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DNA BARCODES IN IDENTIFICATION OF SOME SPECIES OF HAWKMOTHS (LEPIDOPTERA: SPHINGIDAE)

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ABSTRACT

The use of DNA barcodes is a molecular method commonly used for the identification of specimens that are difficult to pinpoint accurately by traditional morphological methods. DNA barcoding uses standard short genomic regions that are universally present in target lineages and has sufficient sequence variation to identify species in the genus. The hawkmoth genus *Cechetra* (Zolotuhin & Ryabov, 2012) was proposed for a group of morphologically similar, stripe-patterned species. Based on the seven specimens of the three species of *Cechetra* genus collected from the Central of Vietnam, we used morphological analysis to initially identified their scientific name of *Cechetra lineosa* (Walker, 1856) for 04 specimens (LB_F01, LB_G01, LB_H01 and LK_G05); 02 specimens (MK_H04 and MK_B07) were identified with the scientific name of *Cechetra minor* (Butler, 1875) and 01 specimen (SG_D7) with scientific name *Cechetra subangustata* (Rothschild, 1920). Then, the *COI* (Cytochrome C oxidase I) sequence was used for molecular analysis. Total DNA was extracted from the legs of specimens, *COI* with over 600 bp in length from each specimen was amplified by PCR reactions. The PCR products are hence purified and sequenced. *COI* gene cloning results showed a very high level of genetic similarity (over 99%) and 7 specimens are of the genus *Cechetra*, Sphingidae family. For three homomorphic species belonging to the genus *Cechetra* Zolotuhin & Ryabov, 2012 including *Cechetra lineosa* (Walker, 1856); *Cechetra minor* (Butler, 1875) and *Cechetra subangustata* (Rothschild, 1920). The studied sequences have been added to the current database at GenBank (NCBI) with accession numbers MT994230, MT994231, MT994232, MT994233, MT994234, MT994235, and MT994236.

Keywords: DNA, cytochrome c oxidase I (*COI*), *Cechetra*, Sphingidae, hawkmoths.

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INTRODUCTION

The family Sphingidae (Insecta: Lepidoptera: Bombycoidea), commonly known as hawkmoths, consists of more than 1600 species of small to very large moths, worldwide except Antarctica (Holloway et al., 2001; Kitching, 2020). Due in part to their large size and great beauty, hawkmoths are also one of the favorite groups for butterfly collectors (Kitching & Cadiou, 2000). According to Zolotuhin & Ryabov (2012), about 174 species including four subfamilies Langiinae, Sphinginae, Smerinthinea, and Macroglossinae of hawkmoths are reported from Vietnam. The hawkmoth genus *Cechetra* Zolotuhin & Ryabov, 2012 was proposed for a group of morphologically similar, stripe-patterned species of the genus *Cechenena* Rothschild & Jordan, 1903 with the type species of *Cechetra subangustata* Rothschild, 1920 (Zolotuhin & Ryabov, 2012). At the present time, a total of six species around the world, including four species of *Cechetra* genus, have been recorded in Vietnam, including *Cechetra bryki* (Ivshin & Krutov, 2018), *Cechetra lineosa* (Walker, 1856); *Cechetra minor* (Butler, 1875) and *Cechetra subangustata* (Rothschild, 1920). Currently, there are 6 species of this genus in the world, among which 5 have been recorded in Vietnam. However, morphological identification of the *Cechetra* species is difficult because of high similarities in wing patterns. Therefore, the use of DNA barcodes, a molecular method commonly used for the identification of specimens that are difficult to pinpoint accurately base on traditional morphological methods.

Recently, DNA barcoding has emerged as a useful tool for identifying difficult specimens (Hebert et al., 2003a). It employs sequence diversity in a standard short DNA sequence as a tool for species discrimination. In animals, a 648 base pair fragment from 5′ end of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene is considered the standard DNA barcode for many species (Hebert et al., 2003a,b), and it has been shown to great success with animal class including insect class (Foottit et al., 2008; Hastings et al., 2008); bird class (Tavares & Baker, 2008); fish class (Hubert et al., 2008; Wong et al., 2009) and crustaceans (Hou et al., 2009), while also proved to be effective on identifying algae (Clarkston & Saunders, 2010). The order Lepidoptera has 1,444,843 sequences of 115,596 species from 177 countries in the world recorded on BOLD, the Barcode of Life Data System, including 5,189 sequences from Vietnam (BOLD Systems, 2020). In particular, Sphingidae has 34,078 sequences of 1,948 species recorded from 117 countries, among which 673 sequences are from Vietnam (BOLD Systems, 2020).

Vietnam is one of the highest biodiversity hotspots in the world, especially for species of the Sphingidae family in Southeast Asia. DNA barcoding allows taxonomists to rapidly sort specimens and highlight divergent taxa that may represent new species. More broadly, DNA barcoding offers taxonomists the opportunity to greatly expand and eventually complete a global inventory of life diversity. In this study, DNA barcoding was used to assign specimens to known species. Therefore, we analyzed the sequence of *COI* (mtDNA) to identify seven hawkmoths specimens of the *Cechetra* genus in Vietnam. *COI* sequences of these species have been added to the existing database.

MATERIALS AND METHODS

According to Zolotuhin & Ryabov (2012), and based on the morphological characters of the collected specimens, there were seven mature hawkmoths specimens belonging to three species of the genus *Cechetra* in the Sphingidae family in this study. All specimens were collected from different areas of the centre of Vietnam, including three specimens from Kon Plong district (Kon Tum province), three specimens from Bach Ma National Park (Thua Thien Hue province), and one from Kon Chu Rang Nature Reserve (Gia Lai province) (Table 1).

Table 1. Detail of species analyzed in the present study

No.	Code of Sample	Species	Collection Places	Latitude and Longitude	Collection Month/Year
1	LK_G05	*Cechetra lineosa* (Walker, 1856)	Kon Plong, Kon Tum province	14,593055N 108,278333E	Jun 2019
2	LB_F01	*C. lineosa* (Walker, 1856)	Bach Ma NP., Thua Thien Hue province	11,464166N 108,0655555E	Jun 2018
3	LB_G01	*C. lineosa* (Walker, 1856)	Bach Ma NP., Thua Thien Hue province	11,464166N 108,0655555E	Jun 2018
4	LB_H01	*C.lineosa* (Walker, 1856)	Bach Ma NP., Thua Thien Hue province	11,464166N 108,0655555E	Jun 2018
5	MK_H04	*C. minor* (Butler, 1875)	Kon Plong, Kon Tum province	14,593055N 108,278333E	Jun 2019
6	MK_B07	*C.minor* (Butler, 1875)	Kon Plong, Kon Tum province	14,593055N 108,278333E	Jun 2019
7	SG_D7	*C. subangustata* (Rothschild, 1920)	Kon Chu Rang NR., Gia Lai province	14,470139N 108,574417E	May 2019

Seven tissue samples (legs) were preserved in Eppendorf tubes 1.5 mL with 70% ethanol, kept at 4 ºC. All adult specimens were spread, identified, labelled, captured, and arranged into the entomological collection of the Vietnam National Museum of Nature.

DNA extraction: DNA was extracted from the specimens' hindleg tissues using the Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. DNA was purified using the Genomic DNA Purification Kit (# K052, Fermentas).

Table 2. List of taxa whose sequences were downloaded from the GenBank (http://www.ncbi.nlm.nih.gov)

No.	Scientific name	Accession Number on Genbank
1	*Cechetra minor*	JN677796
2	*C. subangustata*	JN677799
3	*C. subangustata*	KP20043
4	*C.lineosa*	KC182176
5	*C.lineosa*	JN677795
6	*C.lineosa*	KY962521
7	*Acherontia lachesis*	MG783981

PCR and sequencing: Amplification of the target DNA was performed with 25 µl reaction volumes comprising 12.5 µL Master mix 2X (QIAGEN); 1 µL $MgCl_2$ 25 mM; 0.5 µL Taq polymerase 5 u/µL; 2 µL DNA, using primer LCO2198 (10 pM): 1.25 µL, using primer HCO1490 (10 pM): 1.25µL and 8.5 µL H_2O. A region of *COI* gene was amplified using primers LCO2198 and HCO1490 with 658 bp (Folmer et al., 1994), respectively sequence 5' GGTCAACAAATCATAAAGA TATTGG 3' and 5' TAAACTTCAGGGTGA CCAAAAAATCA 3'. Amplification conditions: (1) Initialization at 94 ºC for 1 minute, (2) Denaturation at 94 ºC for 40 seconds, (3) annealing at 45 ºC in 40 seconds, (4) Elongation at 72 ºC for 1 minute, (5) 35 cycles of steps 2 to 4, (6) Final elongation at 72 ºC for 10 minutes. All PCR reactions were performed on Thermocycler by SensoQuest. The PCR products were preserved at 4ºC, then visualized on 1.2% agarose gel with ethidium

bromide staining under UV Transilluminator camera (Cleaver Sci. Ltd.). After that, the PCR products were purified using the Qiagen purification Kit (Applied Biosystems). The sequencing reactions were performed at Macrogen Corporation in the Netherlands.

Data analysis: The obtained sequences were aligned using ClustalW (Thompson et al., 1997) and GeneDoc2.5 (Nicholas et al., 1997). Attained data was analyzed by MEGA 7 (Kumar et al., 2016). The nucleotide database is available in the Genbank with the accession numbers KY962521, JN677795, KC182176, KP720043, JN677799, JN677796, MG783981 used for comparison in this study (Table 2).

RESULT AND DISCUSSION

The images obtained from PCR products of 7 hawkmoths specimens were clear, without auxiliary bands, and suitable for the size of the *COI* genome of about 650 bp (Fig. 1). After removing the disturbed gene region, we have determined the *COI* sequences for 7 specimens, which were submitted to the GenBank database (Table 3).

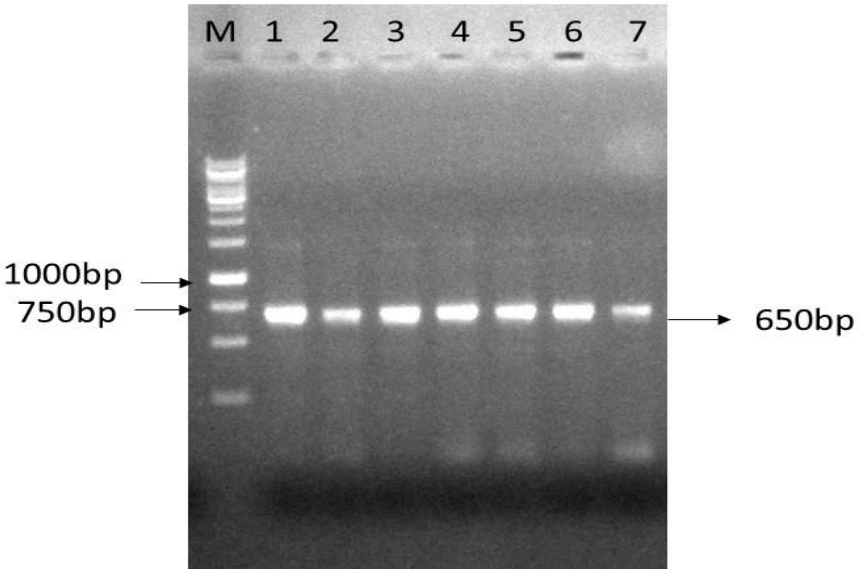

Figure 1. The PCR products of each specimen checked on 1,2% agarose (Lane 1-7: Specimens on table 1; lane M: DNA ladder 1 kb)

Table 3. Detailed of *COI* sequences and accession number on GenBank of 7 hawkmoths specimens

No.	Code of specimens	The study gene range	DNA Size (bp)	Accession Number on Genbank
1	SG_D7	*COI*	657	MT994231
2	MK_H04	*COI*	657	MT994236
3	MK_B07	*COI*	614	MT994230
4	LB_F01	*COI*	634	MT994232
5	LB_G01	*COI*	629	MT994233
6	LB_H01	*COI*	657	MT994235
7	LK_G05	*COI*	657	MT994234

LB_F01 specimen

The *COI* fragment with 634 nucleotides in length was used for analysis. After comparing this sequence against those of the three species of the genus *Cechetra* and one species in the genus *Acherontia* (outgroup), we identified 448 sites useful for analysis, 113 variable sites and 110 parsimony informative sites. The average similarity of the nucleotide of the genus *Cechetra* fluctuated from 96.3% to 100%. Among them, the sample LB_F01 had the highest genetic similarity value of 99.9% among 3 species of the genus *Cechetra* (Table 4). The phylogenetic tree displayed that the species belonging to the genus *Cechetra* formed a clade (Fig. 2). The results also showed that LB_F01, together with other samples of *C. lineosa* downloaded from GenBank, whose respective codes were KC182176, NJ677795, KY962521, formed a group. Bootstrap values were from 81% to 97%. Sub-clade I (Fig. 2). The phylogenetic tree showed that LB_F01 nested in the group with *Cechetra lineosa*; thus it was identified as *C. lineosa*.

Seven PCR product sequences representing three species belonging to the *Cechetra* genus of Sphingidae were identified and obtained.

LB_G01 specimen

The obtained *COI* with 629 nucleotides in length was used for analysis. After comparing this sequence against those of the three species of the genus *Cechetra* and one species in the genus *Acherontia* (outgroup), we identified 449 sites useful for analysis, 112 variable sites and 110 parsimony informative sites. The average similarity of the nucleotide of the genus *Cechetra* fluctuated from 96% to 99.7%. Among them, LB_G01 had the highest genetic similarity value of 99.7% among 3 species of the genus *Cechetra* (Table 4). The phylogenetic tree displayed that the species belonging to the genus *Cechetra* formed a clade (Fig. 2). The results also showed that LB_G01, together with other samples of *C. lineosa* downloaded from GenBank, whose respective codes were KC182176, NJ677795, KY962521, formed a group. Bootstrap values were from 81% to 97%. Sub-clade I (Fig. 2) showed that LB_G01 and LB_F01 had a bootstrap value of 100%, which confirmed that the two samples were *C. lineosa*.

LK_G05 specimen

The obtained *COI* from the LK_G05 specimen with 657 nucleotides in length was used for analysis. After comparing this sequence against those of the three species of the genus *Cechetra* and one species in the genus *Acherontia* (outgroup), we identified 469 sites useful for analysis, 96 variable sites and 110 parsimony informative sites. The average similarity of nucleotides in genus *Cechetra* fluctuated from 96% to 99.9%. Among them, LK_G05 had the highest genetic similarity value of 99.9% among 3 species of the genus *Cechetra* (Table 4). The phylogenetic tree displayed that the species belonging to the genus *Cechetra* formed a clade (Fig. 2). The results also showed that LK_G05, together with other samples of *C. lineosa* downloaded from GenBank, whose respective codes were KC182176, NJ677795, KY962521, formed a group. Bootstrap values were from 81% to 97%. Sub-clade I (Fig. 2) showed that LB_G01, LB_F01 and LK_G05 had a bootstrap value of 100%, which confirmed that the three samples were *C. lineosa*.

LB_H01 specimen

The length of the *COI* amplified from the LB_H01 specimen containing 657 nucleotides was used for analysis. After comparing this sequence against those of the three species of the genus *Cechetra* and one species in the genus *Acherontia* (outgroup), we identified 446 sites useful for analysis, 112 variable sites and 110 parsimony informative sites. The average similarity of nucleotides in genus *Cechetra* fluctuated from 96.3% to 99.9%. Among them, LB_H01 had the highest genetic similarity value of 99% among 3 species of the genus *Cecchetra* (Table 4). The phylogenetic tree displayed that the species belonging to the genus *Cechetra* formed a clade (Fig. 2). The results also showed that

LB_H01, together with other samples of *C. lineosa* downloaded from GenBank, whose respective codes were KC182176, NJ677795, KY962521, formed a group. Bootstrap values were from 81% to 97%. Sub-clade I (Fig. 2) showed that LB_G01, LB_F01, LK_G05 and LB_H01 had a bootstrap value of 100%, which confirmed that the four samples were *C. lineosa*.

SG_D7 specimen

The length of the *COI* containing 657 nucleotides was used for comparing with the three species of the genus *Cechetra* and one species in the genus *Acherontia* (outgroup). We identified 416 sites useful for analysis, 176 variable sites and 120 parsimony informative sites. The average similarity of nucleotides in genus *Cechetra* fluctuated over 99.3%. Study sample SG_D7 had the highest genetic similarity value of 99.9% with the species *C. subangustata* (Fig. 2). The phylogenetic tree displayed that the SG_D7 sample, together with other samples of *C. subangustata* downloaded from GenBank, whose respective codes were KP720043, NJ677799 respectively, formed a group. Bootstrap values were high 99%. In addition to comparing the results with data from GenBank, we also used identification methods based on morphological characteristics and confirmed that the SG_D7 sample was *C. subangustata.*

MK_B07 specimen

The length of the *COI* amplified from the MK_B07 specimen with 614 nucleotides was used for analysis. After comparing this sequence against those of the three species of the genus *Cechetra* and one species in the genus *Acherontia* (outgroup), we identified 418 sites useful for analysis, 134 variable sites and 160 parsimony informative sites. The average similarity of nucleotide in genus *Cechetra* was over 99%. Study sample MK_B07 had the highest genetic similarity value of 99.96% with species *C. minor* belonging to the genus *Cechetra* (Table 4). The phylogenetic tree displayed that the species belonging to the genus *Cechetra* formed a clade (Fig. 2). The results also showed that MK_B07, together with a sample of *C. minor* downloaded from GenBank (code JN677796), formed a group. Bootstrap value was high 97%. Sub-clade III (Fig. 2). It showed that the MK_B07 sample was *C. minor.*

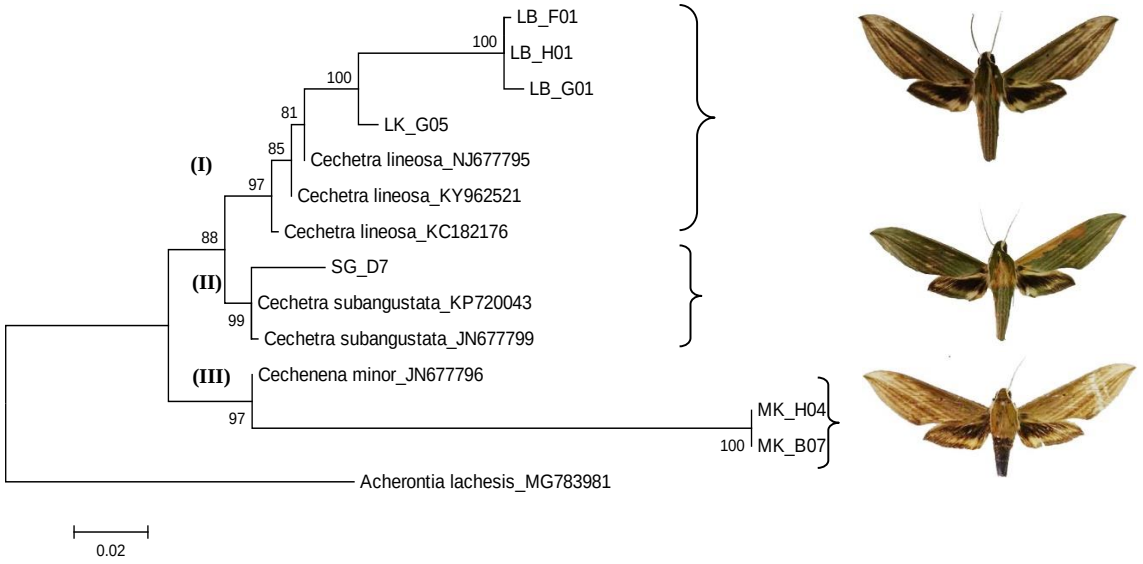

Figure 2. Relationship of 7 studied samples with 6 other genus *Cechetra* species on Genbank based on the *COI* gene region using the ML method. Bootstrap values were obtained with 1,000 replicates. *Acherontia lachesis* (MG783981) was used as an outgroup

Table 4. Nucleotide similarity values (lower triangle) and the percentage genetic distance (upper triangle) of hawkmoths specimens in the genera *Cechetra* and *Acherontia*

No.	Specimens	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*C.lineosa_* KY962521		100.0	99.99	99.98	99.98	99.95	99.82	96.27	99.38	96.37	96.33	96.29	96.38	96.37
2	*C.lineosa_* NJ677795	0.00		99.99	99.98	99.98	99.95	99.82	96.27	99.37	96.37	96.33	96.29	96.37	96.37
3	*C.lineosa_* KC182176	0.01	0.01		99.98	99.98	99.95	99.82	96.26	99.36	96.36	96.32	96.28	96.36	96.36
4	*C.subangustata_* KP720043	0.02	0.02	0.02		100.0	99.96	99.81	96.24	99.35	96.34	96.30	96.26	96.34	96.34
5	*C.subangustata_* NJ677799	0.02	0.02	0.02	0.00		99.96	99.81	96.24	99.35	96.34	96.30	96.26	96.34	96.34
6	*C.minor_* JN677796	0.05	0.05	0.05	0.04	0.04		99.81	96.27	99.37	96.37	96.32	96.29	96.36	96.36
7	*A.lachesis_* MG783981	0.18	0.18	0.18	0.19	0.19	0.19		96.00	96.11	96.11	96.14	96.02	96.17	96.17
8	SG_D7	3.73	3.73	3.74	3.76	3.76	3.73	4.00		99.96	99.96	99.97	99.97	99.95	99.95
9	LB_F01	3.62	3.63	3.64	3.65	3.65	3.63	3.89	0.04		100.0	99.97	99.98	99.94	99.94
10	LB_G01	3.63	3.63	3.64	3.66	3.66	3.63	3.89	0.04	0.00		99.97	99.98	99.93	99.93
11	LK_G05	3.67	3.67	3.68	3.70	3.70	3.68	3.86	0.03	0.03	0.03		99.98	99.94	99.94
12	LB_H01	3.71	3.71	3.72	3.74	3.74	3.71	3.98	0.03	0.02	0.02	0.02		99.94	99.94
13	MK_B07	3.63	3.63	3.64	3.66	3.66	3.64	3.83	0.05	0.06	0.07	0.06	0.06		100.0
14	MK_H04	3.63	3.63	3.64	3.66	3.66	3.64	3.83	0.05	0.06	0.07	0.06	0.06	0.00	

MK_H04 specimen

The length of the *COI* amplified from the MK_B04 specimen with 657 nucleotides was used for analysis. After comparing this sequence against those of the three species of the genus *Cechetra* and one species in the genus *Acherontia* (outgroup), we identified 416 sites useful for analysis, 170 variable sites and 120 parsimony informative sites. The average similarity of nucleotides in genus *Cechetra* fluctuated from 99% to 99.7%. Among them, MK_ H04 had the highest genetic similarity value of 97.5% among one species and subspecies of the genus *Cechetra* (Table 4). The phylogenetic tree displayed that the species belonging to the genus *Cechetra* formed a clade (Fig. 2). The results also showed that MK_ H04, together with a sample of *C. minor* downloaded from GenBank (code JN677796), formed a group. Bootstrap value was high 97%. Sub-clade III (Fig. 2) showed that MK_H04 and MK_B07 had a bootstrap value of 100%, which confirmed that the two samples were *C. minor*.

CONCLUSION

By sequencing the DNA barcode (*COI*) gene region and comparing their results with the data available on Genbank, we have identified exactly the scientific names of 7 hawkmoths specimens belonging to three species of the genus *Cechetra* of the family Sphingidae. Four specimens (LB_F01, LB_G01, LB_H01, and LK_G05) were identified as *C. lineosa*; 02 specimens (MK_H04, MK_B07) were identified as *C. minor*; 01 specimen (SG_D7) was identified as *C. subangustata*. These results were the same as ones obtained by using morphology characteristics to identify.

In addition, seven *COI* sequences from 7 specimens of three hawkmoths species of 614 bp to 657 bp in length were obtained. These sequences were submitted to GenBank databases with accession number codes from MT994230 to MT994236.

The results of the research are the basis to establish the DNA barcodes for the species identification of hawkmoths in particular, as well as the order Lepidoptera in general, using the short DNA sequences (DNA barcodes). DNA barcodes are a useful tool in the identification of hawkmoths species, as well as reliable complementary data for identification.

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