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Evaluating the phylogenetic relationship of a dwarf honey bee through *cytochrome oxidase subunit 1* (*COX1*) gene sequence databases

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ABSTRACT

Dwarf honey bees, *Apis florea*, and *Apis andreniformis* are known as the potential wild pollinator in Asia. *Apis florea* was introduced to Indonesia, West Asia, and Africa. Based on *the cytochrome oxidase subunit 1* (*COX1*) gene, introduced *A. florea* in Iran is suggested to come from India. Although the databases of the dwarf honey bee *COX1* gene are available in GenBank, however, the phylogeny analysis was focused on the restricted area. Thus, this study aims to evaluate the phylogenetic relationship and genetic diversity of dwarf honey bees based on the *COX1* gene sequence from GenBank databases. The *COX1* gene sequence databases are collected from NCBI. The genetic diversity and phylogenetic relationship are analyzed using MEGAX. Alignment of 530 bp *COX1* gene of dwarf honey bee showed that there are 60 nucleotide variations between *A. florea* and *A. andreniformis*. Our analysis indicated that the genetic diversity between *A. andreniformis* is low (0-0.19%). On the other hand, there are 28 nucleotide variations between *A. florea* and eight nucleotide sites that are suggested as a marker to differentiate *A. florea*1 India+Egypt+Iran and *A. florea*2 Thailand+China with 1.7-3.4% genetic distance. The high genetic distance of *A. florea* is supported by the separation sub-clade of *A. florea*1 and *A. florea*2 in the phylogenetic tree.

Introduction

Apis florea and *Apis andreniformis* are well-recognized dwarf honey bees in the world. Based on the morphological appearance, in general, *A. florea* are known as yellow dwarf honey bees while *A. andreniformis* are black dwarf honey bees[1]. Initially, those species were considered the same species, however, *A. andreniformis* was differentiated from *A. florea* based on the different matting drone flight time[2] and the distinct endo-phallus anatomy[1]. Those dwarf honey bees build their nest by wrapping the twigs in open areas and have a role as a wild natural pollinator. *Apis florea* was an important pollinator for *Allium cepa* in India[3], while *A. andreniformis* is a potentially wild pollinator that visited 29 plants in West Sumatera[4]. This species naturally distributed from South Asia to Thailand through Cambodia[5]. The black dwarf honey bee, *A. andreniformis* can be found in the Himalayan, Bhutan, Myanmar, China, Thailand Indochina, Palawan, Philippines, and in the Sundaland region of Indonesia at the lowland below 1000 mdpl[6]

In Indonesia, *A. florea* was found at Jakarta and Surabaya on a ship that was suspected to be transferred by humans[5]. However, the recent distribution of *A. florea* in Indonesia has not been published. This species also was revealed that introduce to some areas in Jordan[7] and Africa[8]. Based on the *cytochrome oxidase subunit 1* (*COX1*) gene, *A. florea* in Egypt was suspected that introduced from India[9]. Thus, the databases of *the COX1* gene sequence of the dwarf honey bee and their phylogeny relationship are important to reveal the genetic diversity and geographical origin of introduced *A. florea*. The whole mitochondrial DNA of *A. florea* (AP0184490) from Thailand has been published, and the sequence was closed related to *A. florea* from China (KC1703003)[10]. Although the *COX1* gene sequences of dwarf honey bees from several locations are available in the open-source GenBank database, the phylogenetic relationship of the dwarf honey bee only focuses on a restricted study area[9]. Thus this study aims to evaluate the phylogenetic relationship and genetic diversity of *A, florea*, and *A. andreniformis* based on the *COX1* gene sequence database from GenBank. This data is needed as basic information for future studies to reveal the geographical origin of the introduced dwarf honey bee which is important for ecological conservation purposes.

Method

The databases of *COX1* gene sequences of *A. florea* and *A. andreniformis* were collected from online open-access databases of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The selection of *the COX1* gene is based on the sequence size. The selected *COX1* gene sequences of those species were aligned with the sequences of another species of the genus *Apis* and *Bombus* as the outgroup by using ClustalX 2.1 program. The genetic diversity of nucleotide variations, amino acid variation, and pairwise genetic distances of each gene was analyzed using MEGAX. The model used for constructing the phylogenetic tree was analyzed using the Find Best DNA/Protein Model implemented in MEGAX. The Neighbor-Joining (NJ) and Maximum likelihood (ML) phylogenetic trees were built using MEGAX with the best model of Tamura-Nei, Gamma distribution (G), and 1,000 bootstrap replications[11].

Results and Discussion

The selected *COX1* sequence database of *A. florea* and *A. andreniformis* from GenBank that was used in the bioinformatic analysis are listed in Table 1. There *is 18 A. Florea* database sequences of partial *COX1* gene and complete mtDNA, and seven database sequence of *A. andreniformis* that was used for nucleotide variation and phylogenetic relationship. The *COX1* gene sequences of *A. florea* are from their natural distribution in India, Bangladesh, Thailand, and China. The *COX1* sequence of *A. florea* was also found in another natural distribution in Iran and Egypt (Table 1). The number of *A. andreniformis COX1* sequences in NCBI was less than *A. florea* and restricted only found in their natural distribution area from India and Thailand. The *A. andreniformis COX1* sequences from Indonesia are available in GenBank[12], however, those sequences are partial and not homolog with other selected sequences, and thus were not included in the phylogenetic relationship analysis.

Table 1. *The Dwarf honey bee sequence dan outgroup species from GenBank were used for phylogenetic analysis using the COX1 gene*

No	Species	Location	Accession Number	References
		Ingroup		
1	*Apis florea*	Punjab, India	KT960841	Unpublished GenBank Makk et al.
2	*Apis florea*	Punjab, India	MN163113	Unpublished GenBank Bajaj et al.
3	*Apis florea*	India	KU666428	Unpublished GenBank
4	*Apis florea*	Kaas Plateau, India	KU737494	Rakshit Ojha et al.
5	*Apis florea*	Bengaluru, India	KR010698	Unpublished GenBank Rakshit Ojha et al.
6	*Apis florea*	Boshehr_Dylam, Iran	MG548252	Najafzadeh et al[18].
7	*Apis florea*	Fars_Khorambid_Iran	MG548255	Najafzadeh et al[18].
8	*Apis florea*	Boshehr Khormoj_Iran	MG548253	Najafzadeh et al[18].
9	*Apis florea*	Red Sea, Safaga, Egypt	OP797664	Unpublished GenBank Ali et al.
10	*Apis florea*	Punjab, India	ON287040	Unpublished GenBank Kaur et al.
11	*Apis florea*	India	KF817578	Unpublished GenBank Reetha et al.
12	*Apis florea*	Bangladesh	MH378769	Unpublished GenBank Aslam et al.
13	*Apis florea*	-	KP259001	Chesters et al[14].
14	*Apis florea*	-	KP259081	Chesters et al[14].
15	*Apis florea*	-	KP259052	Chesters et al[14].
16	*Apis florea*	-	JX982136	Wang et al[19].
17	*Apis florea*	Thailand	AP018491	Takahashi et al[10].
18	*Apis florea*	Yunnan, China	KC170303	Yang et al[16].
19	*Apis andreniformis*	Thailand	NC039709	Takahashi et al[10].
20	*Apis andreniformis*	Mizoram, India	KU212332	Unpublished GenBank Chhakchhuak et al.
21	*Apis andreniformis*	Mizoram, India	KU212333	Unpublished GenBank Chhakchhuak et al.
22	*Apis andreniformis*	Mizoram, India	KU212334	Unpublished GenBank Chhakchhuak et al.
23	*Apis andreniformis*	Mizoram, India	KU212335	Unpublished GenBank Chhakchhuak et al.
24	*Apis andreniformis*	Himalaya, India	MK904788	Unpublished GenBank Kumar et al[11].
25	*Apis andreniformis*	-	KF736157	Wang et al[14].
		Outgroup		
26	*Apis mellifera intermissa*	Morocco	KY926883	Unpublished GenBank Eimanifar et al.
27	*Apis mellifera syriaca*	Syria	KY926882	Unpublished GenBank Eimanifar et al.
28	*Apis koschevnikovi*	Sabah, Malaysia	AP017643	Wakamiya et al[23].
29	*Apis koschevnikovi*	-	KY348372	Unpublished GenBank Eimanifar et al.
30	*Apis cerana*	Russia	LC640351	Unpublished GenBank Takahashi and Ilyasov [10,21]
31	*Apis cerana*	Russia	AP018450	Ilyasov et al[21].
32	*Apis cerana*	Korea	KX908206	Wang et al[20].

33	*Apis nuluensis*	Sabah, Malaysia	AP018157	Takahashi et al[15].
34	*Apis dorsata*	-	NC037709	Unpublished GenBank Wang et al[20].
35	*Bombus terrestris*	France	NC045179	Cejas et al[22].

Alignment of *COX1* gene sequences from all dwarf honey bee databases resulted in the 530 bp homologous sequences. Based on this alignment, there are 60 sites of nucleotide variation (Supplementary 1a) and 10 amino acid site variations (Supplementary 1b) between *A. florea* and *A. andreniformis*. From the total of 10 deductive *COX1* amino acid variations, there is three amino acids that could differentiate *A. andreniformis* from *A. florea* in the position of 66, 111, and 152 that showed mutation of Leucine to Isoleucine (L→I), Methionine to Isoleucine (M→I), and Isoleucine to Valine (I→V), respectively (Supplementary 1b).

Between intra species of *A. andreniformis COX1* gene sequences from India and Thailand showed almost no nucleotide variation, excluding the singleton transversion of T→G in the site 433 of *Apis andreniformis* from Mizoram, India (KU212335) (Supplementary 1a). This transversion leads to the mutation of the amino acid Phenylalanine to Tryptophan (F→W). In parallel with the genetic distance result that showed 0-0.19% genetic diversity among *the A. andreniformis* sequences database (Table 3). Those results indicated that the genetic diversity of *the COX1* gene in the intra-species of *A. andreniformis* is low, thus, the *COX1* gene was less appropriate as a genetic marker for genetic diversity analysis in the intra-species level of *A. andreniformis*. The *cytb* gene could be the alternative gene for this analysis, due to this gene showed 5.7% genetic diversity (Table 3) between *A. andreniformis* in Thailand[13].

Based on nucleotide variation analysis of all *A. florea COX1* gene sequences database revealed 28 site substitutions (Table 2). The partial COX1 sequences of *A. florea* from Thailand and China have the same sequences. Table 2 also showed that the *A. florea* COX1 sequences that have no location information with the Accession number of KP259001, KP259081, and KP259052[14] also have similar nucleotide sequences with those from Thailand (AP018491)[15] and China (KC170303, Yang et al., with the low genetic diversity range from 0% to 0.7%[16]. Thus, those *A. florea* sequences might be from East or Southeast Asia. This analysis also revealed that *A. florea* from introduced locations in Iran and Egypt had a similar sequence with those from India with genetic diversity range between 0-0.97%. It indicated that the introduction of *A. florea* to Western Asia and Africa might be derived from locations around India. This analysis supported a previous study that found *A. florea* in Egypt was suspected introduce from India based on *the COX1* gene[9]. From 530 bp, there are eight sites (nucleotide in grey highlight) that were proposed as a genetic marker to distinguish *A. florea* from India and *A. florea* from Thailand and China. The genetic diversity of the partial *COX1* gene sequence database between *A. florea* from India, Iran, Egypt and the *A. florea* from Thailand and China is 1.7-3.4%.

The phylogenetic tree of dwarf honey bee based on *COX1* gene sequence was built using Neighbour-Joining and Maximum Likelihood methods. The topological appearance of NJ and ML-based phylogenetic trees are similar, except for the placement of *A. mellifera* and *A. dorsata* clade (Fig. 1). The dwarf honey bee is clustered into a monophyletic clade with 93-94 bootstrap value and *A. koschevnikovi* as the sister clade. The position of *A. koschevnikovi* as the sister clade is unstable due to the low bootstraps value of 37-43%. Based on the analysis of three mitochondrial (*rrnL, cox2, nad2*) and one nuclear gene (*itpr*) the position of the dwarf honey bee is always in the basal of the tree[17].

Table 2. *The nucleotide variation of A. florea based on the COX1 gene from GenBank*

No	Sequences	Nucleotide site																											
		3	9	78	90	111	121	177	186	189	192	205	208	213	225	243	246	252	267	324	332	333	420	426	456	472	474	507	522
1	KP259001 *Apis florea* 0508-A2-02	A	A	T	G	A	T	A	C	A	A	T	T	A	C	A	A	T	A	A	T	T	T	A	T	C	C	A	A
2	AP018491 *Apis florea* Thailand	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	KC170303 *Apis florea* China	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	KP259081 *Apis florea* 0507-D2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
5	KP259052 *Apis florea* 0515-A2-03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
6	JX982136 *Apis florea*	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.
7	KT960841 *Apis florea* India	.	.	A	.	.	C	.	T	.	G	.	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
8	MN163113 *Apis florea* India	.	.	A	.	.	C	.	T	.	G	.	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
9	MH378769 *Apis florea* Bangladesh	G	.	A	.	.	C	.	T	.	G	.	C	G	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
10	KR010698 *Apis florea* India	.	.	A	.	.	C	.	T	.	G	C	C	.	T	.	.	A	T	G	.	.	C	.	A	.	T	.	.
11	MG548252 *Apis florea*	.	.	A	.	.	C	.	T	.	G	C	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
12	OP797664 *Apis florea* Safaga Egypt	.	.	A	.	.	C	.	T	.	G	C	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
13	MG548253 *Apis florea* Boshehr Khormoj Iran	.	.	A	.	.	C	.	T	.	G	C	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
14	MG548255 *Apis florea* Fars Khorambid Iran	.	.	A	A	.	C	.	T	.	G	C	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
15	KU737494 *Apis florea* India	.	.	A	.	.	C	.	T	.	G	C	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	.
16	KF817578 *Apis florea* India	.	.	A	.	.	C	.	T	.	G	C	C	.	T	.	.	A	T	.	.	.	.	.	A	.	T	.	G
17	KU666428 *Apis florea* India	.	.	A	.	.	C	.	T	.	G	C	C	.	T	C	.	A	T	.	.	.	.	.	A	.	T	G	.
18	ON287040 *Apis florea* Punjab India	.	T	A	.	T	C	T	T	C	G	.	C	.	T	.	T	A	T	.	.	.	.	T	.	T	T	.	.

Table 3. *The genetic distance of dwarf honey bee based on COX1 gene sequences databases from GenBank*

No	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]
[1]																								
[2]	0,0000																							
[3]	0,0000	0,0000																						
[4]	0,0000	0,0000	0,0000																					
[5]	0,0000	0,0000	0,0000	0,0000																				
[6]	0,0000	0,0000	0,0000	0,0000	0,0000																			
[7]	0,0019	0,0019	0,0019	0,0019	0,0019	0,0019																		
[8]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898																	
[9]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898	0,0000																
[10]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898	0,0000	0,0000															
[11]	0,0899	0,0899	0,0899	0,0899	0,0899	0,0899	0,0923	0,0019	0,0019	0,0019														
[12]	0,0851	0,0851	0,0851	0,0851	0,0851	0,0851	0,0874	0,0019	0,0019	0,0019	0,0038													
[13]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898	0,0057	0,0057	0,0057	0,0077	0,0077												
[14]	0,0850	0,0850	0,0850	0,0850	0,0850	0,0850	0,0874	0,0196	0,0196	0,0196	0,0196	0,0176	0,0216											
[15]	0,0850	0,0850	0,0850	0,0850	0,0850	0,0850	0,0874	0,0196	0,0196	0,0196	0,0196	0,0176	0,0216	0,0000										
[16]	0,0900	0,0900	0,0900	0,0900	0,0900	0,0900	0,0924	0,0236	0,0236	0,0236	0,0236	0,0216	0,0256	0,0039	0,0039									
[17]	0,0924	0,0924	0,0924	0,0924	0,0924	0,0924	0,0948	0,0259	0,0259	0,0259	0,0259	0,0239	0,0279	0,0058	0,0058	0,0097								
[18]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898	0,0217	0,0217	0,0217	0,0217	0,0197	0,0237	0,0019	0,0019	0,0058	0,0038							
[19]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898	0,0217	0,0217	0,0217	0,0217	0,0197	0,0237	0,0019	0,0019	0,0058	0,0038	0,0000						
[20]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898	0,0217	0,0217	0,0217	0,0217	0,0197	0,0237	0,0019	0,0019	0,0058	0,0038	0,0000	0,0000					
[21]	0,0874	0,0874	0,0874	0,0874	0,0874	0,0874	0,0898	0,0217	0,0217	0,0217	0,0217	0,0197	0,0237	0,0019	0,0019	0,0058	0,0038	0,0000	0,0000	0,0000				
[22]	0,0899	0,0899	0,0899	0,0899	0,0899	0,0899	0,0923	0,0237	0,0237	0,0237	0,0237	0,0217	0,0257	0,0038	0,0038	0,0078	0,0058	0,0019	0,0019	0,0019	0,0019			
[23]	0,0898	0,0898	0,0898	0,0898	0,0898	0,0898	0,0922	0,0257	0,0257	0,0257	0,0257	0,0237	0,0277	0,0057	0,0057	0,0097	0,0077	0,0038	0,0038	0,0038	0,0038	0,0058		
[24]	0,0850	0,0850	0,0850	0,0850	0,0850	0,0850	0,0874	0,0237	0,0237	0,0237	0,0237	0,0217	0,0257	0,0038	0,0038	0,0078	0,0058	0,0019	0,0019	0,0019	0,0019	0,0039	0,0058	
[25]	0,0972	0,0972	0,0972	0,0972	0,0972	0,0972	0,0997	0,0318	0,0318	0,0318	0,0340	0,0298	0,0339	0,0155	0,0155	0,0194	0,0214	0,0174	0,0174	0,0174	0,0174	0,0194	0,0214	0,0194

Aan: *Apis andreniformis*, Afl: *A. florea*, [1] KU212332 Aan_Mizoram_India, [2] KU212334 Aan_Mizoram_India, [3] NC_039709 Aan Thailand, [4] MK904788 Aan India, [5] KU212333 Aan Mizoram India, [6] KF736157 Aan, [7] KU212335 Aan Mizoram_India, [8] KP259001 Afl, [9] AP018491 Afl Thailand, [10] KC170303 Afl China, [11] KP259081 Afl, [12] KP259052 Afl, [13] JX982136 Afl, [14] KT960841 Afl India, [15] MN163113 Afl India, [16] MH378769 Afl Bangladesh [17] KR010698 Afl India, [18] MG548252 Afl, [19] OP797664 Afl Safaga_Egypt, [20] MG548253 Afl Boshehr Khormoj Iran, [21] KU737494 Afl India, [22] KF817578 Afl India, [23] KU666428 Afl India, [24] MG548255 Afl Fars_Khorambid_Iran, [25]ON287040 Afl _Punjab_India

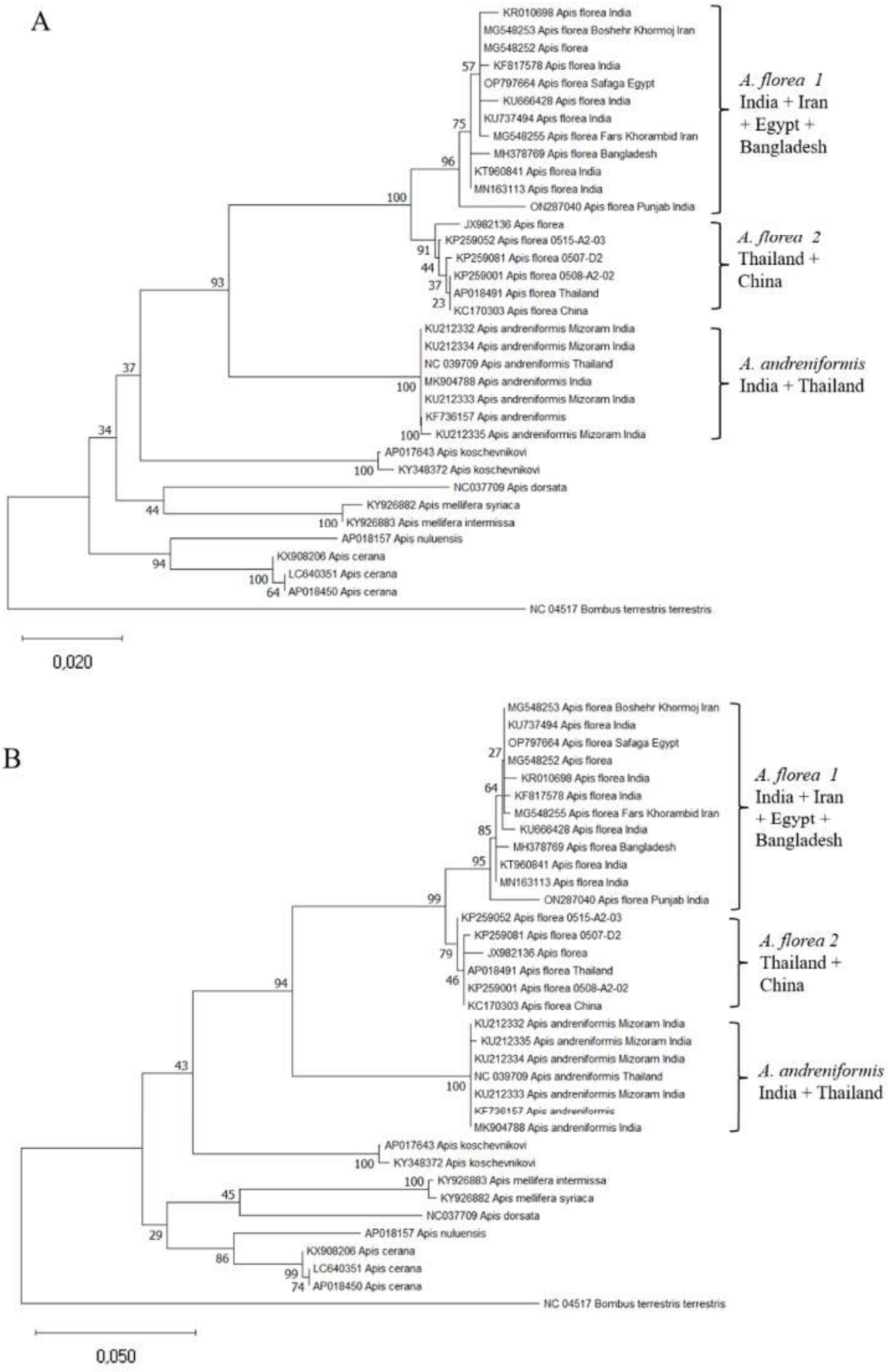

Figure 1. Phylogenetic tree of dwarf honey bee inferred from COX1 gene sequences from GenBank database using (A) Neighbor-Joining method and (B) Maximum Likelihood method

The position of *A. andreniformis* clade is monophyletic (99-100 bootstraps value) and separated from *A. florea* (Fig. 1). In concordance with nucleotide variation and genetic distance, both phylogenetic trees separated *A. florea* into two subclades i.e. sub-clade of *A. florea* 1 that composed by honey bee from India, Egypt, and Iran, and sub-clade *A. florea* 2 that consisted of honey bee from Thailand, China, and unknown location. The sub-clade of *A. florea*1 has supported the conclusion that based on the *COX1* gene sequence, *A. florea* in Egypt is similar to *A. florea* from India with 99%- 100% similarity and query of 91-100%[9].This study suggested that *the COX1* gene is the potential as a molecular marker to detect the geographical origin of *A. florea*, however, for *A. andreniformis* this gene has low genetic diversity thus more polygenic markers are needed to explore in *A. andreniformis* for future study.

Conclusion

The *COX1* sequence databases of dwarf honey bees from NCBI showed the low genetic diversity of *A. andreniformis*, while this gene could differentiate A. florea into two sub-clade of *A. florea* 1 (India+Egypt+Iran) and sub-clade *A. florea* 2 (Thailand+China+unknow location). Thus, this study proposed that *the COX1* gene is the potential as a molecular marker to detect the geographical origin of introduced *A. florea,* however, need more exploration of another marker for *A. andreniformis*

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