RESEARCH ARTICLE

DNA Barcoding of Key Storage Pests Using Mitochondrial Cytochrome Oxidase I

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ABSTRACT

Stored grain insect pests are of economic importance, and they spread rapidly through the grain trade. Hence, it is important to identify these pests accurately. Although several morphological methods exist, it is tedious and time-consuming. DNA barcoding using *mitochondrial COI* is an alternative approach that aids in precise species identification. In this study, 13 stored grain insect pests belonging to the order Coleoptera and Lepidoptera were collected from different storage structures in Coimbatore. A fragment of *mt-COI* was amplified and sequenced. Sequence analyses were carried out with BOLD (Barcode of Life Data System) and ABGD (Automatic Barcode Gap Discovery) tools. The barcoding gap analysis revealed that the inter-species genetic distance is greater than the intra-species genetic distance. The ABGD analysis for species delimitation partitioned the Coleopteran and Lepidopteran datasets into 9 and 4 putative species, respectively. The barcode gap was absent in more closely related species. However, analysis of their sequences revealed significant variations. Our results showed the ability of the *mt-COI* to discriminate between the species, thus provide a complementary technique for the diagnosis of stored grain insect pests.

Keywords: *DNA Barcoding; Stored grain pests; Mitochondrial COI; Barcoding gap*

INTRODUCTION

Stored grain insect pests cause loss in both quality and quantity, leading to the contamination of stored grain products. Generally, stored products are attacked by more than 600 species of coleopterans, 70 species of lepidopterans, and about 355 species of mites (Rajendran and Sriranjini, 2008). Hence, rapid identification is highly essential for preventing and controlling these pests. Traditionally, morphological features are used for the identification of insect pests. The stored grain insect pests are small and difficult to identify with morphological features alone. Usually, only the fragments of the insects are found in the stored products that lack exclusive information required for identification. Hence, DNA barcoding provides an alternative approach for identification and biodiversity assessment as it meets the need for fast, efficient, and reliable species identification (Hebert and Gregory, 2005; Valentini *et al.,* 2009). A standard 650 bp mitochondrial COI fragment is being used as a universal marker for species identification. The molecular identification of species over morphological identification has several advantages. DNA is more resistant to degradation and more stable than the morphological characters (Bohmann *et al.,* 2014). Also, molecular identification does not require complete specimens (Sinha and Watters, 1985). In addition, molecular identification helps differentiate species with similar morphological characters (Mayr, 1999). Aside from species identification, molecular identification is frequently employed in various other disciplines such as biological invasions and biodiversity monitoring (Ruppert *et al.,* 2019). In the present study, we have provided an efficient method for the identification of stored grain insect pests with mitochondrial COI that will be helpful for accurate diagnosis.

MATRIALS AND METHODS

Collection of stored grain insect pest

The stored grain insect pest specimens used for the DNA barcoding study were collected in grain storages and households across Coimbatore. 13 stored grain insect pests species belonging to the order Coleoptera and Lepidoptera were collected (Table 1). The collected specimen was kept in 70 per

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cent ethanol and stored at -80 °C. Three specimens from each species were used for analysis.

Genomic DNA extraction

Genomic DNA was isolated from individual insects using the HotSHOT method (Montero-Pau *et al.,* 2008). Two buffers were used in this method: alkaline lysis buffer (pH 8.0) containing 25 mM NaOH, 0.2 mM Na2EDTA, and neutralizing solution containing 40mM Tris-HCl. Individual adults were homogenized with 100 µl alkaline Lysis buffer and incubated at 95 ˚C in a hot water bath for 30 mins. After incubation, the samples were removed from the hot water bath and were allowed to cool at 4 ˚C in a refrigerator for 5-10 mins. Then 100 µl neutralizing solution (pH 5.0) was added to each tube with brief vortex and spin to settle the debris. The extracted DNA samples were stored at -20 ˚C.

PCR amplification

PCR amplification was done with universal barcoding primer, mitochondrial Cytochrome Oxidase I (mt-COI) as suggested by the International Barcode of Life (https://ibol.org/). The fragments of the mitochondrial gene (Cytochrome oxidase I) COI, LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3' were used for PCR amplification (Folmer *et al.,* 1994).

Polymerase chain reactions were performed in SureCycler 8800 (Agilent technologies) that involved an initial denaturation step of 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 56°C, extension for 30 S at 72°C with a final extension at 72°C for 10 min. Polymerase chain reactions were performed in 25 µL reactions, containing 15.7 µL water, 2.5 µL of 10X Taq Buffer, 2.5 µLof 250 µM dNTPs, 1.5 µL of 10 µM forward primer, 1.5 µL of 10 µM reverse primer, 0.3 µL of 5 U/µL Taq polymerase (TaKaRa™) and 2 µLof template DNA. Amplified products of *COI* gene was separated using Agarose gel electrophoresis (1.5% - 1.5 g in 100 ml 1X TBE buffer), 5 µL of PCR product, and 2.5 µL of loading dye and visualized using the gel documentation unit (GELSTAN 1312, Mediccare Scientific, Chennai).

mtDNA sequencing

Amplified PCR products (20 µl) and their respective forward and reverse primers (10 µl each per sample) were labelled appropriately and sent to Agrigenome labs Pvt. Ltd., Cochin, Kerala for sequencing. The PCR products were sequenced by double pass method in both forward and reverse directions. The PCR products were purified using PureLink PCR Purification Kit and the sequencing PCR was set up using the BigDye Terminator V3.1 Cycle Sequencing Kit. The resulting sequencing information was retrieved from the client database of Agrigenome labs online portal.

Table 1. Details of the stored grain pests used for barcoding study

S. No.	Order	Family	Insect Species
1.	Coleoptera	Chrysomilidae	*Callosobruchus maculatus*
2.	Coleoptera	Laemophloeidae	*Cryptolestes pusillus*
3.	Coleoptera	Chrysomelidae	*Caryedon serratus*
4.	Coleoptera	Tenebrionidae	*Tribolium castaneum*
5.	Coleoptera	Silvanidae	*Oryzaephilus surinamensis*
6.	Coleoptera	Bostrichidae	*Rhyzopertha dominica*
7.	Coleoptera	Ptinidae	*Lasioderma serricorne*
8.	Coleoptera	Curculionidae	*Sitophilus oryzae*
9.	Coleoptera	Ptinidae	*Stegobium paniceum*
10.	Lepidoptera	Pyralidae	*Cadra cautella*
11.	Lepidoptera	Pyralidae	*Corcyra cephalonica*
12.	Lepidoptera	Gelechiidae	*Sitotroga cerealella*
13.	Lepidoptera	Gelechiidae	*Phthorimaea operculella*

Molecular data analysis

The barcode sequences were trimmed and aligned using Geneious Ver. 11.1.3 (https://www.geneious.com; Kearse *et al.,* 2012). Aligned sequences were then submitted to BOLD (Barcode Of Life Database) and NCBI-GenBank databases. The COI barcodes were identified using the BLAST and BOLD databases. In addition, sequence analyses were performed with BOLD Ver. 4 analytical tools. The distance summary analyses with the parameter setting included BOLD alignment option and pairwise deletion (ambiguous base/gap handling) to evaluate the Kimura 2-parameter (K2P) distances (Kimura, 1980) at species, genus, and family levels. Barcode gap analysis was performed

with the following parameters: K2P; BOLD alignment option, and pairwise deletion (ambiguous base/gap handling) to identify the intra and interspecific genetic distances. Also, in order to differentiate between the species, barcode gap analyses in Automatic Barcode Gap Discovery (https://bioinfo.mnhn.fr/abi/public/abgd/) was performed. ABGD was used with K2P along with the transition/transversion ratio and with other parameters set with default values (Pmin = 0.001; Pmax = 0.1; Nb bins = 20). In addition, barcode gap analysis for closely related species was also analyzed by retrieving sequences from GenBank.

RESULTS AND DISCUSSION

The partial (650 bp) mt-COI fragment from 13 stored grain insect species, including 9 species belonging to the order Coleoptera and 4 species belonging to the order Lepidoptera, were successfully amplified using mt-COI. The trimmed sequences showed that there was no ambiguous site or stop codons present in these sequences indicating that these sequences were not nuclear pseudo genes. The sequences identified using NCBI-Genbank and BOLD databases represented 13 different species. Identification of the stored grain pests based on NCBI-Genbank (BLASTn) showed similarities that ranged from 99.28 to 100 per cent whereas the identification based on BOLD database showed similarities ranging from 97.84 to 100 per cent (**Table 2**). The mean nucleotide frequencies of Coleopteran insect species were A (31.36%), T (34.68%), G (15.75%) and C (18.21%). Whereas the mean nucleotide frequencies of the insect species belonging to the order Lepidoptera were A (29.80%), T (39.59%), G (15.12%) and C (15.49%). The examination of the barcodes revealed AT-biased with a mean AT content of more than 60 per cent in all the insect species, which is a common feature of the animal mitochondrial DNA (Pentinsaari *et al.,* 2016). AT bias in Lepidopteran insect pests (69.39%) was significantly higher than the Coleopteran insect pests (66.04%).

The mean K2P distances within species, within the genus, and within families in Coleoptera were 0.59%, 0.00%, and 19.03%, whereas in Lepidoptera, 0.53%, 0.00% and 18.86%, respectively. The average genetic distance between the families was greater than the congeneric and conspecific distances. The barcode gap analysis revealed that the mean interspecies K2P distance was greater than the mean intra-species K2P distance (> 2) in all the insect species used in this study. This indicated a barcode gap that was essential for discriminating the COI barcodes (Čandek and Kuntner, 2015). The ABGD tool was used for species delimitation. All the 10 partitions with the prior maximal distance ranging from P = 0.010 to 0.10 delimited the Coleopteran dataset into 9 putative species and Lepidopteran data set into 4 putative species. All these species were clearly delimited through ABGD which were consistent with the observations of neighbour-joining analyses. However, a comparison of closely related species revealed that the inter-species K2P distance was lesser than two (**Table 3**) and lacked barcode gap. Incomplete sorting by lineage associated with recent speciation might be the reason for the inability of the barcodes to identify species (Ramon *et al.,* 2003). This can be overcome by using mitochondrial control region or the first internal transcribed ribosomal DNA spacer, which aided in better identifying the closely related species (Sheraliev and Peng, 2021). In addition, multiple sequence alignment (**Figure 1a-d**) of the closely related species revealed variations between the species that can be used for designing species-specific markers based on the presence of single nucleotide polymorphism (SNPs) or the intraspecific uniformity in the barcode region for more reliable identification (Varadínová *et al.,* 2015). The partial COI region was initially chosen for species identification because of its DNA variation patterns and relative simplicity of getting the sequence. This region was sufficiently conserved within species and varied between species to allow for accurate taxon identification (Hebert *et al.,* 2003). The most widely used barcode gene, mitochondrial (mt) DNA cytochrome c oxidase I (COI), thus serves as a reliable and cost-effective technique for identifying organisms of various taxa at all phases of their lives.

Table 3. Details of comparison between species (Inter-species comparison)

Insect species	Intra-sp. distance	Nearest species	Inter-sp. distance
T. castaneum	0.00	*T. confusum*	1.11
C. maculatus	0.00	*C. chinensis*	2.44
C. maculatus	0.00	*C. analis*	0.96
S. oryzae	0.00	*S. zeamais*	1.34
C. pusillus	0.00	*C. ferrugineus*	2.11
O. surinamensis	0.00	*O. mercatus*	1.22

Table 2. Identification of stored grain pests using GenBank and BOLD databases

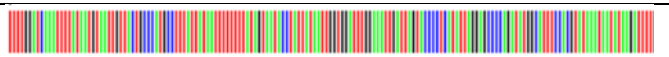
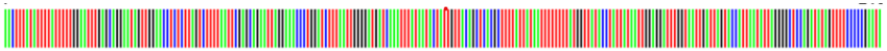
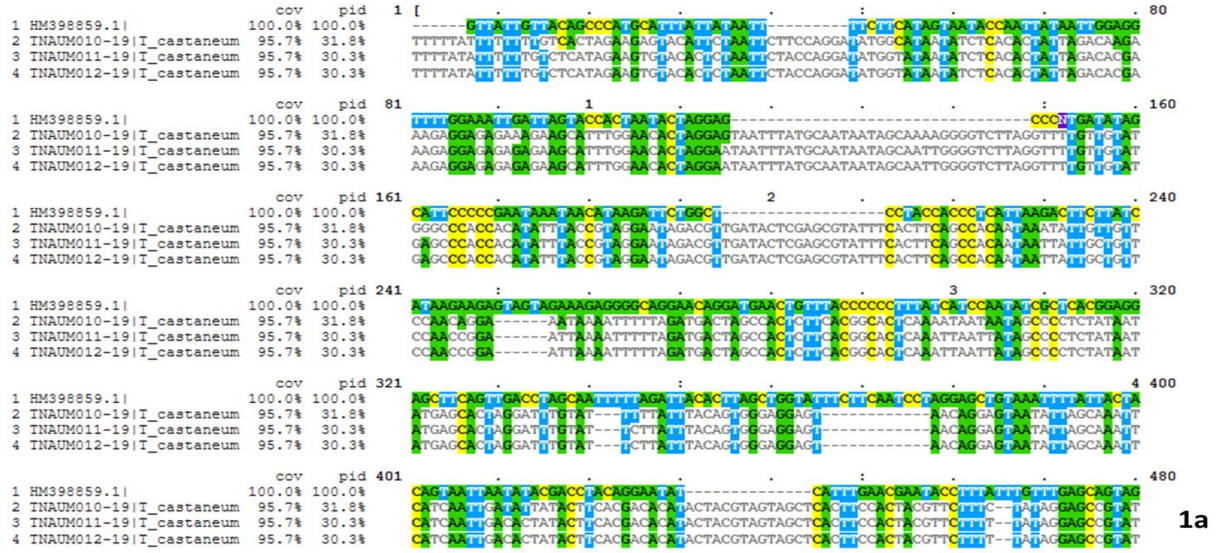
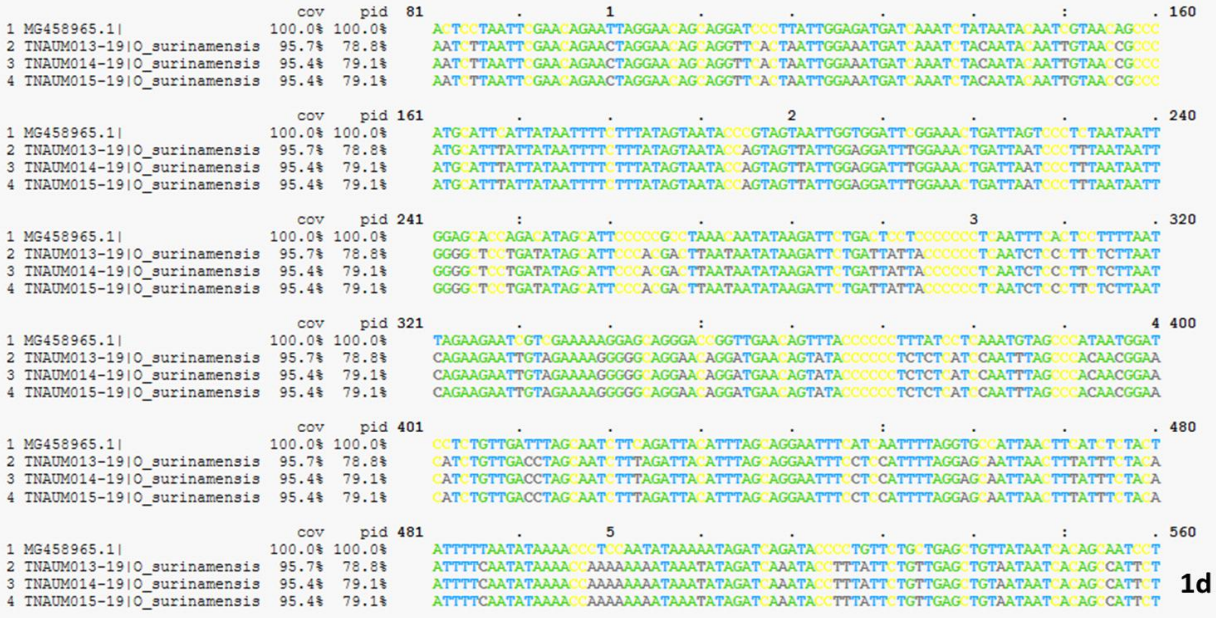
S. No.	Insect Species	BOLD Similarity %	GenBank Similarity %	Barcode Index Number	GenBank Accession	Illustrative Barcode
1.	*Callosobruchus maculatus*	100.00	99.82	ACH4854	MN658890.1	
2.	*Cryptolestes pusillus*	100.00	99.82	ACD2055	MN658935.1	
3.	*Caryedon serratus*	100.00	100.00	ACG5956	MN658893.1	
4.	*Tribolium castaneum*	100.00	96.59	AAH8019	MN658907.1	
5.	*Oryzaephilus surinamensis*	100.00	100.00	AAF0496	MN658889.1	
6.	*Rhyzopertha dominica*	97.84	99.28	ACB4329	MN658901.1	
7.	*Lasioderma serricorne*	100.00	100.00	ACG7582	MN658897.1	
8.	*Sitophilus oryzae*	100.00	100.00	AAJ6841	MN658909.1	
9.	*Stegobium paniceum*	100.00	100.00	AAH9980	MN658892.1	
10.	*Cadra cautella*	100.00	99.82	AAB9605	MN658875.1	
11.	*Corcyra cephalonica*	99.82	98.73	AAY8077	MN658895.1	
12.	*Sitotroga cerealella*	100.00	100.00	AAD0546	MN658905.1	
13.	*Phthorimaea operculella*	100.00	99.82	AAB9396	MN658899.1	

Figure 1a-d. Multiple sequence alignment showing variable regions in the COI sequences between the closely related species. 1a. *T. castaneum* and *T. confusum*; 1b. *C. pusillus* and *C. ferrugineus*; 1c. *S. oryzae* and *S. zeamais* and 1d. *O. surinamensis* and *O. mercatus*





Conclusion

The study indicated that DNA barcoding, a DNA-based species identification system is a promising additional technique for identifying stored grain insect pests.

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Ethics statement

No specific permits were required for the described studies because no human or animal subjects were involved in this research.

Originality and plagiarism

Authors should ensure that they have written and submit only entirely original works, and if they have used the work and/or words of others, that this has been appropriately cited. Plagiarism in all its forms constitutes unethical publishing behavior and is unacceptable.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There were no conflict of interest in the publication of this content

Data availability

All the data of this manuscript are included in the MS. No separate external data source is required. If anything is required from the MS, certainly, this will be extended by communicating with the corresponding author through corresponding official mail; smktnau@gmail.com

Author contributions

Research grant- SM, Idea conceptualization-SM, Experiments- SU , Writing original draft - SU Writing-reviewing &editing -SM & SU

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