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In Silico Primer Design for geographical detection of Apis florea using Cytochrome c oxidase subunit 1 (COX1) gene

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Abstract

The yellow dwarf honey bee, *Apis florea* is well distributed in South Asia to South East Asia. This species is expanded and introduced area from their original distribution. However, the distribution of this honey bee in Indonesia is unexplored. The cytochrome c oxidase subunit 1 (*COX1*) gene is successful to detect the original geographic of introduced A. florea found in Egypt. The *A. florea* specific primer of *COX1* gene is needed to produce the molecular marker for geographical origin detection. Thus, this study aims to in silico design the *COX1* gene primer of *A. florea* using Primer3 and Primer-BLAST. This study results in the best candidate primer of Af6_COX1_F and Af6_COX1_R primers that start from 212 to 983 regarding the *A. florea COX1* gene (NC_021401) and produce 772 bp of the amplicon. The melting temperature of the forward primer is 54. 63 and 55.58°C, GC content of 40 and 45%, GC clamp of 3 and 2 for forward and reverse primer, respectively. There are no secondary structures of those primers and 100 % homolog with *A. florea COX1* sequences, thus those primer is the potential as the geographical origin marker of A. florea.

Keywords: Dwarf honeybee, Geographic origin, Primer3, Primer-BLAST, Primer design

Introduction

Apis florea are recognized as yellow or red dwarf honey bees based on their basic morphological appearance they also construct their nests in open spaces by wrapping the twigs (Wongsiri et al. 1996). In India, this honey bee is well explored to play a significant pollination role for onion (*Allium cepa*) (Abrol 2010), and 35 crop plants including cauliflower (*Brassica oleracea*), radish (*Raphanus sativum*, lemon (*Citrus limon*), and others (Sihag 2019). Its native range extends from South Asia, India via Cambodia to Thailand, while in Indonesia this was thought to have been transported by people via ship to Surabaya and Jakarta (Otis 1996). Although *A. florea* has an important role in pollination, however, the information of the recent distribution of *A. florea* in Indonesia is unclear, thus the exploration of this species in Indonesia is needed to know the ecological impact of this species in Indonesia. Additionally, it was discovered that this species had been introduced to various regions of Jordan (Haddad et al. 2009) and Africa (El-Niweiri et al. 2019). According to the *cytochrome c oxidase subunit 1* (*COX1)* mitochondrial gene data, it was assumed that *A. florea* in Egypt had been imported from India (Salem et al. 2020). This gene also successfully confirms that *A. cerana* from West Papua and the Moluccas were introduced from Java (Raffiudin et al. 2022). Thus, this gene is a potential marker to detect the geographical origin of *A. florea* in Indonesia.

The *COX1* gene is mitochondrial DNA (mtDNA) that has been used as a DNA barcode in a wide range of animal species (Hebert et al. 2003). Another study also found that this DNA barcode of *COX1* could detect the geographical detection of *A. florea* (Salem et al. 2020). Although the *COX1* gene for *A. florea* is well explored, however, the primer used for molecular-based geographical detection (Salem et al. 2020), applied the universal primer from corbicula bees. Those primers included the forward primer of BarbeeF (Françoso and Arias 2013) and the reverse primer of MtD9 (Simon et al. 1994), with 50 °C annealing temperature. This universal primer could amplify several corbicula species (Françoso and Arias 2013) and this was proved by the BLAST-N analysis that revealed BarbeeF primers are 100% identic with several mtDNA sequences of corbicula bees. However, this primer is also a homolog for another gene in *A. florea* according to the BLASTN results. For specific amplification results to geographical origin analyse, a specific primer is needed to design based on the *A. florea* sequence databases. For designing *A. florea* primer, the complete *COX1* gene from the whole *A. florea* mtDNA sequences are needed. The whole mtDNA reference sequence of *A. florea* is

established with the accession number NC_021401 (Wang et al. 2013). Another whole mtDNA of *A. florea* from Thailand (AP0184490) that was closed related to *A. florea* from China (KC1703003) has been revealed (Takahashi et al. 2018). Thus, this study aims to design the *COX1* gene primer for *A. florea* original geographical detection using open access program of Primer3 and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). By using the new primer of *COX1* gene, hopefully, the origin geographical of *A. florea* that is found in Indonesia can be detected, thus, the information on the vast distribution of this species can be revealed.

Materials and Methods

The template of *COX1* gene used for primer designing was obtained from the whole mtDNA *A. florea* sequence (17694 bp) with the Accession number NC_021401 (Wang et al. 2013). This nucleotide sequence data was searched by the National Center for Biotechnology Information or NCBI (http://www.ncbi.nlm.nih.gov) with the resource option of "nucleotide". The position of *COX1* gene in this *A. florea* whole mtDNA sequence is 2302-3862. The primer targeting *COX1* gene was designed using the online free access software program Primer3 (https://primer3.ut.ee/) (Koressaar and Remm 2007, Untergasser et al. 2012) and Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al. 2012). The amplification product size was targeted in the range of 500 to 800 bp, thus this primer also can use as a DNA barcode (Kress and Erickson 2008). For the "general picking primer condition", several parameters were set for ideal primer conditions such as the range of primer size were 18-24, melting temperature (Tm) were 50-60 ^{o}C, and GC content was 40-60 %. The GC clamp was analyzed manually by counting the number of GC bases in 5 last bases at the 3' end of the candidate primer. The secondary structure of candidate primers such as hairpin, self-dimer, and hetero dimer was analyzed using the integrated DNA technologies' (IDT) oligo analyzer (https://www.idtdna.com/calc/analyzer) (Owczarzy et al. 2008). The homology of the best primer candidate was examined using BLAST-N (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990) to recognize the similarity of the primer with another organism.

Results and Discussion

The *COX1* gene is well known as a marker for DNA barcode in corbicula bees (Françoso and Arias 2013), and successfully differentiate honey bee at the intraspecies level based on their geographical distribution (Raffiudin et al. 2022), as well as for *A. florea* (Salem et al. 2020). In this study, the species-specific primers were designed in silico to detect the geographical origin of *A. florea* that was introduced to the adjacent region of its original distribution (Haddad et al. 2009, El-Niweiri et al. 2019).

This study found five pair of candidate primers obtained from the Primer3 Program (Table 1), and 10 pair of candidate primers from the Primer-BLAST program (Table 2). This study used two primer design programs in order to produce more candidate primers. This study also uses those two programs, to compare the best program for primer designing. Based, on the results Primer-BLAST produce twice more primer candidates than Primer3, with varied primer location and primer length. The target PCR product of all primer range from 542 to 772 bp, this size is optimal based on (Dieffenbach *et al*. (1993) which recommend the ideal amplification product of 150-1000 bp. The length of the PCR product could have enough information for the research goal. Based on (Kress and Erickson 2008) the ideal length for a short DNA barcode is 400-800 bp or around 640 bp for corbicula Apidae bees (Françoso and Arias 2013)

The parameters that should be of concern for primer designing are primer length, melting temperature, GC content, GC clamp, and secondary structure such as hairpin, self-dimer, hetero-dimer, etc. (Borah 2011). The primer length from Primer3 Program is consistent with 20 bases while using Primer-BLAST the length is varied from 19 to 21 bases. This range of primer length has fulfilled the optimal primer length of 18-24 (Borah 2011). The primer that is shorter than 18, will be less specific to the targeted gene, while primer with a long size not significantly affected the specificity of primer. Based on Table 1 and Table 2, the melting temperature (Tm), GC content (%), and GC clamp range from 53.07 to 56.25, 36.36 to 52.63, and 0 to 4, respectively. All candidate primer has an ideal Tm as mentioned by Borah (2011) that the Tm are 52-58 °C or up to 60 °C (Sharma 2021), commonly generating the best amplification results. The higher temperature will tend to anneal again. The ideal CG

content is 40-60% (Borah 2011, Sharma 2021). The previous study explained that if the GC content is too low, the primer will not efficient to anneal the DNA template, while the high GC content in the primer resulting the obstacle of the primer separating from the DNA template in the PCR. The reason is due to G and C are bonding with the three hydrogen Cain, while A and T have only two bonds. All candidate primer resulting from Primer3 has ideal CG content of 40-45%, while some candidate primers by Primer-BLAST have GC content below 40%. This result showed that although Primer3 produce less candidate primer than Primer-BLAST, however, Primer3 produce candidate primer with more precise or ideal parameter. The CG clamp is the number of presenting G and C nucleotides in the last five bases from the 3' end of primers which should be 2-3 GC bases and no more than 3 bases (Patel and Prakash 2013). In this result, the GC clamp is varied from 0 to 4, thus only candidate primer with 2-3 GC clamp was chosen as the best primer. The GC content in the 3' end of the primer supports the specific binding due to the stronger bonding of G and C bases than the A and T bases. The position and GC content in the 3'-terminal of primer is important for controlling mispriming (Kwok et al. 1990).

Hairpins, self-dimers, and hetero dimers are examples of primer secondary structures that should be avoided in the primer design. Based on Borah (2011), a hairpin is an intramolecular contact within the primer that prevents primer attachment to the template. The bonds between similar primers either in forward or reverse primers will create self-dimers. This condition can occur when the primer has a self-complementary base sequence. Dimer or bonding between forward and reverse primers are called hetero dimer or cross dimer (Borah 2011). According to IDT (https://www.idtdna.com/) (Owczarzy et al. 2008), the ΔG of harpin greater than -3 kcal/mole is tolerance. ΔG of the hairpin is energy that needs to break the bonding of hairpins. The ΔG for internal self-dimer and hetero dimer should be greater than -6 kcal/mole and -9 kcal/mole respectively. Table 3 shows that ΔG of the hairpin of all candidate primer are greater than -3 kcal/mole, thus there is no hairpin in all candidate primer. However, there are five primer pairs that have ΔG of self-dimer below -6 kcal/mole, i.e., Af2, Af3, Af7, Af10, Af11, and Af12 (Table 3) that causing those primers could not be selected as the best primer. Based on all parameters the pair of Af1_COXI forward and reverse primers are chosen as the best primer candidate based on Primer3 and the pair of Af6_COXI forward and reverse primers are the best-recommended primer based on the Primer-BLAST program. However, the best primer is near the beginning or 5' end targeted gene, thus the Af6_COXI forward reverse primer is selected as the best primer candidate. Although there are 4 run bases in Af6_COX1_F primer, however, this can be tolerated. The number of run bases or the number of repeating similar bases have no more than 5 bases, for example, AAAAA. Fortunately, there is no repeat in both Af6_COX1 forward and reverse primer (Table 4). The accepted repeat for primer is 4 bases, for example ATATATAT (Borah 2011).

Table 1. The *COX1* gene primer candidate of *Apis florea* designed using Primer3 program.

No	Primer Name	Sequence (5' --> 3')	Length	Start	Product Size	Tm (°C)	GC%	GC Clamp
1	Af1_COX1_F	TTTTACCCATCACCAGGAAC	20	346	638	55,23	45	3
2	Af1_COX1_R	GCTCCGTGATAAGTTGCTAA	20	983		55,58	45	2
3	Af2_COX1_F	ATTATTGCCGTTCCTACAGG	20	925	577	55,21	45	3
4	Af2_COX1_R	AGTGTTCAATTGGTGGTGTT	20	1501		55,67	40	2
5	Af3_COX1_F	ATCTTTACCAGTTCTCGCAG	20	585	737	55,19	45	4
6	Af3_COX1_R	AATCTGAATAACGTCGAGGT	20	1321		54,22	40	3
7	Af4_COX1_F	TTGGAGGATTCGGAAATTGA	20	218	770	54,63	40	1
8	Af4_COX1_R	TTTTGCTCCGTGATAAGTTG	20	987		54,23	40	2
9	Af5_COX1_F	TTGATTGGAGGATTCGGAAA	20	214	730	54,63	40	2
10	Af5_COX1_R	CTGTAGGAACGGCAATAATT	20	943		53,38	40	0

Table 2. The *COX1* gene primer candidate of *Apis florea* designed using Primer-BLAST program.

No	Primer Name	Sequence (5' --> 3')	Length	Start	Product Size	Tm (°C)	GC%	GC Clamp
1	Af6_COX1_F	TTTTGATTGGAGGATTCGGA	20	212	772	54,63	40	3
	Af6_COX1_R	GCTCCGTGATAAGTTGCTAA	20	983		55,58	45	2
2	Af7_COX1_F	TGATTGGAGGATTCGGAAAT	20	215	735	54,39	40	1
	Af7_COX1_R	TAATTCCTGTAGGAACGGCA	20	949		56,25	45	4
3	Af8_COX1_F	ATTTTACCCATCACCAGGAA	20	345	639	54,17	40	2
	Af8_COX1_R	GCTCCGTGATAAGTTGCTA	19	983		54,72	47.37	2
4	Af9_COX1_F	ATCTTTACCAGTTCTCGCAG	20	585	573	55,19	45	4
	Af9_COX1_R	GCAAATACAGCTCCTATTGA	20	1157		53,07	40	1
5	Af10_COX1_F	TAGCAACTTATCACGGAGC	19	965	542	54,72	47,37	3
	Af10_COX1_R	ATGTGAGTGTTCAATTGGTG	20	1506		54,1	40	3
6	Af11_COX1_F	AGATGTTGATACACGAGCTTA	21	885	623	54,51	38.10	2
	Af11_COX1_R	CATGTGAGTGTTCAATTGGT	20	1507		54,1	40	2
7	Af12_COX1_F	CCAGGAACAGGATGAACTG	19	358	546	55,19	52,63	2
	Af12_COX1_R	AGCTCGTGTATCAACATCTAA	21	903		54,51	38,1	1
8	Af13_COX1_F	ATTGGAGGATTCGGAAATTGA	21	217	564	55,25	38,1	1
	Af13_COX1_R	TAAGCTCGTGTATCAACATCT	21	905		54,41	38,1	2
9	Af14_COX1_F	ATCACCAGGAACAGGATGA	19	354	564	55,26	47,37	2
	Af14_COX1_R	GCTGATGTAAAATAAGCTCGT	21	917		54,4	38,1	3
10	Af15_COX1_F	CCTGATATAGCATTTCCACG	20	262	642	53,67	45	4
	Af15_COX1_R	AGCTCGTGTATCAACATCTAAT	21	903		55,09	36,36	1

The results of nucleotide base BLAST showed that primer Af6_COX1_F has 100% identity not only for *A. florea COXI* gene (MN163113.1) but also homolog with *Chironomidae sp.* (LC494944.1) and *Plexippus paykulli* (MK392810.1). Our BLAS-N results revealed that this forward primer is potentially as general primer. In the other hand, the BLAST-N analysis show that Af6_COX1_R primer are specific for *Apis florea*, and has 100% identity with *A. florea* partial *COX1* gene sequence of AP018491.1, MH138080.1, and MF363140.1. Although the forward primer is not specific to *A. florea*, however, the pairing of Af6_COX1_F and Af6_COX1_R primers could be the potential specific primer to amplify *COX1* gene in *A. florea*.

The start position of Af6_COX1_F based on *A. florea* whole mtDNA sequences (NC_021401) are 212, while the start position of Af6_COX1_R is 983. This position is overlapping with the position of *COX1* primer used for *A. florea* original molecular detection (Salem et al. 2020) that used Barbee (forward) and MTD9 (reverse) primers. The Barbee forward primer (Françoso and Arias 2013) and MTD9 reverse primer (Simon et al. 1994) target the corbicula bee *COX1* gene in the position of 20 to 743 base. Salem et al. (2020) resulted from the 690bp amplicon of *A. florea COX1* sequences, and those sequences could detect that the introduced *A. florea* in Egypt is 99-100% identical to the *A. florea* from India. Thus, they assumed that *A. florea* in Egypt is transferred from India via Jordan and confirmed that *COX1* gene is the potential marker for *A. florea* geographical detection. Finally, our study concludes that Primer3 and Primer-BLAST is a promising tools for primer designing. Although the Primer-BLAST produced more candidate primers with varied primer locations than Primer3, however, both programs can result in the primer with ideal criteria. The best candidate primer for specific is *A. florea* Af6_COX1_F and Af6_COX1_R primers. Future research and PCR process are needed to confirm the efficiency and potential of this primer targeting *COX1* gene as geographical origin detection marker of *A. florea*.

Table 3. The primer parameter and secondary structure result from the integrated DNA technologies' (IDT) oligoanalyzer.

No	Primer Name	Length	Tm (°C)	Hairpin ΔG (kcal/mole)	Self-Dimmer ΔG (kcal/mole)	Hetero-Dimmer ΔG (kcal/mole)
1	Af1_COX1_F	20	52,4	-0,28	-3,07	-6,35
	Af1_COX1_R	20	52,4	0,61	-3,61	
2	Af2_COX1_F	20	52,3	-0,13	-4,67	-5,37
	Af2_COX1_R	20	52,6	0,25	-9,27	
3	Af3_COX1_F	20	52,1	0,47	-3,61	-6,78
	Af3_COX1_R	20	51	1,3	-6,76	
4	Af4_COX1_F	20	51,6	0,7	-5,36	-8,26
	Af4_COX1_R	20	50,9	1,28	-3,61	
5	Af5_COX1_F	20	51,6	1,01	-3,61	-5,37
	Af5_COX1_R	20	50,2	0,69	-5,36	
6	Af6_COX1_F	20	51,6	1,01	-3,61	-8,26
	Af6_COX1_R	20	52,4	0,61	-3,61	
7	Af7_COX1_F	20	51,5	0,71	-3,61	-6,59
	Af7_COX1_R	20	53,4	-2,05	-8,19	
8	Af8_COX1_F	20	51,4	-0,28	-3,07	-6,35
	Af8_COX1_R	19	51,9	0,61	-3,61	
9	Af9_COX1_F	20	52,1	0,74	-0,361	-3,89
	Af9_COX1_R	20	50	-0,33	-6,34	
10	Af10_COX1_F	19	51,9	0,61	-3,61	-4,87
	Af10_COX1_R	20	50,9	0,07	-9,27	
11	Af11_COX1_F	21	51,1	-0,08	-6,34	-5,47
	Af11_COX1_R	20	50,9	0,07	-9,27	
12	Af12_COX1_F	19	52,8	-0,87	-3,55	-5
	Af12_COX1_R	21	51,1	-0,16	-6,34	
13	Af13_COX1_F	21	52	0,7	-5,36	-5,47
	Af13_COX1_R	21	51,1	-0,16	-6,34	
14	Af14_COX1_F	19	53	-0,37	-3,53	-5
	Af14_COX1_R	21	50,8	-0,37	-6,34	
15	Af15_COX1_F	20	50,6	0,83	-3,91	-6,91
	Af15_COX1_R	22	51,4	-0,16	-6,34	

Table 4. BLAST Nucleotide results of *COX1* gene best primer

No	Primer name	Sequen (5'-3')	Start	Description	Query Cover (%)	E-value	Ident. (%)	Acc. Number
1	Af6_COX1_F	TTTTGATTGGAG GATTCGGA	212	*Chironomidae sp.* 1, partial COI	100	1,9	100	LC494944.1
				Apis florea, partial COI	100	1,9	100	MN163113.1
				Plexippus paykulli , partial COI	100	1,9	100	MK392810.1
2	Af6_COX1_R	GCTCCGTGATAA GTTGCTAA	983	*Apis florea*, mtDNA complete genome	100	1,9	100	AP018491.1
				Apis florea YN4, partial COI	100	1,9	100	MH138080.1
				Apis florea XM6, partial COI	100	1,9	100	MF363140.1

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