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Full Length Research Article

DNA BARCODING ANALYSIS OF PUMPKIN BEETLES *AULACOPHORA FOVEICOLLIS* (LUCAS) AND *AULACOPHORA NIGRIPENNIS* (MOTSCHULSKY) (COLEOPTERA: CHRYSOMELIDAE)

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ABSTRACT

DNA Barcoding reveals that identification of adult insects and parasitoids using *cytochromeoxidase I* gene and also provide molecular markers that may be used for simple rapid species identification. In the present study was carried out to identify the pumpkin beetles, *A.foveicollis* and *A.nigripennis* sp with use of molecular markers. The genomic DNA of *A.foveicollis* generated six bands (23130, 9416, 6557, 4361, 2322 and 2027 bp) and *A. nigripennis* the PCR product generated nine base pairs (10000, 6000, 3000, 2000, 1500, 1000, 750, 500 and 250 pb). The graphic summary shows the distribution of 100 BLAST hits on the query sequence with color key for alignment scores <40, 40-50, 50-80, 80-200 and > = 200. The alignments ranged from 1 to 658 and the number of matches and sequence ID-gb/KP898252.1. In *A. nigripennis* BLAST results showed the nucleotide sequence 647 letters,RID-SSYS870J101R, Query ID-209171 and the color key for alignment scores represents <40, 40-50, 50-80, 80-200 and >= 200. The alignment ranged 27-655 and the sequences are matched 86% for ID-gb/ KP 851141.4 in *Aulacophora foveicollis* (DNA sequence). The matching and mismatching of percentage may indicate the phylogenetic relationship and transformation of species level. The *A. nigripennis* sequences were not matched BLASTed against the gene bank public database (www.ncbi.nih.gov/blast). In this case (*A. nigripennis*) the present study had been the first to report sequence of *A. nigripennis* from chrysomelidae species.

Key words: *A foveicollis, Aulacophora foveicollis, Aulacophora nigripennis*

INTRODUCTION

Among the major insect pests of curcubit vegetables, pumpkin beetle, A. *foveicollis* and A.*nigripennis* (Coleoptera: Chrysomelidae) are important pests. The pumpkin beetle is widely distributed throughout India, Bangladesh, Pakistan, Afghanistan, Ceylon, Burma, Indo-china, Iraq, Iran, Greece, Turkey, Israel, South Europe, Algeria, Egypt, Cyprus and Andaman Island (Singh and Gill, 1979; Burke *et al*., 2005; Lim, 2012; Mukherjee *et al*., 2013). DNA Barcoding reveals that identification of adult insects and parasitoids using cytochromeoxidase I gene and also provide molecular markers that may be used for simple rapid species identification. Here an attempt has made to identify the pumpkin beetles, A.*foveicollis* and A.*nigripennis* sp with use of molecular markers. DNA barcoding has two main goals. The first is to use the barcode sequence to identify specimens (distinguish between known species) and the second one is to discover new species (species delimitation or description) (De Salle *et al*., 2005; Frezal and Leblois, 2008). After suitable laboratory procedures, obtained sequences are compared to reference sequences of known species, already deposited on an online database (Hebert and Gregory, 2005). For these purposes, two databases are commonly used: Gen Bank (www.ncbi.nlm.nih.gov/genbank) and BOLD (www.barcodinglife.com).

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However, the use of a divergence threshold to distinguish between intra and interspecific variations should be done carefully, since these thresholds can suffer from statistical problems, compromising species identification (Valentini *et al*., 2008). Phylogenetic reconstruction could also be used as a complementary analysis in species identification (Dawnay *et al*., 2007). Usually, DNA barcodes do not have sufficient phylogenetic signal to resolve evolutionary relationships, especially at deeper levels, so barcode-based trees should not be interpreted as phylogenetic trees (Hajibabaei *et al*., 2007). Nevertheless, they can be used to reveal the specimens closest relatives and narrow the choice of possible species (Benecke and Wells, 2001). Since DNA barcoding is a relatively recent approach, there is a large number of undescribed species, which may constrain the representation of the overwhelming insect diversity on DNA barcode databases (Frezal and Leblois, 2008; Virgilio *et al*., 2010). Nevertheless, although DNA barcoding does not allow a complete taxonomic resolution, several studies demonstrate that the success in species identification exceeds 95% of the cases. Even when it fails it will reduce the option to a small number of congeneric taxa (Hebert and Gregory, 2005; Waugh, 2007). Therefore, several studies showed that DNA barcoding is a reliable, cost effective and easy molecular identification tool for Diptera and Coleoptera species identification. This approach would be beneficial in the application of forensic insect evidences, since it allows an accurate identification in all life stages, which are often impossibleto identify based on morphological characteristics (Nelson *et al*., 2007; Frezal and Leblois, 2008; Virgilio *et al*., 2010). DNA barcoding does not

replace morphological taxonomists, but build alliances between molecular and morphological areas. In fact, DNA barcoding requires fully described voucher species to match the sequence of an unknown specimen to a reference sequence on a database (Hebert and Gregory, 2005; Dawnay *et al*., 2007; Waugh, 2007). The mitochondrial gene has some characteristics which make it particularly suitable to be used as molecular marker. The universal primers for this gene are very robust, which enables the routine amplification of this specific segment (Hebert *et al*., 2003). Several studies demonstrate that COI gene has successfully been used for species identification of a large range of animal taxa (Danway *et al*., 2007; Hajibabaei *et al*., 2007; Frezal and Lebois, 2008; Raupach *et al*., 2010). However, a lack of reference sequences in online databases prevents a match for a large number of species, limiting the use of COI. In addition, COI based identifications sometimes fail to distinguish closely related species, due to shared barcode sequence (Nelson *et al*., 2007). The aim of the present study is to identification of pest species of A.*foveicollis* and A.nigripennis.

MATERIALS AND METHODS

DNA extraction

Whole insect specimens were preserved in 95–100% ethanol then stored at -20°C until DNA extraction. DNA extractions using Qiagen DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. All DNA samples were electrophoresed in 0.7% agarose gel and visualized under UV transluminator. DNA concentrations were standardized to 50 ng/ml and stored at-200C until PCR analysis. 603 bp COI fragment was amplified using universal HCO-LCO primer pairs (Folmer *et al*., 1994).

PCR-amplification

PCR reactions were performed in total volumes of 50 ml by using 1 ml of DNA template and GoTaq Flexi DNA polymerase (Promega) according to manufacturer's instructions: 5x buffer, 10 mM of each dNTP, 10 mM of each primer and 1.25 u/ml DNA polymerase. PCR thermal cycling conditions consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 52°C for 45 s and 72°C for 1 min, followed by an extension at 72°C for 10 min. All the PCR products were visualized on 2% agarose gel to confirm the band corresponding to amplification product and purified with Wizard SV Gel and PCR Clean up System (Promega). The purified PCR fragments were cloned into PGEM T vector systems. Sequencing reactions were performed with DTCS Quick Start Kit (Beckman Coulter), cleaned with Agencourt CleanSeq Kit (Agencourt Bioscience) and analyzed with the GenomeLab GeXP Genetic Analysis System (Beckman Coulter).

DNA Sequencing

Electropherograms of all sequences were assembled into contigs and proofread manually using the program Chromas, v.1.41. The DNA and deduced amino acid sequences were analyzed using the BLAST tool at the National Center for Biotechnology Information (NCBI) (www.ncbi.nih. gov/ BLAST), the BOLD Identification System (http://www. boldsystems.org) and EXPASY (http://expasy.org). Sequence alignments were performed using the CLUSTALW v.1.82 software (Thompson *et al*., 1994). Conserved residues in the alignments were highlighted with Box shade 3.21 (http://www.ch.embnet.org/software/ BOX form. html). All sequences have been deposited in the Gen Bank database.

RESULTS

Polymerase chain reaction (PCR)

In molecular biology, the polymerase chain reaction (PCR) is a technique to amplify a single few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. As PCR progresses, the DNA generated is self used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

The genomic DNA and PCR product of A. *foveicollis*

The primer pair yielded three lanes viz. Lane-1-lambda DNA/ Eco RI + Hind IIIDigest marker and genomic DNA band (Fig. 1) and lane-2- DNA of A. *foveicollis*.The genomic DNA generated six bands (23130, 9416, 6557, 4361, 2322 and 2027 bp).The LCO primer of DNA sequence of A. *foveicollis* in the electro pherograms sharp peaks are shown in Fig. 3. The HCO primer of DNA sequence are shown in Fig. 4.The PCR product yielded three lane viz. Lane-1-1kb DNA ladder and lane-2- PCR product of A. *foveicollis*. The sequences were matched in the gene bank public database (www.ncbi.nih.gov/blast).

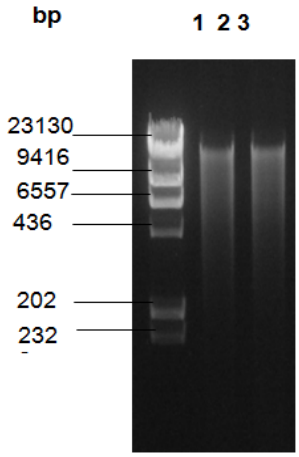

Fig.1. PCR gel banding of A.*foveicollis* primer pair yield three lane Lane 1 : Lamda DNA/ Eco RI+Hind III digest marker- genomic DNA Lane 2 and 3 : DNA band of A.*foveicollis* PCR product generated six band

Cytochrome C oxidase I Primers

The COI forward (LCO) and backward (HCO) primers are used for this study. Amplification of a ~ 658bp fragment of the COI gene was obtained from A. *foveicollis* (21-422). In A.*nigripennis* fragment of the COI gene was ~ 800 bpobtained

(Fig. 7 and 8). The primer was three nucleotides shorter on the 3'-5' end.A. *foveicollis* and A. *nigripennis* produced viable DNA in amounts quantitatively comparable to the other insects.

The genomic DNA and PCR product of A. *nigripennis*

Genomic DNA contains Agarose Gel (1%) showing Lambda DNA. Lane-3-genomic DNA of A. *nigripennis* and Agarose Gel (1%) shows 1Kb DNA ladder and PCR product.Lane-3-PCR product of A. nigripennis. The PCR product generated nine base pairs (10000, 6000, 3000, 2000, 1500, 1000, 750, 500 and 250 pb) (Fig. 2).

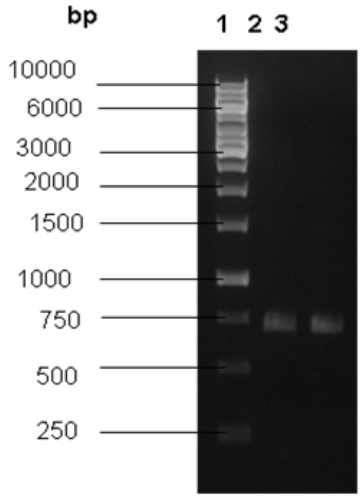

Fig. 2. PCR gel banding of A.nigripennis. PCR product generated nine base pairs – A portion of the reaction was subjected to Agarose gel electrophoresis

Obtained sequences were compared with reference sequences of known species contained in online databases (Gen Bank and BOLD). These databases allow species identification when the present sequence did not match. Sequences from A. nigripennis, had been the first to submit the sequence of A. *nigripennis* (Fig. 7and 8).

DNA Barcoding of A. *foveicollis*

DNA barcoding aims to both endorse global standards and coordinate research in DNA barcoding. For animals, the gene region proposed for the standard barcode is a 658 base pair region in the gene encoding the mitochondrial cytochrome coxidaseI (COI). Single-species identification is the historical fundament of DNA barcoding. Finally, the unprecedented potential of DNA barcoding is simultaneous multiple-species identification from a single taxonomy sample, for biodiversity. COI sequences were correctly amplified and sequenced. With BOLD-IDS tool, the specimen were correctly identified at the species level with a high specimen similarity percentage (99 – 100%). Sequences were compared with the online BLAST search tool in order to confirm BOLD results and to ensure that there was no misidentification. BLAST also identified the specimens sequences with 99 – 100% of maximum identity. These molecular identifications allowed to correctly group the specimens in the same order obtained with morphological identification, with a great assurance. The DNA sequences from A. *foveicollis* were BLASTed the insect reference sequences to make an identification of the chrysomelidae species of A.*foveicollis*. In morphological identification sequences were 100% homologous with a sequence from the insect reference collections of NCBI/BLAST formatting results- C65D69W6014, Nucleotide sequence (683 letters), Query ID- 232977 and Molecular type-nucleic acid. The graphic summary shows the distribution of 100 BLAST hits on the query sequence with color key for alignment scores <40,40-50,50-80,80-200and > = 200 (Fig. 5). The descriptions of sequences producing significant alignments are presented (Table 1). The sequence alignments are showed in Fig. 6. The alignments ranged from 1 to 658 and the number of matches and sequence ID-gb/KP898252.1. Therefore this result has clearly showed the chrysomelidae species of A. *foveicollis*. This result has matches with the NCBI database.

DNA Barcoding of A. nigripennis

In A. *nigripennis* BLAST results showed the nucleotide sequence 647 letters, RID-SSYS870J101R, Query ID-209171 and Molecular type- nucleic acid. Graphic summary shows the distribution of 100 BLAST hits on the query sequence presented. The color key for alignment scores represents <40, 40-50, 50-80, 80-200 and >= 200 (Fig. 9). Description of sequence producing significant alignment is presented in Table. 2. The sequence alignments are showed in Fig. 10. The alignment ranged 27-655 and the sequence are matched 86% for ID-gb/ KP 851141.4 in Aulacophora *foveicollis* (DNA sequence). The matching and mismatching of percentage may indicate the phylogenetic relationship and transformation of species level. The A. *nigripennis* sequences were not matched, BLASTed against the gene bank public database (www.ncbi.nih.gov/blast). In this case (A. nigripennis) the present study had been the first to report sequence of A. *nigripennis* from chrysomelidae species. In this study, online database regarding species identification is attempted for the first time. Indeed, the quantity of sequences for comparison deposited in online databases reflects the quality and assurance of such databases in species identification.

DISCUSSION

One important technical challenge to the success of a barcoding approach is the availability of universal primers (Ekrem *et al*., 2007). The present study demonstrated the uncritical application of Coleopteran-specific COI primers (Hebert *et al*., 2004a). Hebert's COI primers were originally modified from the LCO1490 and HCO2198 COI primers of Folmer *et al*., (1994). These primers were designed for 11 invertebrate phyla and have also been shown to amplify the Wolbachia genome (Deans *et al*., 2006). This result underscores a major problem with the widespread application of universal primers for DNA barcoding i.e. non-specific species amplification. In this case, the extreme conservation of the COI barcode primer region inadvertently led to the amplification of homologous sequences across very distantly related taxa, despite the fact that these primers were designed to be ''Coleopteran-specific" (Hebert *et al*., 2004a,b). In the present investigation, the Coleoptera: Chrysomelidae family that comprises pumpkin beetles A. *foveicollis* and A. *nigripennis* species DNA sequence varied. A. *foveicollis* sequencing has been marginally recorded in previous study.

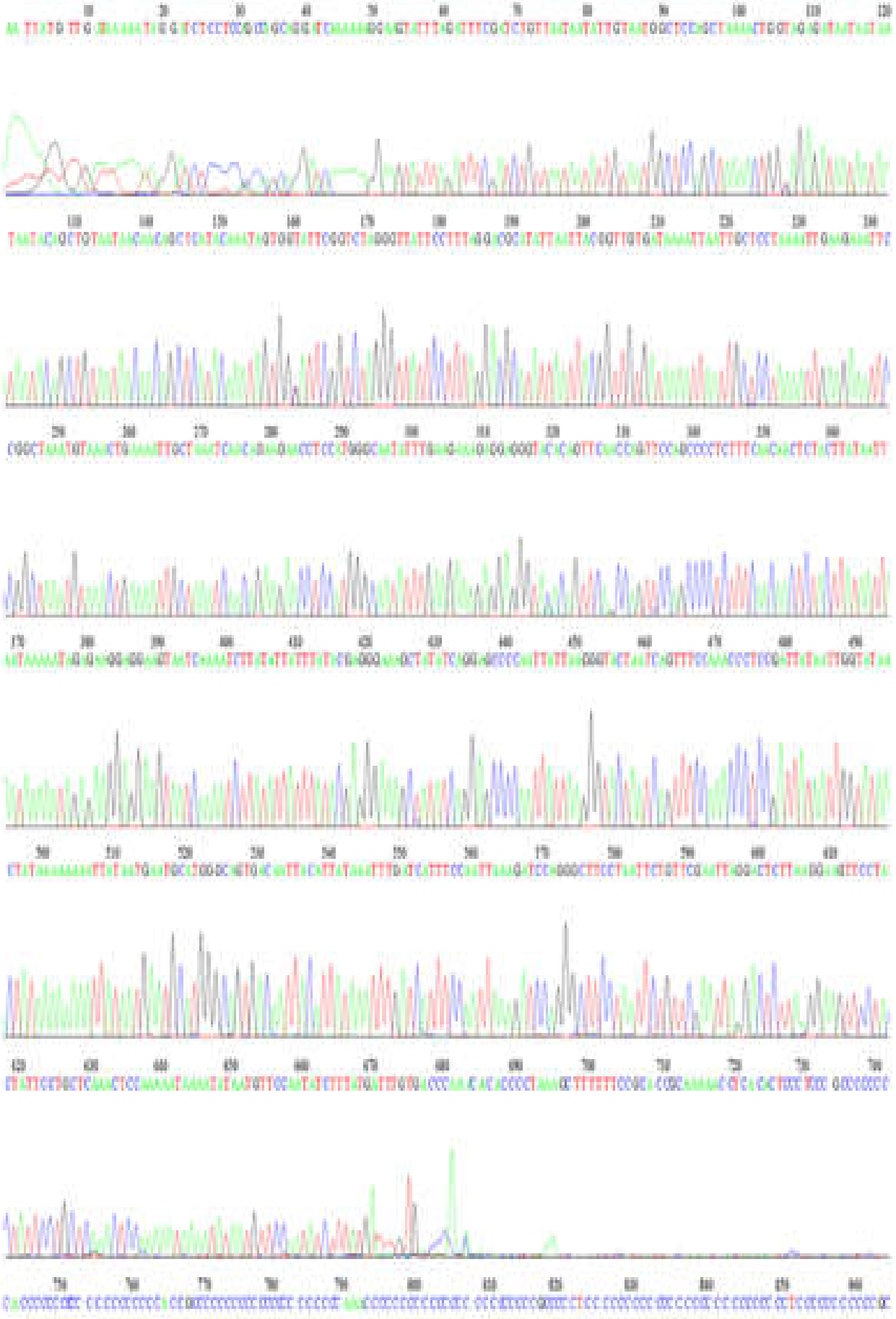

Fig. 3. LCO forward primer DNA sequences of Aulacophora foveicollis

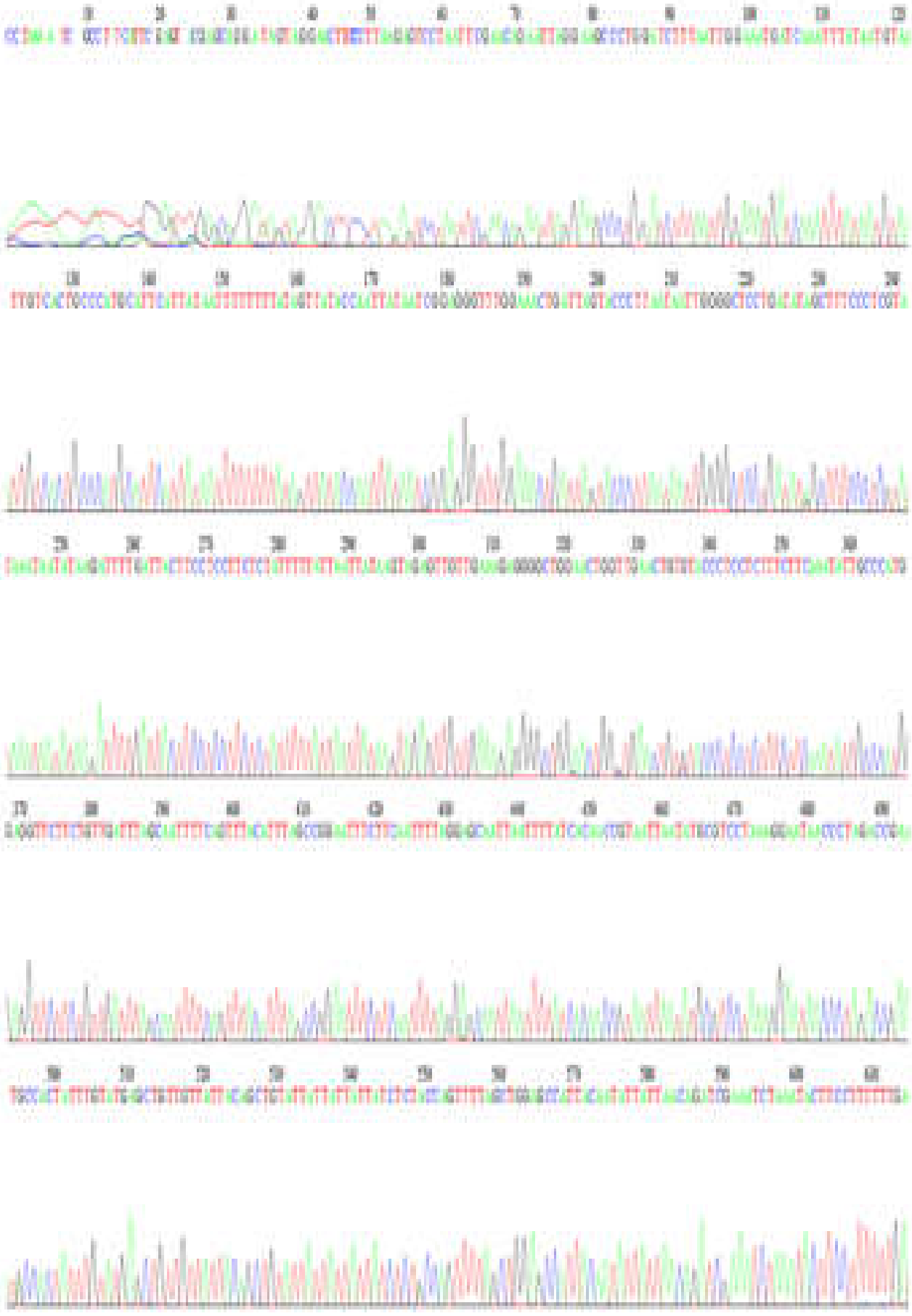

Fig. 4. HCO backward primer DNA sequences of A. foveicollis
DNA amplification of 658bp fragment

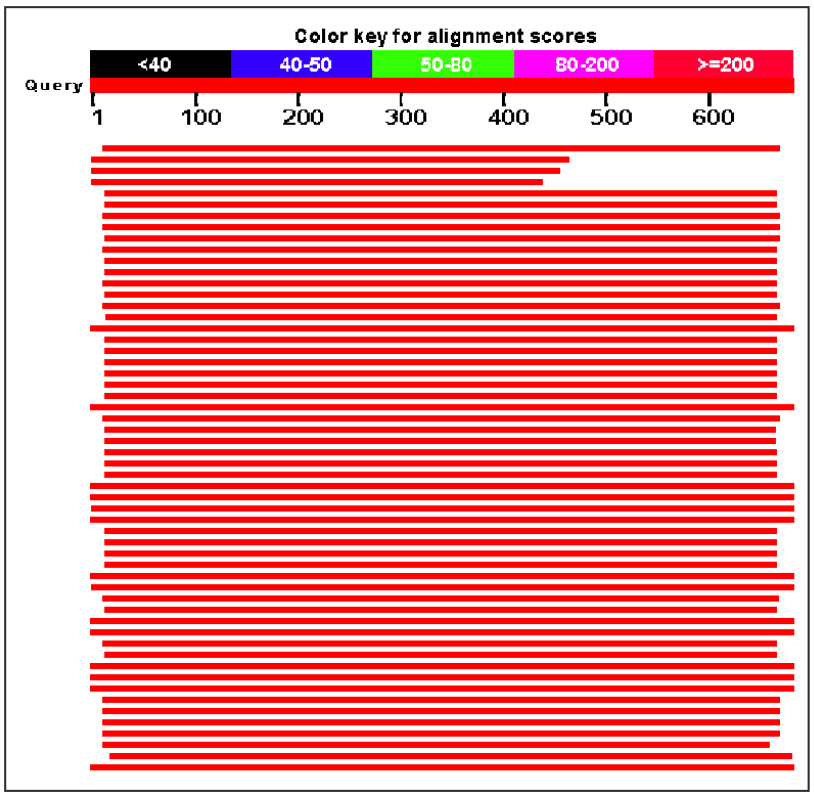

Fig. 5. Colour key alignment score DNA sequences of A. foveicollis

Score	Expect	Identities	Gaps	Strand	Frame
1166 bits(631)	0.0()	649/658(99%)	0/658(0%)	Plus/Minus	

Features:

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Query  13   AAATAAATGTTGATATAAAATAGGATCTCCCCCGCCAGCAGGATCAAAAAAGGAAGTATT  72
            ||||||||||||||||||||||||||| || || ||||||||||||||||||||||||||
Sbjct  658  AAATAAATGTTGATATAAAATAGGATCACCTCCACCAGCAGGATCAAAAAAGGAAGTATT  599

Query  73   TAGATTTCGATCTGTTAATAATATTGTAATGGCTCCAGCTAAAACTGGTAGAGATAATAA  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  598  TAGATTTCGATCTGTTAATAATATTGTAATGGCTCCAGCTAAAACTGGTAGAGATAATAA  539

Query  133  TAATAATACAGCTGTAATAACAACAGCTCATACAAATAGTGGCATTCGGTCTAGGGTTAT  192
            |||||||||||||||||||||||||||||||||||||||||||| |||||||||||| |||||
Sbjct  538  TAATAATACAGCTGTAATAACAACAGCTCATACAAATAGTGGTATTCGGTCTAGTGTTAT  479

Query  193  TCCTTTAGGACGCATATTAATTACGGTTGTGATAAAATTAATTGCTCCTAAAATTGAAGA  252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  478  TCCTTTAGGACGCATATTAATTACGGTTGTGATAAAATTAATTGCTCCTAAAATTGAAGA  419

Query  253  AATTCCGGCTAAATGTAAACTGAAAATTGCTAAATCAACAGAAGAACCTCCATGGGCAAT  312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  418  AATTCCGGCTAAATGTAAACTGAAAATTGCTAAATCAACAGAAGAACCTCCATGGGCAAT  359

Query  313  ATTTGAAGAAAGAGGAGGGTACACAGTTCAACCAGTTCCAGCCCCTCTTTCAACAACTCT  372
            |||||||||||||||||||| |||||||||||||| ||||||||||||||||||||||||
Sbjct  358  ATTTGAAGAAAGAGGAGGGTAAACAGTTCAACCAGTCCCAGCCCCTCTTTCAACAACTCT  299

Query  373  ACTTATAATTAATAAAAATAGAGAAGGAGGAAGTAATCAAAATCTTATATTATTTATACG  432
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  298  ACTTATAATTAATAAAAATAGAGAAGGAGGAAGTAATCAAAATCTTATATTATTTATACG  239

Query  433  AGGGAAAGCTATATCAGGAGCCCCAATTATTAAGGGTACTAATCAGTTTCCAAACCCTCC  492
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  238  AGGGAAAGCTATATCAGGAGCTCCAATTATTAAGGGTACTAATCAGTTTCCAAACCCTCC  179

Query  493  GATTATAATTGGTATAACTATaaaaaaaaTTATAATGAATGCATGGGCAGTGACAATTAC  552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  178  GATTATAATTGGTATAACTATAAAAAAAATTATAATGAATGCATGGGCAGTGACAATTAC  119

Query  553  ATTATAAATTTGATCATTTCCAATTAAAGATCCAGGGCTTCCTAATTCTGTTCGAATTAG  612
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118  ATTATAAATTTGATCATTTCCAATTAAAGATCCAGGGCTTCCTAATTCTGTTCGAATTAG  59

Query  613  GACTCTTAAGGAAGTTCCTACTATTCCTGCTCAAACTCCAAAAATAAAATATAATGTT  670
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  58   GACTCTTAGGGAAGTTCCTACTATTCCTGCTCAAACTCCAAAAATAAAATATAATGTT  1
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Fig. 6. DNA sequence identities in NCBI datas list of A. foveicollis

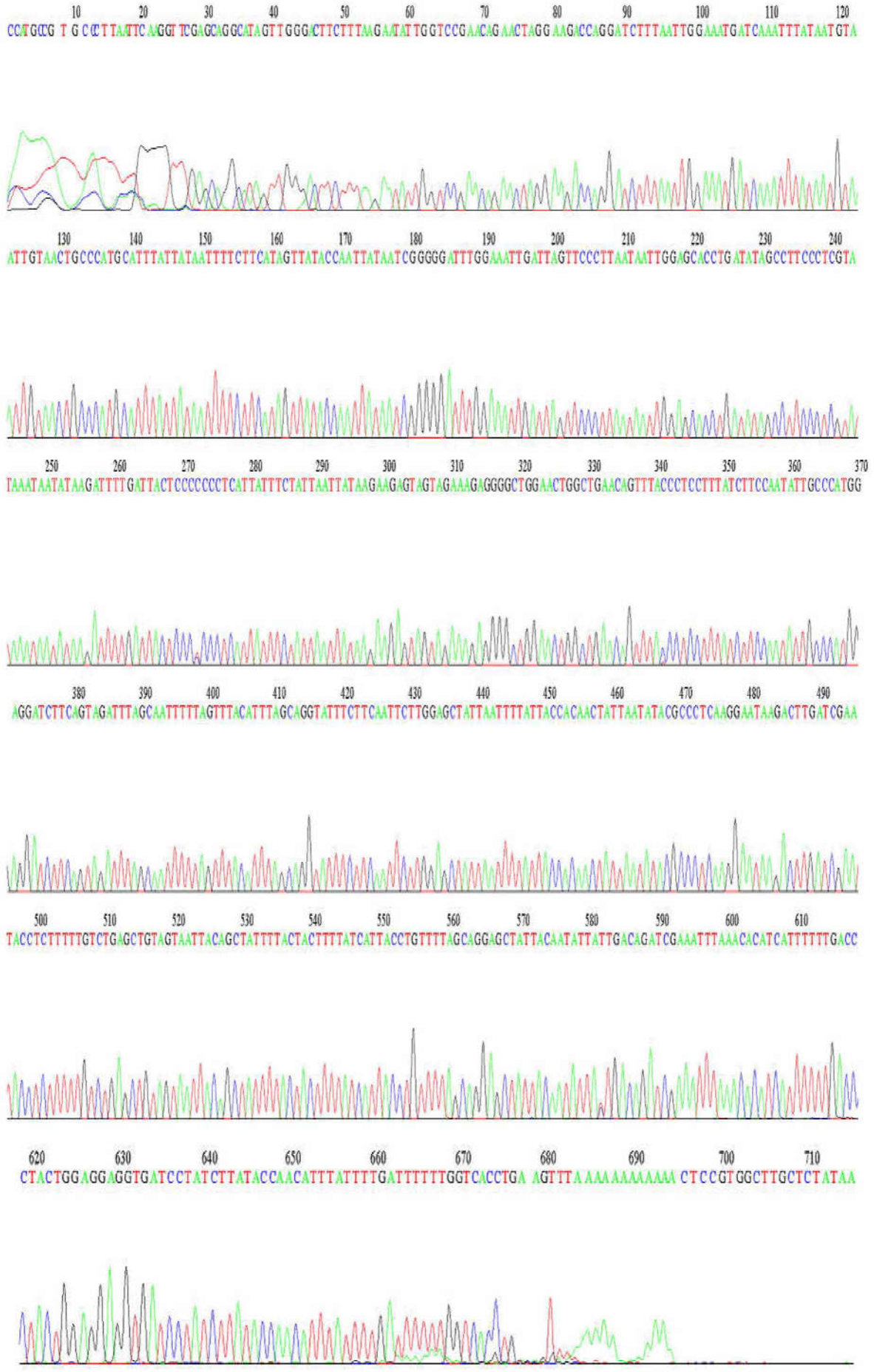

Fig. 7. LCO forward primer DNA sequences of A. nigripennis

Fig. 8. HCO backward primer DNA sequences of A. nigripennis

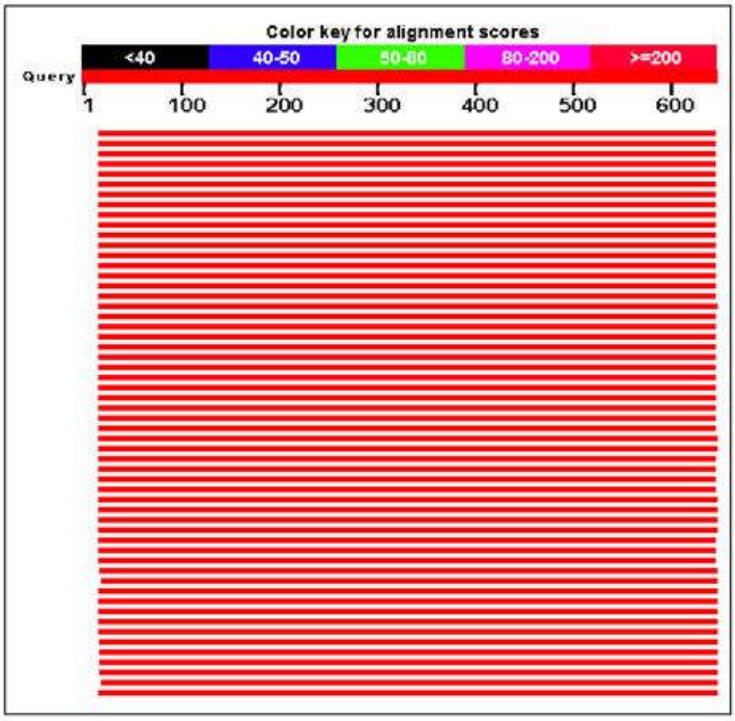

Fig. 9. Colour key alignment score of DNA sequences in A. nigripennis

Table 1. DNA sequence matching 99% ident of A. foveicollis in NCBI references

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aulacophora foveicollis voucher RO_AF2015 cytochrome oxidase subunit I (Cox1) gene, partial cds; mitochondrial	1166	1166	96%	0.0	99%	KP898252.1
Aulacophora indica cytochrome oxidase subunit I gene, partial cds; mitochondrial	841	841	67%	0.0	99%	AY796207.1
Aulacophora indica COI gene, partial cds; mitochondrial gene for mitochondrial product	797	797	66%	0.0	98%	AY171417.1
Aulacophora indica isolate JJG220 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	791	791	63%	0.0	99%	AY242435.1
Longitarsus atricillus voucher BMNH:850392 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	736	736	95%	0.0	87%	KF134569.1
Longitarsus bedeli voucher BMNH:849184 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	736	736	95%	0.0	87%	KF134558.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_LTCH180	734	734	96%	0.0	87%	AB794751.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_LTCH178	734	734	96%	0.0	87%	AB794749.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_LTCH098	732	732	96%	0.0	87%	AB794748.1
Dinoptera collaris voucher BFB_Col_FK_10214 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	730	730	95%	0.0	87%	KM449303.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_LTCH016	730	730	95%	0.0	87%	AB794742.1
Longitarsus atricillus voucher BMNH:847898 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	730	730	95%	0.0	87%	KF134545.1
Dinoptera collaris voucher GBOL_Col_FK_6727 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	728	728	95%	0.0	87%	KM445325.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_LTCH063	728	728	95%	0.0	87%	AB794746.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_KC004	728	728	96%	0.0	87%	AB794741.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_LTCH042	726	726	95%	0.0	87%	AB794745.1
Gaurotes tuberculicollis voucher HNAU.PP 005 cytochrome oxidase subunit I gene, complete cds; mitochondrial	725	725	100%	0.0	86%	KF737784.1
Monolepta sp. KKY2013-4 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo5_LTCH017	725	725	95%	0.0	87%	AB794743.1

Table 2. DNA sequence matching 86% indent of in NCBI reference of Galerucinae and *A. foveicollis* sp

Description	Max score	Total score	Query cover	E value	Ident	Accession
Longitarsus atricillus voucher BMNH:847898 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	669	669	97%	0.0	86%	KF134545.1
Haliplus flavicollis voucher BMNH835590 mitochondrion, partial genome	669	669	100%	0.0	85%	JX313677.1
Galerucinae sp. 72 BT-2014 voucher BT_1294 cytochrome c oxidase subunit 1 (COI) gene, partial cds; mitochondrial	667	667	97%	0.0	86%	KJ677804.1
Galerucinae sp. 72 BT-2014 voucher BT_0434 cytochrome c oxidase subunit 1 (COI) gene, partial cds; mitochondrial	665	665	97%	0.0	86%	KJ677805.1
Aulacophora foveicollis voucher Af Ma3 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	665	665	97%	0.0	86%	KP851141.1
Tricholochmaea cavicollis voucher 08BBCOL-0038 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	665	665	97%	0.0	86%	KM842073.1
Galerucinae sp. 5 ACP-2013 voucher BMNH:1040542 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	665	665	97%	0.0	86%	KF946261.1
Tricholochmaea sp. CHU1 voucher 07PROBE-05010 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	665	665	97%	0.0	86%	KJ204192.1
Monolepta sp. KKY2013-5 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo7_LTCH169	665	665	97%	0.0	86%	AB794753.1
Diabrotica barberi isolate 1656-BC cytochrome oxidase subunit I (cox1) gene, partial cds; mitochondrial	665	665	99%	0.0	85%	EU498290.1
Diabrotica barberi isolate 1742-BC cytochrome oxidase subunit I (cox1) gene, partial cds; mitochondrial	665	665	99%	0.0	85%	EU498295.1
Diabrotica barberi isolate 1877-BC cytochrome oxidase subunit I (cox1) gene, partial cds; mitochondrial	665	665	99%	0.0	85%	EU498292.1
Psylliodes chalcomerus voucher BMNH:849362 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	664	664	97%	0.0	86%	KF653557.1
Longitarsus atricillus voucher BMNH:848276 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	664	664	97%	0.0	86%	KF652624.1
Disonycha procera voucher 08BBCOL-0278 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	664	664	97%	0.0	86%	KM844807.1
Monolepta sp. KKY2013-5 mitochondrial COI gene for cytochrome c oxidase subunit I, partial cds, isolate: Mo7_LTCH154	664	664	97%	0.0	86%	AB794752.1
Longitarsus atricillus voucher BMNH:855569 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	664	664	97%	0.0	86%	KF134581.1
Longitarsus atricillus voucher BMNH:849513 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	664	664	97%	0.0	86%	KF134566.1
Longitarsus bedeli voucher BMNH:849184 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial	664	664	97%	0.0	86%	KF134558.1
Psylliodes chalcomerus voucher GBOL_Col_FK_5539 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	662	662	97%	0.0	86%	KM444655.1

DNA sequences are characterized by summary statistics like length and base composition. Prior to comparison of nucleotide sequences in phylogenetic analysis, several additional parameters like overall rate of nucleotide substitution, ratio of two specific instantaneous rates of substitution rate at which transitions and transversions occur and the rate variation among sites play a significant role and are necessary for accurate reconstruction of phylogeny (Dwivedi and Gadagkar, 2009).

Results indicated that the COI-based pest identification was extremely effective for insects because these species were accurately and successfully identified based on the COI marker profile. Most of the phylogenetic information has been derived from mitochondrial DNA variations (Avise, 2000) and recently DNA sequence data have been employed successfully to elucidate the relationships of many groups of insect species at generic level (Wang *et al*., 2009; Ruo *et al*., 2006). The composition of the mitochondrial sequence of the COI gene in

the present study successfully achieved the DNA sequences of two pumpkin beetles A. *foveicollis* and A. nigripennis. Molecular identification was done for several pests worldwide, in Orius (Hemiptera: Anthocoridae) (Gomez-Polo *et al*., 2013) and potato flea beetles (Coleoptera: Chrysomelidae) (Germain *et al*., 2013). Moreover, discrimination of aphids of 32 species collected in various host plants in South India was also reported (Rebijith *et al*., 2013). The present study was carried out DNA sequence of insect pests attacking field crops in South India and hence it was a varied and wide combination of individuals from distantly related taxa. This could be an essential breakthrough and unique difference from earlier studies. This investigation of COI barcoding could potentially be applied in agricultural and horticultural researches to rapid identification of pests. The phylogenetic signal is a direct function of the length of the branch (in units of the expected number of substitutions per site), which sheds light over the evolutionary relationship.

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