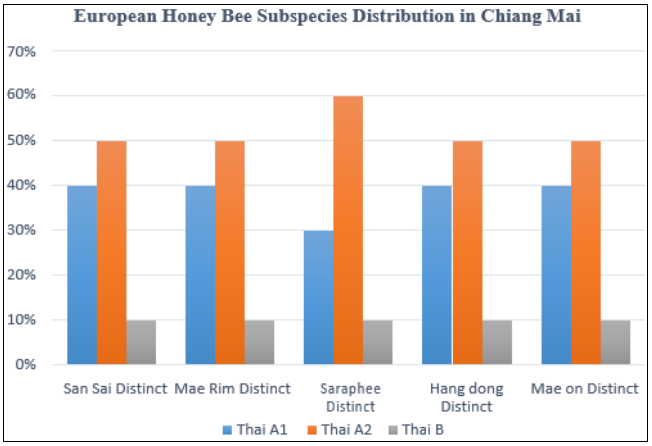

Fig 2: Percentage of European Honey Bee Subspecies distribution in Chiang Mai Province, Thailand

Fig 3: PCR-RFLP patterns of Dra *I* and *Hinf I* enzymes digestion at tRNAleu-COII region. Lane 1 is 100bp marker, Lane 2 to lane 6 are digested by Dra I enzyme and Lane 7 to lane 10 are digested by Hinf I enzyme

4. Discussion

From 1940's European Honey Bees were introduced to Bangkok, Thailand for research in Chulalongkorn and Kasetsart Universities. In the 1970s large numbers of *A. mellifera* were imported from Taiwan to northern Thailand Chiang Mai and Lamphun for commercial purposes (Wongsiri *et al*., 1995) [27]. From 1970, the European honeybees are distributed in Chiang Mai Provence. Suppasat. T, *et al*., 2007 [24], studied matrilineal origins of European Honeybees *Apis mellifera* in Thailand. The three subspecies of *A. mellifera* were distributed around the country. The group Thai A1 (22%), the group of Thai A2 (60%) and the group of Thai B (18%). And the result of European Honeybees distribution in Chiang Mai have three different subspecies in Chiang Mai. There have (1.5%) of Thai A1, (14%) of Thai A2 and (1.9%) of Thai B of European Honeybees Sub species distribution in Thailand. In this studied subspecies distribution in Chiang Mai are (38%) of Thai A1, (52%) of Thai A2 and (10%) of Thai B. After 10 year, later from 2007 to 2017 in Chiang Mai European Honeybee keeping didn't change for the new Honeybees Species. It can be concluded the European honey Beekeeping in Chiang Mai have the group Thai A2 species distribution are increased. Thai A2 is very similar with *A.m. ligustica* and *A.m. carnica*. Although the bee colonies were increased more than two times the honey production and honeybee product production are didn't increase too much. Thai European Honey Bee sub species can be resistant for climate changes and local diseases problems, but the production and breeding are not so good like European honeybees. To the best quality of beekeeping in Thailand the keeping bees are needed to upgrade the best honey production quality bees, the worker bees foraging activities and the best breeding quality of queen.

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