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Genetic variability in *Callosobruchus maculatus* (Fabricius) populations collected from major pulse growing districts of Andhra Pradesh

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Abstract
Molecular characterisation of the *Callosobruchus maculatus* (Fabricius) populations collected from major pulse growing districts of Andhra Pradesh *viz.*, Chittoor, Kurnool, Guntur, East Godvari and Srikakulam was carried out at Department of Entomology, Institute of Frontier Technology, RARS, Tirupati during 2020-21by using partial mitochondrial cytochrome B (*Cyt*B) primer. The nucleotide sequence obtained were blasted in NCBI database and they were confirmed as *C. maculatus* with 98 per cent similarity. Intra specific variability of *C. maculatus* sequences assessed through multiple sequence alignment revealed that there were no nucleotide variations in the nucleotide sequences of the populations. The present study confirmed the genetic identity of *C. maculatus* populations collected from five surveyed location in Andhra Pradesh.

Keywords: *Callosobruchus maculatus,* Polymerase chain reaction, Primer, Phylogenetic tree.

1. Introduction
Pulses are cost-effective and considerable source of protein and they can be seen of as a lifeline for India's vast vegetarian population. Pulses include a significant amount of minerals, vitamins, crude fibre, and other nutrients in addition to being a rich source of protein. Pulses dominate both area and production in India, accounting for 34% (>293 lakh hectares) and 26% (234 lakh tonnes), respectively. The country's productivity 823 $Kgha^{-1}$ in 2019-20. Pulses are produced on 1.25 Mha in Andhra Pradesh, with 1.17 MT of production and 932 $Kgha^{-1}$ overall productivity (Directorate of Economics and Statistics, 2020). In India, yearly post-harvest losses of pulses range from 25 to 50%, with storage losses accounting for 5 to 10% of the total (Lal and Verma, 2007) [11].

Pulse beetles *viz., Callosobruchus chinensis* (Linnaeus), *Callosobruchus maculatus* (Fabricius), and *Callosobruchus analis* (Fabricius), are the most common pests in stored pulses (Kedia *et al.*, 2015) [8], accounting for 90% of damage in cowpea, chickpea, green gramme, garden pea, black gramme, lentil, and *C. maculatus* is the most dominant of the three species in South India (Raina, 1970) [15].

To identify the insects, traditional taxonomy is utilised, which focuses mostly on external morphology (Rebijith *et al*., 2012) [17]. However, taxonomic keys are typically provided for specific life stages or genders, and phenotypic changes in taxonomically significant traits can make species identification difficult (Ball and Armstrong, 2006) [1]. According to the taxonomic analysis, there is a lot of morphological diversity across species, making it difficult to identify insects, especially stored grain pests (Ball and Armstrong, 2006; Singh and Singh, 2014) [1, 20]. Deep genetic divergences within a species are viewed as a potential indicator of cryptic species and molecular diversity studies can be an important first step in identifying cryptic species (Hebert *et al.*, 2004; Schmidt *et al.*, 2015; Toon *et al.*, 2016 and Hereward *et al.*, 2017) [5, 18, 22, 6]. Phylogeographic studies are viewed as a valuable tool for pest species management and to be taken up to know the diversity of insects in a particular region (Ochando *et al*., 2010 and Porretta *et al.*, 2007) [13, 14]. With that background, in the current study *C. maculatus* samples from five geographical sites in Andhra Pradesh were genetically examined using the mitochondrial marker *Cyt*B (*i.e.* CB1 and CB2).

2. Material and Methods
2.1 Sample collection
Adults of pulse beetle, *C. maculatus* were collected from warehouses storing pulses located at major pulse growing districts of Andhra Pradesh (Table 1.).

The collected bruchids were transferred to 70 per cent ethyl alcohol and were stored in deep freezer (-20 ^{0}C).

Table 1: *Callosobruchsus maculatus* populations collected from different geographical locations of Andhra Pradesh.

S. No.	District	Sampling Locations	Latitude	Longitude	Population code
1	Chittoor	Renigunta	15.4746 N	78.4728 E	CH. Rng
2	Kurnool	Nandyal	13.6347 N	79.5163 E	KRL. Ndl
3	Guntur	Sattenapalli	16.4042 N	80.1471 E	GTR. Stp
4	East Godavari	Kakinada	16.9508 N	82.2666 E	EG. Knd
5	Srikakulam	Amadalavalasa	18.1044 N	83.4019 E	SKL. Ads

2.2 Extraction of genomic DNA from pulse beetle, *Callosobruchus maculatus.*

Total genomic DNA was extracted from whole individual adult beetle by using CTAB method (Doyle and Doyle, 1990) [3]. The whole adult pulse beetle was homogenized by using pre-sterilized micropestle in 2 ml of pre-heated (65 ºC) extraction buffer (1M Tris (pH 8.0), 5 M NaCl, 0.5M EDTA, 2% CTAB, 1% PVP, 0.1% Mercapto ethanol). These samples were incubated in water bath at 65 ºC for 1 hour. After incubation the tubes were centrifuged in refrigerated centrifuge (Eppendorf, USA) at 10,000 rpm for 10 min at room temperature and the supernatant was collected into 2 ml fresh eppendorf tubes. Equal volume of phenol-chloroform (1:1 ratio) was added to the supernatant and mixed gently and centrifuged at 10,000 rpm for 10 min and the supernatant was transferred to the 2 ml fresh eppendorf tubes. Equal volumes of chloroform and Isoamyl alcohol (24:1 ratio) was added and centrifuged at 10,000 rpm for 10 min. The supernatant was collected into separate eppendorf tube and added with 0.6 volume of ice-cold isopropanol, 0.1volume of sodium acetate (0.3M, p^H 5.0) then incubated for 24 hours at -20 ^{0}C. After incubation, the tubes were taken out and centrifuged at 13,000 rpm for 20 min at 4 ^{0}C. The supernatant was discarded and the pellet was washed with 70 per cent ethanol and again centrifuged at 13,000 rpm at 4 ^{0}C for 10 min. After discarding the supernatant, the pellets were air dried and dissolved in 50 µl of sterile distilled water. The DNA samples were stored at -20 ^{0}C until use.

2.3 Quality and quantity check of genomic DNA

The concentration and quality of DNA was estimated using nanodrop spectrophotometer at 260 nm (ND-1000, USA). Nanodrop spectrophotometric method was based on measuring the amount of ultraviolet radiation that is absorbed by the bases. The ratio of optical density (OD) 260/280 was determined to assess the purity of the sample. If the ratio is 1.8: 2.0, the absorption is due to nucleic acids. The quantity of DNA was calculated by using the below mentioned formula. The quantity and purity of DNA in all five samples were listed here under

1-OD = 50 mg DNA and taking the dilution factor

DNA mg/ml= A260 × dilution factor

Purity of DNA sample was verified by running DNA sample (2 ml) from each isolate mixed with 2 ml of 1 per cent loading dye on 0.8 per cent agarose gel along with 50 bp marker**.**

2.4 PCR amplification of Cytochrome B region

The genomic DNA was subjected to Polymerase chain reaction (PCR) amplification by using cytochrome B primer *i.e.,* CB1/CB2 (Table 2). PCR reaction was performed in 25 µl volume of mix containing the components of 10x PCR reaction buffer, 2.5 mM of $MgCl_2$, 10 mM of dNTPs, 10 pM of each primer, 2.5 units of Taq DNA polymerase and 100 ng of DNA template (Table 3**)**. The amplification was performed in a PCR machine (Eppendorf Pro S).

Table 2: Primers used for *C. maculatus*

Primer	Sequence
CB1 (Forward)	5′-TATGTACTACCATGAGGACAAATATC-3′
CB2 (Reverse)	5′-ATTACACC TCCTAATTTATTAGGAAT-3′

Table 3: The components of PCR mixture used for DNA amplification

PCR Components	Volume (µl)
Taq buffer (10X)	2.5
$MgCl_2$ (2.5 Mm)	2
dNTP (10 mM)	0.5
Taq DNA polymerase (5U/µl)	0.3
Forward primer (10 pM)	1
Reverse primer (10 pM)	1
Template DNA (50 ng/µl)	2
Nuclease free water	15.7
Total	**25 µl**

The conditions for amplification of target DNA sequence are**,** one cycle of 94 ^{0}C for 4min for initial denaturation, 94 ^{0}C for 30s denaturation, 48 ^{0}C for 45s annealing, 72 ^{0}C for 1 min extension (35 cycles) and 1 cycle of 72 ^{0}C for 10 min final extension. PCR products were analyzed on 1 per cent agarose gel in 1x TBE buffer.

2.4 Phylogenetic Tree and Sequences Analysis.

Sequence assembling and nucleotide alignment were done with Bio Edit version 7.0 software (Hall, 1999) [4]. Sequences obtained were analyzed using BLAST program in National center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.org) to confirm identification of *Callosobruchus maculatus*. The nucleotide sequences are compared with other *C. maculatus*. sequence mined from National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.org) and a phylogram was constructed from aligned sequences using neighbor-joining method using boot strap option using Mega 7.0 software (Kumar *et al*., 2016) [10].

3. Results and Discussions

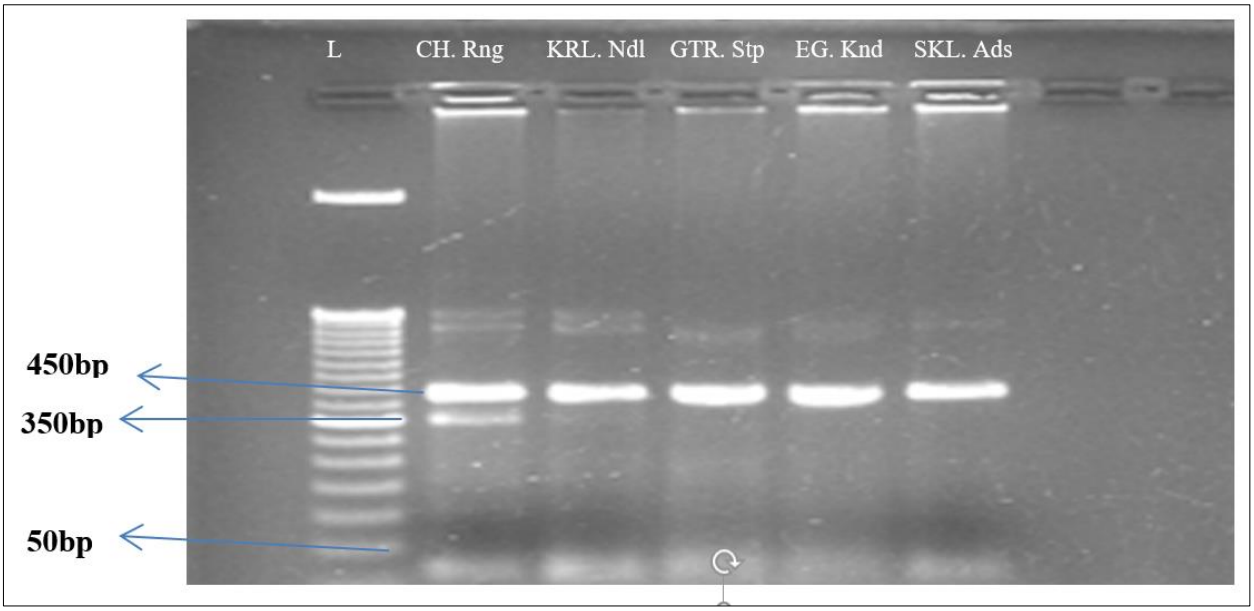
C. maculatus samples from the Andhra Pradesh districts of Chittoor, Kurnool, Guntur, East Godavari, and Srikakulam were employed to test intra-specific changes in the Cytochrome B region (*Cyt*B). The amplification of the *Cyt*B gene yielded a 450-bp product (Fig 1). The nucleotide and amino acid sequences of a representative PCR product from each region, namely Chittoor, Kurnool, Guntur, East Godavari, and Srikakulam District, were sequenced and presented. Using n-blast, the nucleotide sequence was compared to the NCBI database and confirmed as *C. maculatus* with 98.00% similarity. Multiple sequence alignment was used to examine intra-specific variation in the *C. maculatus* population (Fig 2). There were no nucleotide

differences in the nucleotide sequences of the populations obtained from the Andhra Pradesh districts of Chittoor, Kurnool, Guntur, East Godavari, and Srikakulam, confirming the identity of the *C maculatus* populations.

Besides nucleotide sequences of the present study, the sequences of five specimens originating from different geographical regions were retrieved from NCBI and BOLD database for comparative analysis. Following that, all of the samples were subjected to multiple sequence alignment. The phylogenetic analysis of *C. maculatus* sequences with NCBI accession revealed that all five populations were closely related and clustered together. The Chittoor, Kurnool, Guntur, East Godavari, and Srikakulam populations of *C. maculatus* were included in the cluster, as were accessions from Nepal (KY995263.1), Madagascar (KY995259.1), Yemen (KY995283.1), China (KY995256.1), Togo (KY995271.1), Uganda (KY995281.1), Burkina Faso (KY995253 (AY 625465.1) (Fig 3).

The resultant amplicon size of *C. maculatus* was 450 bp which was in line with the Ndong *et al.* (2012) [12] who employed the same primer (*Cyt*B) to test the climate influence (agro-ecological zones) on genetic diversity of *C. maculatus* and obtained the amplicon size of 448 bp.

The results of the present study are in concurrence with Sembene *et al.* (2008) [19] who reported that geographical distances between localities did not influence the genetic structure of *C. serratus* populations from a given host plant. Krishnega *et al.* (2021) [9] characterized fourteen different populations of *C. maculatus* from distinct geographical locations of Tamil Nadu using mt-COI sequences and reported that there is no significant influence of geographical locations on population structure whereas the present results are in contrary with Raja *et al.* (2009) who reported that the genetic variation was significant among six different populations (Madurai, Nagapattinam, Coimbatore, Thiruvannamalai, Chennai and Vellore) of *C. maculatus* analyzed using *COI* primer.



CH. Rng: Chittoor-Renigunta KRL. Ndl: Kurnool-Nandyal
GTR. Stp : Guntur-Sattenapalli EG. Knd: East Godavari-Kakinada
SKL. Ads: Srikakulam-Amadalavalasa L: Ladder 50bp

Fig 1: Agarose gel electrophoresis of PCR amplified DNA of mitochondrial partial cytochrome B region of five populations of *C. maculatus* collected from different locations of A.P., India.

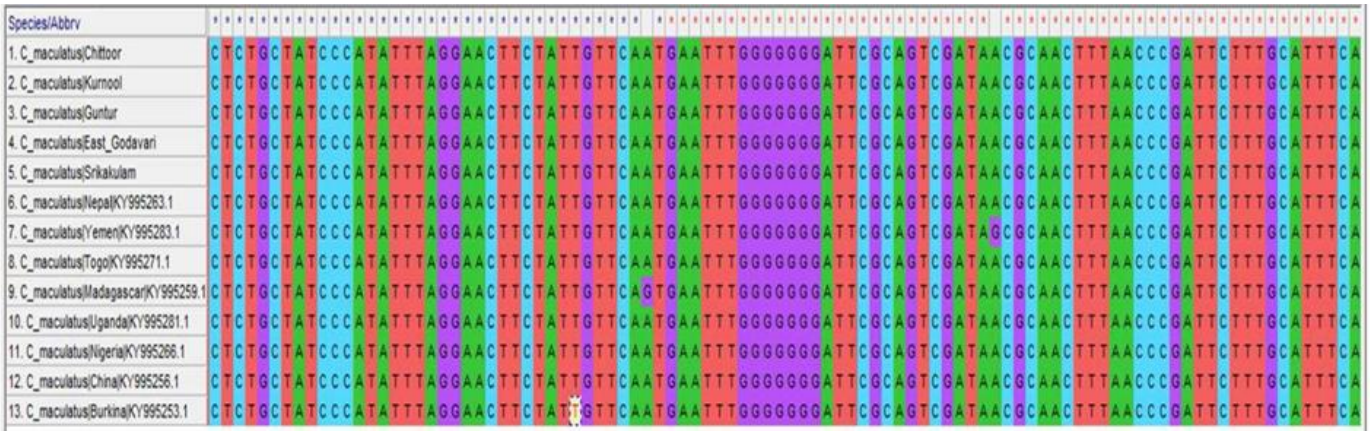


Fig 2: Multiple sequence alignment of *CytB* gene of *C. maculatus* samples collected from different districts of A.P. and accessions from NCBI and BOLD data base

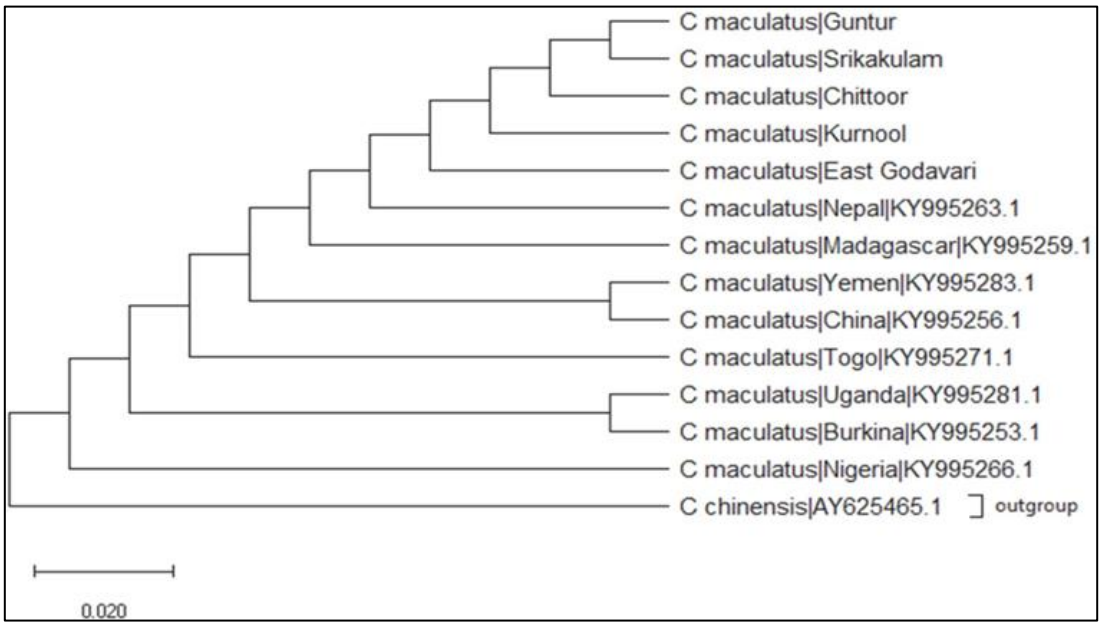


Fig 3: Phylogenetic analysis of *C. maculatus* samples collected from different districts of A.P and accessions from NCBI

4. Conclusion

Phylogenetic analysis of five molecular sequences of pulse beetle obtained from Andhra Pradesh mined from GenBank through BLAST analysis proved that all the five populations as *C. maculatus* with 98 per cent similarity. There were no nucleotide differences in the nucleotide sequences of the populations and they are identical.

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