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Molecular Characterization of *Callosobruchus maculatus* (Fabricius, 1775), DNA Barcoding and Development of Multiplex PCR for Detection of Stored Grain Pests

Pothukuchi SPANDANA[1a]* Mandali RAJASRI[1b]
Gonchikara Siddanna PANDURANGA[1c] Yellari AMARAVATHI[2]

[1]Department of Entomology, S.V. Agricultural College, Tirupati, 517 502, ANGRAU, Andhra Pradesh, INDIA
[2]Department of Genetics and Plant Breeding, Institute of Frontier Technology, Regional Agricultural Research Station, Tirupati, 517 502, Andhra Pradesh, INDIA
e-mails: [1a]spandanapothukuchi@gmail.com, [1b]m.rajasri@angrau.ac.in,
[1c]gs.panduranga@angrau.ac.in, [2]y.amaravathiangrau@ac.in
ORCID IDs: [1a]0009-0005-3460-080X, [1b]0000-0002-0930-1868, [1c]0000-0001-5713-2010
*Corresponding author

ABSTRACT

The present study aimed to characterize *Callosobruchus maculatus* populations collected from five distinct locations in Andhra Pradesh, India (Tirupati, Lam, Guntur, Krishna and Nandyal), using mt*CO1*-based DNA barcoding, with genomic DNA extracted from approximately 10 adult beetles per population. Additionally, the research sought to develop a molecular diagnostic tool for detecting hidden infestations in stored pulses. PCR amplification employing universal primers yielded fragments of approximately 750 bp. Subsequent nBLAST analysis confirmed these sequences exhibited >99% similarity with *C. maculatus* entries in GenBank. The generated sequences were submitted to the National Center for Biotechnology Information (NCBI) and unique DNA barcodes were registered in the Barcode of Life Data (BOLD) systems. Notably, this represents the first documented report of *C. maculatus* DNA barcodes from Andhra Pradesh. Phylogenetic analysis indicated low intraspecific divergence (<1%) among the examined *C. maculatus* populations from Andhra Pradesh, with populations from Krishna and Tirupati demonstrating complete sequence identity. Furthermore, a multiplex PCR assay targeting the mt*CO1* gene was developed to enable simultaneous detection of *C. maculatus*, *Sitophilus oryzae* and *Rhyzopertha dominica*. The assay produced distinct amplicons of 119 bp, 95 bp and 143 bp for the respective species, indicating its potential utility for molecular identification of hidden infestations in stored pulses. The findings demonstrate the usefulness of DNA barcoding for genetic characterization of pulse beetle populations and suggest that multiplex PCR may serve as a promising approach for the simultaneous detection of major stored grain pests.

Keywords: Barcode Index Number (BIN), Hidden infestation, Molecular identification, mtCO1 gene, Phylogenetic analysis, Species-specific primers, Stored product pests.

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INTRODUCTION

Pulses, the dried edible seeds of the Leguminosae family, are renowned for their high nutritional value, containing 20-30% protein approximately three times that of cereals (Divya et al., 2018). Often termed “poor man’s meat,” pulses offer an affordable protein source (Umbarkar, Parsana, & Jethva, 2010). India stands as the world’s largest producer, importer and consumer of pulses. However, insect pests pose significant constraints, causing substantial losses to pulses both in field conditions and during storage. Among these pests, *Callosobruchus* spp. (Coleoptera: Bruchidae), commonly known as pulse beetles, inflict serious damage on various pulses including chickpea, cowpea, green gram, lentil and pigeon pea. Key species include *Callosobruchus chinensis* (adzuki bean weevil), *C. maculatus* (cowpea bruchid) and *C. analis* (pulse weevil) (Singh & Boopathi, 2021). These beetles are primary pests initiating infestation in the field and continuing damage in storage, with larvae boring into seeds and causing considerable losses (Tuda et al., 2004). Economic damage by *Callosobruchus* spp. can range from 30-40% within six months, potentially escalating to 100% if unchecked (Dongre, Pawar, Thakare, & Harwalker, 1996; Akinkurolere, Adedire, & Odeyemi, 2006; Sharma, Yadav, Singh, & Kumar, 2013). Accurate species identification is critical for targeted and timely pest management interventions.

The mitochondrial cytochrome oxidase I (mt*CO1*) gene is valuable for DNA barcoding due to its high copy number, maternal inheritance, lack of recombination, and relatively higher evolutionary rate, facilitating species identification and study of genetic diversity (Centre for Biodiversity Genomics, University of Guelph, 2021). DNA barcoding employing Barcode Index Numbers (BINs) enhances taxonomic resolution. Several studies across different countries have utilized mt*CO1*-based DNA barcoding to study genetic diversity, phylogenetic relationships, and geographic variation in stored grain pests including *C. maculatus*. Previous reports from Africa, Asia, Europe, and America demonstrated that mt*CO1* is an efficient molecular marker for accurate species identification and population level characterization due to its conserved primer regions and comparatively higher mutation rate. Molecular characterization studies have also revealed low to moderate intraspecific divergence among geographically separated populations of *C. maculatus*, suggesting possible dispersal through grain trade and storage practices.

Despite the availability of global studies on DNA barcoding and phylogenetic analysis of stored grain pests, molecular information on *C. maculatus* populations from South India, particularly Andhra Pradesh, remains limited. In addition, studies focusing on simultaneous molecular detection of major hidden infestation pests of pulses through multiplex PCR are scarce. Therefore, generation of regional DNA barcode data and development of rapid molecular diagnostic tools are essential for accurate pest identification and effective management of stored grain pests.

Additionally, lesser grain borer, *Rhyzopertha dominica* and rice weevil, *Sitophilus oryzae* also cause damage to broken pulses besides *Callosobruchus* spp. (Vijay et al., 2025). Conventional morphological identification of stored grain pests is often difficult during immature stages and hidden infestation conditions. Molecular approaches such as DNA barcoding and multiplex PCR provide rapid, reliable and species-specific

identification of insect pests. Although several molecular diagnostic assays have been reported for stored-product insects, information on multiplex PCR-based identification of major pulse storage pests remains limited for Indian populations. Therefore, development of a multiplex PCR assay capable of simultaneously detecting multiple pest species would facilitate rapid molecular identification. Therefore, the present study was undertaken to characterize *C. maculatus* populations collected from different regions of Andhra Pradesh using mt*CO1*-based DNA barcoding and phylogenetic analysis. The study also aimed to develop a multiplex PCR assay for simultaneous detection of *C. maculatus*, *S. oryzae* and *R. dominica* associated with stored pulses

MATERIAL AND METHODS

Survey and Sample Collection

A survey was undertaken in public and private warehouses and storage facilities across Andhra Pradesh, India to collect adult pulse beetles (*C. maculatus*) from five locations spanning four districts: Tirupati (79.4192°E, 16.5385°N), Guntur (80.4365°E, 16.3067°N), Krishna (80.7982°E, 16.5385°N), Nandyal (78.0373°E, 15.8281°N), and LAM (80.4347°E, 16.3610°N) (Fig. 1). Adults were preserved in 90% ethyl alcohol and stored at –20°C for subsequent molecular analysis.

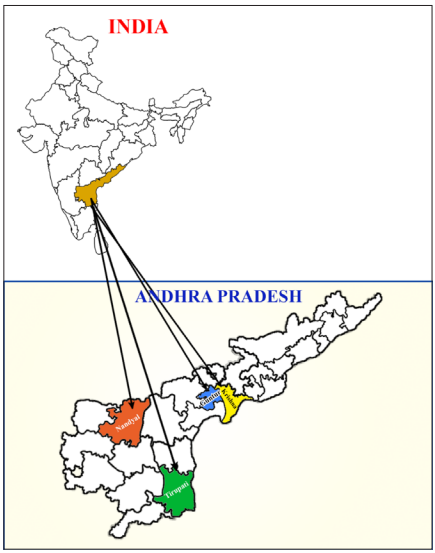


Figure 1. Survey map showing the collection locations of *C. maculatus* populations from Tirupati, Lam-Guntur, Guntur, Krishna and Nandyal districts of Andhra Pradesh, India.

DNA Extraction of Pulse Beetles

Genomic DNA was extracted using a modified CTAB protocol based on Murray and Thompson (1980). Approximately 10 adult beetles collected from each population were homogenized in CTAB extraction buffer and incubated at 65°C for 1 h. The

homogenate was subjected to sequential purification using phenol: chloroform (1:1) followed by chloroform: isoamyl alcohol (24:1). DNA was precipitated using ice-cold isopropanol and incubated overnight at −20°C. The DNA pellet was washed with 70% ethanol, air dried and finally resuspended in sterile distilled water.

PCR Amplification, Gel Electrophoresis, and Sequencing of *CO1*

Extracted DNA (50 ng) was subjected to PCR amplification targeting the mitochondrial cytochrome oxidase I (mt*CO1*) gene using universal primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGAC-CAAAAAATCA-3′) (Folmer et al., 1994). PCR reactions (25 µL) contained 10× PCR buffer, 2 mM $MgCl_2$, 10 mM dNTPs, 10 pM of each primer, 2.5 U Taq DNA polymerase, and 50 ng template DNA. Thermal cycling conditions included initial denaturation at 94°C (4 min), followed by 35 cycles of 94°C (30 s), 53.3°C (45 s), 72°C (1 min) and a final extension at 72°C (10 min). PCR conditions were optimized and reactions were repeated whenever necessary to obtain clear and reproducible amplification products. Amplicons were resolved via 1.5% agarose gel electrophoresis and documented using a gel documentation system (Alpha Innotech, USA). PCR products were sequenced via Sanger sequencing (Grada & Weinbrecht, 2013) at Eurofins Genomics India Pvt. Ltd., Bengaluru.

Data Analysis

Obtained sequences were compared with *Callosobruchus* spp. entries in the NCBI GenBank database. Forward and reverse sequences were checked for homology, insertions/deletions, stop codons and frame shifts. Trimmed mt*CO1* sequences were submitted to NCBI; accession numbers were obtained. Sequence assembly and alignment utilized BioEdit version 7.0 (Hall, 1999). A phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA version 11.0 and evolutionary distances were computed using the Tamura 3-parameter (T92) model (Kumar, Stecher, & Tamura, 2016). DNA barcodes were generated for the five Andhra Pradesh populations using BOLD Systems.

Multiplex PCR Assay for *C. maculatus*, *S. oryzae* and *R. dominica*

Maintenance of Insect Culture

S. oryzae and *R. dominica* (10 pairs each) were cultured in plastic containers (11 cm × 8 cm) containing 500 g of broken pigeon pea grains, while *C. maculatus* was cultured in whole green gram seeds under ambient conditions (25 ± 2°C, 75% RH). Emerged adults were preserved in 95% ethanol (-20°C) for DNA extraction.

Species-Specific Primer Designing

Species-specific primers targeting mt*CO1* were designed for *C. maculatus*, *S. oryzae* and *R. dominica* using primer3plus software, optimizing for primer length (20-26 bp), Tm (55-60°C), and GC content (25-50%). Expected amplicon sizes were 119 bp (*C. maculatus*), 95 bp (*S. oryzae*) and 143 bp (*R. dominica*). Primers were BLAST-validated for specificity. Primers were checked for complementarity to avoid hairpin loop formation. Details of the designed primers are presented in the Table 1.

S. No.	Stored grain pests of pulses	NCBI accession number	Primer name	Primer sequence	Length (bp)	AT	GC	Tm (℃)	PCR Product size (bp)
1.	*Sitophilus oryzae*	NC_030765.1	MSoF	AGCAGGAGCAATCACTATACTTCT	24	14	10	57.9	95
			MSoR	AATGTTGATAGAGAATTGGGTCTC	24	15	9	57.7	
2	*Rhyzopertha dominica*	NC_042820.1	MRdF	ATCAATTCTTGGAGCAGTAAACTT	24	16	8	57.7	143
			MRdR	CAGCTAGAACCGGAAGAGATAATA	24	14	10	58.2	
3	*Callosobruchus maculatus*	KY942062.1	MCmF	TCCTTCTTTCACTCCCAGTATTAG	24	14	10	57.4	119
			MCmR	TAAGTGTTGATAGAGAATGGGGTC	24	14	10	57.7	

Table 1. Species-specific primers designed for multiplex PCR-based identification of major stored-product pests of pulses.

Validation of Multiplex PCR

DNA from each species was pooled; multiplex PCR (25 µL) included 10× buffer, $MgCl_2$, dNTPs, primers, Taq polymerase, and 100 ng pooled DNA. Cycling conditions were 94°C (4 min), 35 cycles of 94°C (30 s), 52.9°C (45 s), 72°C (1 min), and 72°C (10 min final extension). Products were resolved on 3% agarose gel and UV-visualized.

RESULTS AND DISCUSSION

The PCR amplification of the mt*CO1* gene with universal primers (LCO1490 and HCO2198) produced ~750 bp amplicons for the five *C. maculatus* populations (Fig. 2). The nBLAST analysis of the mitochondrial genome of these populations showed maximum similarity of >99% confirming that all pulse beetle samples from Andhra Pradesh belonged to *C. maculatus*. The trimmed sequences were submitted to NCBI and accession numbers for the five populations were obtained *viz*., PV569548 (Tirupati), PV595833 (Lam), PV650458 (Guntur), PV595857 (Krishna) and PV715814 (Nandyal).

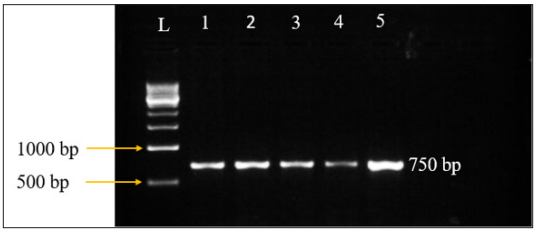


Figure 2. Agarose gel electrophoresis of PCR-amplified mtCO1 fragments (~750 bp) of *C. maculatus* populations collected from different locations of Andhra Pradesh, India. M: 500 bp DNA ladder; Lane 1: Tirupati; Lane 2: LAM, Guntur; Lane 3: Guntur; Lane 4: Krishna; Lane 5: Nandyal.

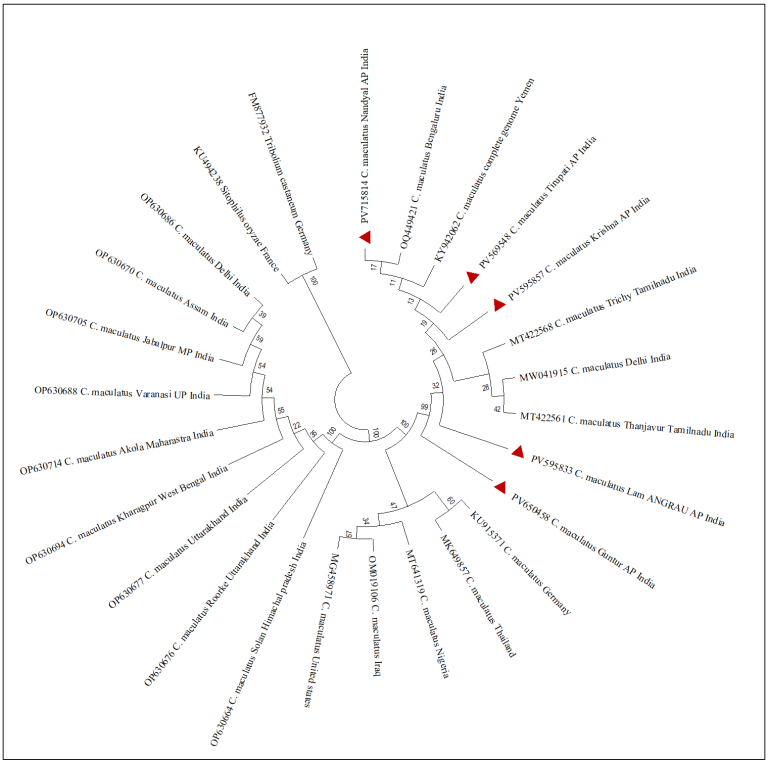
The nucleotide sequences of the five populations, along with 19 other *C. maculatus* sequences retrieved from NCBI GenBank, were aligned using BioEdit version 7 and MEGA version 11. The similarity percentage coefficients of *C. maculatus* sequences retrieved from GenBank are presented in Table. 2. Among the five populations under study, Krishna and Tirupati showed the highest similarity (100%), while the Guntur, Lam and Nandyal populations showed <1% divergence from Krishna and Tirupati, indicating very low genetic divergence among the Andhra Pradesh populations. The

similarity matrix revealed a high degree of sequence conservation among the Andhra Pradesh populations, with pairwise similarity values exceeding 99%. Krishna and Tirupati populations showed complete sequence identity, whereas the remaining populations exhibited less than 1% divergence. The low genetic divergence observed among Andhra Pradesh populations may be attributed to movement of infested pulse grains through trade and storage channels, facilitating gene flow among geographically separated populations.

Phylogenetic analysis was carried out using the neighbor-joining method with mt*CO1* gene sequences of the five *C. maculatus* populations, along with 19 other *C. maculatus* sequences and two outgroup species, *Tribolium castaneum* and *Sitophilus oryzae*, obtained from NCBI GenBank (Fig. 3). The phylogenetic tree grouped the sequences into three major clusters (I, II and III). Cluster I was further divided into subgroups IA and IB based on clade branching patterns. The *C. maculatus* populations from Tirupati, Nandyal, Guntur, Lam and Krishna clustered under IA, showing close relatedness to *C. maculatus* sequences from Bengaluru, Delhi, Tamil Nadu and the Yemen complete genome. Populations from Germany, Thailand, Nigeria, Iraq and the United States clustered under IB. Sequences from parts of North India grouped separately under Cluster II. The mt*CO1* sequences of *Tribolium* and *Sitophilus* were highly divergent and as expected, were clearly out grouped into Cluster III. Bootstrap values generally supported the major clustering pattern observed among the analyzed sequences, indicating consistency in the inferred evolutionary relationships. The close clustering of Andhra Pradesh populations with previously reported Indian and Yemen sequences is consistent with the observations of Kebe et al. (2017), suggesting that human-mediated movement of infested legumes has contributed to the genetic structure and dispersal of *C. maculatus* across geographical regions.

Table 2. Sequence Identity Matrix of *C. maculatus* mtCO1 sequences in the study with other *C. maculatus* mtCO1 sequences mined from NCBI.

S.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	ID																							
2	0.998	ID																						
3	0.998	0.996	ID																					
4	1	0.998	0.998	ID																				
5	0.998	0.996	0.996	0.998	ID																			
6	1	0.998	0.998	1	0.998	ID																		
7	0.978	0.977	0.977	0.978	0.977	0.978	ID																	
8	1	0.998	0.998	1	0.998	1	0.978	ID																
9	0.982	0.984	0.98	0.982	0.982	0.982	0.964	0.982	ID															
10	0.982	0.984	0.98	0.982	0.982	0.982	0.964	0.982	0.989	ID														
11	0.978	0.98	0.977	0.978	0.978	0.978	0.961	0.978	0.989	0.985	ID													
12	0.832	0.83	0.83	0.832	0.834	0.832	0.825	0.832	0.835	0.837	0.834	ID												
13	0.834	0.832	0.832	0.834	0.835	0.834	0.827	0.834	0.837	0.839	0.839	0.992	ID											
14	0.834	0.832	0.832	0.834	0.835	0.834	0.827	0.834	0.837	0.839	0.835	0.996	0.996	ID										
15	0.984	0.985	0.982	0.984	0.984	0.984	0.966	0.984	0.991	0.991	0.987	0.834	0.835	0.835	ID									
16	0.832	0.83	0.83	0.832	0.834	0.832	0.823	0.832	0.83	0.832	0.832	0.985	0.989	0.989	0.828	ID								
17	0.834	0.832	0.832	0.834	0.835	0.834	0.827	0.834	0.837	0.839	0.835	0.996	0.996	1	0.835	0.989	ID							
18	0.998	0.996	0.996	0.998	0.996	0.998	0.98	0.998	0.98	0.98	0.977	0.832	0.834	0.834	0.982	0.832	0.834	ID						
19	0.832	0.83	0.83	0.832	0.834	0.832	0.825	0.832	0.835	0.837	0.834	0.994	0.994	0.998	0.834	0.987	0.998	0.832	ID					
20	0.98	0.982	0.978	0.98	0.98	0.98	0.962	0.98	0.987	0.991	0.984	0.834	0.835	0.835	0.989	0.828	0.835	0.978	0.834	ID				
21	0.834	0.832	0.832	0.834	0.835	0.834	0.825	0.834	0.837	0.839	0.839	0.992	0.996	0.996	0.835	0.989	0.996	0.832	0.994	0.835	ID			
22	0.834	0.832	0.832	0.834	0.835	0.834	0.827	0.834	0.837	0.839	0.835	0.996	0.996	1	0.835	0.989	1	0.834	0.998	0.835	0.996	ID		
23	0.834	0.832	0.832	0.834	0.835	0.834	0.827	0.834	0.837	0.839	0.835	0.996	0.996	1	0.835	0.989	1	0.834	0.998	0.835	0.996	1	ID	
24	0.991	0.989	0.989	0.991	0.989	0.991	0.973	0.991	0.973	0.973	0.97	0.828	0.83	0.83	0.975	0.828	0.83	0.989	0.828	0.971	0.83	0.83	0.83	ID



▲ Five populations of *C. maculatus* under present investigation are marked as red.

Figure 3. Neighbor joining circular phylogenetic tree illustrating the relationships among mtCO1 sequences collected from different districts of Andhra Pradesh in comparison with sequences mined from NCBI and BOLD databases using MEGA 11.0 software. Evolutionary distances were computed using the Tamura 3-parameter (T92) model and branch support was assessed using 1000 bootstrap replicates.

The average nucleotide composition of the mt*CO1* gene fragments of the five *C. maculatus* populations (Tirupati, Lam, Guntur, Krishna and Nandyal) showed high A+T content *i.e.*, 62.69%, 62.56%, 63.71%, 63.12% and 61.49%, respectively and low GC content *i.e.*, 37.04%, 37.44%, 36.29%, 36.88% and 38.51% respectively (Table. 3). The mt*CO1* region in arthropods is frequently characterized by high AT and low GC composition (Lunt, Zhang, Szymura, & Hewltt, 1996; Navajas, Gutierrez, Lagnel, & Boursot, 1996; Shashank et al., 2018). The high A+T content observed in the present study is consistent with the findings of Zhang et al. (2018), who reported an AT-rich mitochondrial genome in *C. maculatus*. Such nucleotide composition is a characteristic feature of insect mitochondrial DNA and supports the suitability of mtCO1 as a molecular marker for DNA barcoding and phylogenetic studies.

Table 3. Nucleotide composition (%) and ATGC content of mtCO1 sequences of *C. maculatus* populations collected from Andhra Pradesh, India.

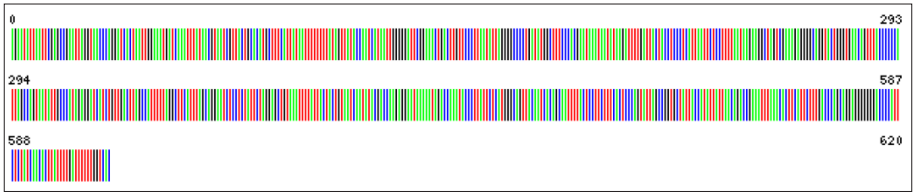
S. No.	*C. maculatus* sequences	A (%)	T (%)	G (%)	C (%)	AT (%)	GC (%)
1.	Tirupati (PV569548)	30.27	32.69	16.43	20.61	62.69	37.04
2.	LAM, Guntur (PV595833)	30.02	32.54	16.43	21.01	62.56	37.44
3.	Guntur (PV650458)	31.00	32.71	16.20	20.09	63.71	36.29
4.	Krishna (PV595857)	30.27	32.85	16.43	20.45	63.12	36.88
5.	Nandyal (PV715814)	30.10	31.39	18.12	20.39	61.49	38.51

Multiple sequence alignment revealed minor nucleotide changes when compared with the *C. maculatus* reference genome from Yemen (KY942062). The Nandyal population showed a transition (A↔G) at position 114, LAM showed a transition at position 261 (A↔G) and Guntur showed a transversion at position 567 (A↔T). No variations were observed in the Tirupati and Krishna populations, which showed complete identity with the reference genome, indicating very low intraspecific genetic variation.

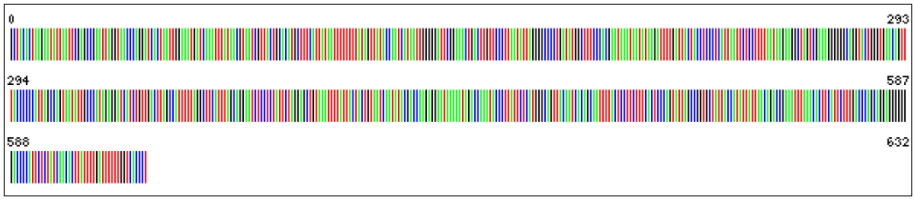
DNA Barcoding of *C. maculatus* Populations

Unique DNA barcodes for all the five *C. maculatus* populations were successfully generated and submitted to the Barcode of Life Data Systems (BOLD) along with corresponding metadata, photographs and sequence trace files (Fig. 4). The mt*CO1* DNA barcodes generated for the five populations were assigned process IDs: Tirupati (PBTP002-25), Lam (PBLAM001-25), Guntur (PBGNT001-25), Krishna (PBKN001-25), and Nandyal (PBNDL001-25). A Barcode Index Number (BIN: BOLD: ACX1369) was also obtained (https://doi.org/10.5883/BOLD:ACX1369). To the best of our knowledge, this is the first report of *C. maculatus* DNA barcodes from Andhra Pradesh, India. Similar DNA barcoding studies were also conducted by different researchers and generated the DNA barcodes of different insects from Andhra Pradesh *viz., Rhyzopertha dominica* (Anu, 2023) and *Maruca vitrata*, *Spodoptera litura*, *Euchrysops cnejus* and *Eublemma dimidialis* (Bhowmik, Rajasri, Devaki, & Reddy, 2025).

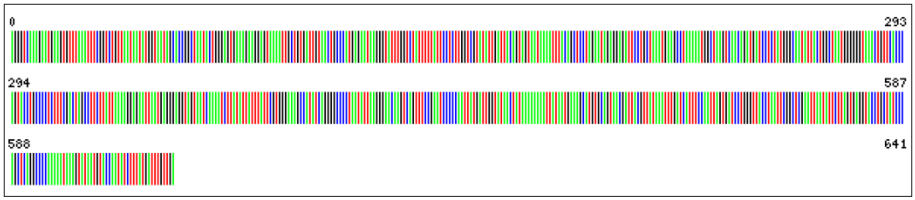
1. *Callosobruchus maculatus* from Tirupati, Andhra Pradesh, India. Sample code: PBTP, NCBI Accession no.: PV569548, Process ID: PBTP002-25 BIN: BOLD: ACX1369



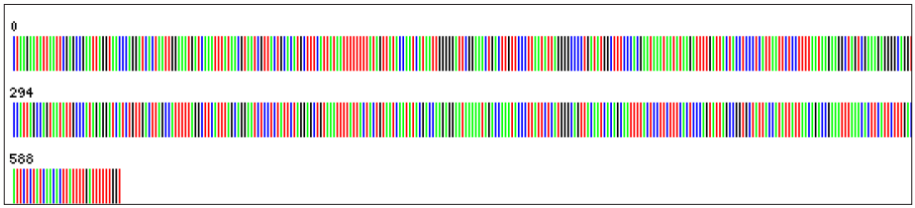
2. *Callosobruchus maculatus* from LAM, Guntur, Andhra Pradesh, India. Sample code: PBLAM, NCBI Accession no.: PV595833, Process ID: PBLAM001-25 BIN: BOLD: ACX1369



3. *Callosobruchus maculatus* from Guntur, Andhra Pradesh, India. Sample code: PBGNT, NCBI Accession no.: PV650458, Process ID: PBGNT001-25 BIN: BOLD: ACX1369



4. *Callosobruchus maculatus* from Krishna, Andhra Pradesh, India. Sample code: PBKN, NCBI Accession no.: PV595857, Process ID: PBKN001-25 BIN: BOLD: ACX1369



5. *Callosobruchus maculatus* from Nandyal, Andhra Pradesh, India. Sample code: PBNDL, NCBI Accession no.: PV715814, Process ID: PBNDL001-25 BIN: BOLD: ACX1369

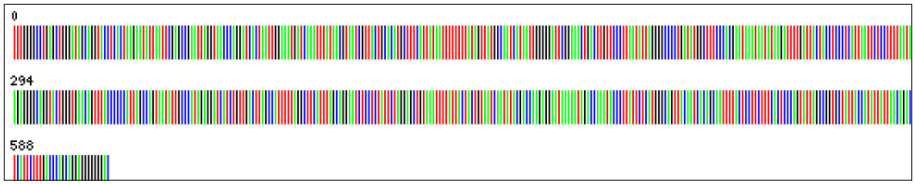


Figure 4. DNA barcode records of *C. maculatus* populations generated in the present study and registered in the Barcode of Life Data Systems (BOLD), showing corresponding sample identifiers and Barcode Index Number (BIN) assignments.

Multiplex PCR

The designed primers targeting the mt*CO1* gene of *C. maculatus*, *S. oryzae*, and *R. dominica* were validated through simplex PCR for each insect species. Multiplex PCR at an annealing temperature of 52.9°C produced distinct amplification products *i.e.*, 95 bp for *S. oryzae*, 119 bp for *C. maculatus* and 143 bp for *R. dominica* (Fig. 5). Negative control (water plus primers) showed no amplification, indicating the absence of contamination during PCR amplification.

Similar studies were also conducted by Sola, Agusti, & Riudavets (2015) who developed multiplex PCR for simultaneous detection and discrimination of the three morphologically similar storage insects of the genus *Sitophilus granarius, Sitophilus oryzae, Sitophilus zeamais* present in stored grain facilities. Sola, Agusti, & Riudavets (2018) also developed five specific primer pairs corresponding to *S. zeamais, S. oryzae, S. granarius, R. dominica and Sitotroga cerealella* from the *CO1* (Cytochrome Oxidase 1) mitochondrial region. The optimal conditions for the multiplex PCR were established to detect the target species in a PCR. The approach for the detection and identification of the five most concerning primary pests that develop inside the grain kernels and cause hidden infestation.

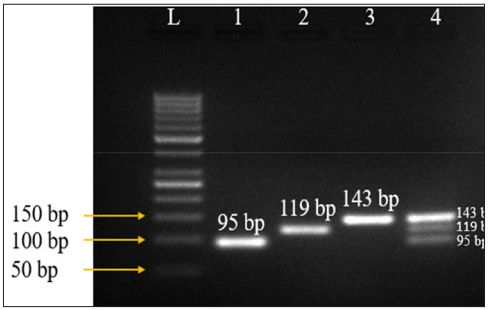


Figure 5. Agarose gel electrophoresis showing singleplex and multiplex PCR amplification of mtCO1 gene fragments using species-specific primers for *S. oryzae, C. maculatus* and *R. dominica.* L: 50 bp DNA ladder; Lane 1: *S. oryzae* (95 bp); Lane 2: *C. maculatus* (119 bp); Lane 3: *R. dominica* (143 bp); Lane 4: multiplex PCR amplification; Lane 5: negative control.

Leelavathi (2024) developed the multiplex PCR markers for mt*CO1* region suitable for the identification of hidden stored grain pests *viz*., rice weevil, *S. oryzae*., red flour beetle, *Tribolium castaneum*., lesser grain borer, *R. dominica*., rice moth, *Corcyra cephalonica* and Angoumois grain moth, *S. cerealella*. A set of five species specific markers *viz*., multiplex PCR primers were designed in such a way that all the five stored grain pests of rice can be identified in a single PCR reaction.

Multiplex PCR offers distinct advantages over simplex PCR, including simultaneous detection of multiple target species in a single reaction. The assay developed in the present study successfully amplified the target species under optimized laboratory conditions and demonstrated its potential utility for molecular identification of hidden infestations in stored pulses.

The multiplex PCR assay developed in the present study may have practical applications in grain storage facilities, seed testing laboratories and quarantine inspection programs where rapid identification of hidden infestations is required. Early detection of *C. maculatus*, *S. oryzae* and *R. dominica* can assist in timely implementation of pest management measures and help reduce post-harvest losses. However, additional validation under field and storage conditions is necessary before routine adoption.

The present study has certain limitations. Molecular characterization was based on a limited number of populations from Andhra Pradesh and utilized only the mtCO1 marker for phylogenetic analysis. Furthermore, specificity testing against additional non-target stored grain pests and closely related Bruchidae species was not performed. Detection limit (LOD), limit of quantification (LOQ) and sensitivity analyses through serial DNA dilution experiments were also beyond the scope of the present investigation. Future studies involving larger population samples, additional molecular markers, and broader validation of the multiplex PCR assay will further strengthen its applicability for stored grain pest diagnostics and management.

CONCLUSIONS

The present study successfully characterized *C. maculatus* populations from five locations in Andhra Pradesh using mtCO1-based DNA barcoding. PCR amplification with universal primers yielded ~750 bp fragments, and nBLAST analysis confirmed >99% similarity with known *C. maculatus* sequences. Unique DNA barcodes were generated and submitted to BOLD Systems, marking the first report of *C. maculatus* barcodes from Andhra Pradesh.

Comparative sequence analysis and phylogenetic reconstruction revealed minimal genetic divergence (<1%) among the populations, with Krishna and Tirupati showing complete identity. All Andhra Pradesh populations clustered closely with Indian and Yemen sequences, while those from Germany, Nigeria, Thailand, Iraq, and the USA formed separate groups, reflecting minor geographic differentiation.

Overall, this study demonstrates the reliability of mt*CO1*-based DNA barcoding for accurate identification and phylogenetic assessment of *C. maculatus*. The low intraspecific variation suggests a lack of strong geographic isolation among Andhra Pradesh populations and underscores the need for uniform management strategies across the state.

In addition, a multiplex PCR assay targeting the mt*CO1* gene was developed to simultaneously detect *Callosobruchus maculatus*, *Sitophilus oryzae* and *Rhyzopertha dominica*. The assay successfully differentiated the target species and demonstrated its potential for molecular identification of hidden infestations in pulses. Furthermore, the multiplex PCR approach enabled simultaneous detection of multiple species in a single reaction and may serve as a useful tool for stored grain pest identification under laboratory conditions.

The protocol may have potential applications in pest management, grain storage monitoring and quarantine inspections; however, further validation studies are required before large-scale implementation and molecular studies incorporating whole-genome sequencing are required to better understand the genetic diversity and evolutionary relationships of pulse beetle populations.

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