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Assessment of genetic diversity of *Callosobruchus maculatus* populations of Tamil Nadu through mt-COI maker

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Abstract

Callosobruchus maculatus is a serious pest in Indian subcontinent, which infests wide range of legumes and causes huge losses in the stored pulse grains. As the larvae, pupae and pre-emergence adults live inside the seeds, the initial infestations are hard to detect and hence the pest build up and movement. The understanding of genetic structure and pest movement is of prime importance in confining the pest and deploying management practices, but there have not been many studies, focusing on the genetic structure at regional level in Tamil Nadu. Hence, in our study we have characterized 14 different populations from distinct geographical locations using mt-COI sequences. Our analysis showed that there are more haplotypes, low nuclear diversity and distinct structuration among populations. This is further supported by AMOVA and high *F*_{st} value. However, we did not find any significant influence of geographical locations on population structure.

Keywords: *Callosobruchus maculatus*, South India, mt-COI, diversity analysis

Introduction

Pulses are the major protein sources of vegetarian diet. In India, pulses occupy about 23 percent of total area under food crops cultivation. Globally, India is the leading producer, consumer and importer of pulses. About 34 percent of the total area, and 26 percent of global production is contributed by India^[1]. The low productivity and high consumer market have resulted in net import of pulses. However, Indian Institute of Pulses Research reported that cutting down the post-harvest losses alone will save as much as import equivalent of pulses. The post-harvest losses in pulses range between 25 – 30 percent of which 5 percent is due to storage insect pest^[2]. *Callosobruchus* spp. is the most common and serious insect storage pest of pulses that has reached cosmopolitan distribution through international trade of its host seeds^[3]. In India about 14 *Callosobruchus* spp. have been reported thus far with subtle differences in biology^[4]. The adults are generally aphagous and lays egg individually on the whole grains^[5]. The pest form is the larva, that bores into the seed and completely feeds on the contents of the grain, impairing germination, nutritive and economic value. Pupation occurs inside the seeds. The rapid population build up can cause up to cent percent storage loss in six generation cycles if left unchecked^[6]. *C. maculatus* is adapted to a range of legume seeds as hosts and exhibits a great deal of plasticity in bionomic traits depending on larval density, temperature and grain moisture content during larval stage^[6-8] that makes species identification a difficult task. Besides, the pre-adult forms of the pest also exhibit constraints on taxonomic identification. The use of DNA sequence, resolves these constraints and allows delimitation of even cryptic species^[9-11] and have further been used to understand population structure and divergence patterns. With that background, the present study aimed to characterize populations of *Callosobruchus maculatus* using mt-COI barcodes.

Materials and Methods

Collection of *Callosobruchus maculatus* populations

C. maculatus infested pulse seeds were collected from Tamil Nadu (11 locations), Karnataka, Gujarat and Delhi during 2019 - 2020. The cultures were established at Molecular Ecology lab, Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore (Table.1).

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Genomic DNA isolation

Genomic DNA was isolated individually for a total of 42 insects, three representing each population by conventional method with CTAB buffer^[12]. The DNA extraction buffer had 100 mM Tris. HCl, 10 mM EDTA, 1.4M NaCl and 2 per cent CTAB, with pH adjusted to 8 units. Individual insects were homogenized with 200 µl CTAB buffer in an eppendorf tube and incubated in water bath at 65°C for an hour. The tubes were then allowed to cool till room temperature and centrifuged for 10 minutes at 12,000 rpm. The supernatant was transferred to a fresh tube to which equal volume (200µl) of chloroform: isoamyl alcohol (24: 1 v/v) was added. This was gently shaken for five minutes and centrifuged at 12,000 rpm for 10 minutes. The top clear aqueous layer supposedly containing the DNA was transferred to a fresh tube and incubated overnight at -20 °C with ice cold isopropanol (0.7 volume) and 3M sodium acetate (0.1 of total volume). After 12h, samples were centrifuged at 12,000 rpm for 15 minutes to pellet the DNA. The pellets were washed with 70 per cent ethanol, air dried, resuspended in 25 µl of nuclease free water, labelled and stored at - 20 °C until further use.

Polymerase chain reaction (PCR) amplification and product detection

Polymerase Chain Reaction (PCR) was carried out with forward and reverse primers (mt-COI Forward primer^[13] - CTTTATCAACATTTATTTTGGATTTTTT and Reverse primer^[13] - TACTCCAATAAATATTATAATAAATTG) amplifying partial sequence of mitochondrial Cytochrome C oxidase subunit I (mt-COI). The reaction volume comprised a total of 25 µl (11.5 µl of nuclease free water, 2.5 µl of 10X reaction buffer containing 25 mM MgCl₂, 2.5 µl of 2 mM dNTPs, 1.5 µl each of forward and reverse primers, 1.0 µl each of 25mM MgCl₂ and 10% DMSO, 0.5 µl of Taq polymerase with activity 3U/ µl, and 4 µl of DNA diluted to 50 ng/µl). PCR amplification was done in Sure cycler 8000 (Agilent technologies) with profile conditions optimised to 3 min of initial denaturation, 35 cycles of denaturing, annealing and extension at 94°C, 51°C and 72°C respectively for 1 min each and 10 min of final extension at 72°C followed by storage at 4°C. The amplified fragments were run on 1.5% agarose gel (Fig.1) and the PCR products were sent to Eurofins, Bengaluru for double pass sequencing by Sanger's method, after purification of PCR products.

Sequence analysis

The sequences obtained were assembled using Geneious.v.11.1.3^[14] and analyzed through BLAST of NCBI database for species confirmation. The 42 sequences were then submitted to GenBank after assigning the reading frames based on the genetic codes of invertebrate mt-DNA.

The sequences were aligned and trimmed to an equal length of 601 nucleotides using Geneious.v.11.1.3 and further analysed. Pairwise genetic distances were calculated using MEGA X.v.1.52^[15]. Total number of haplotypes, haplotype diversity and nucleotide diversity were analysed using DNAsp.v.6.12.03^[16]. Arlequin.v.3.5.12^[17] was used to calculate AMOVA where each population was represented by three sequences. Phylogenetic tree was generated with Neighbour Joining (NJ) method using Geneious.v.11.1.3.

Further, Isolation by distance was calculated for the 42 sequences with IBD.v.1.52^[18] using genetic distance calculated from MEGA X.v.10.0.5 and Euclidian geographical distance(km) calculated using Franson

CoordTrans.v.2.3^[19]. All the above analyses were done for a total of 66 sequences, that included 42 sequences of this study and 24 other sequences retrieved from GenBank representing 24 countries.

Results and Discussion

South Indian (SI) strain of *C. maculatus* collected from Tirunelveli during July 1979^[20] had been characterized in various studies to understand behavioural and genetic differences, between globally sampled populations. But less has been known about the genetic diversity of *C. maculatus* populations of Tamil Nadu. The present study with populations sampled from different hosts and different districts have revealed high genetic diversity. A total of 42 sequences (GenBank accession numbers given in Table 2) formed 30 haplotypes. The haplotype diversity of individual populations varied between 0.00 and 1.00, and the nucleotide diversity varied between 0.00 and 0.0375 respectively (Table 3). An overall haplotype diversity of 0.978±0.011 was observed, which is comparable to haplotype diversity calculated for global (0.97±0.007)^[21] and African population (0.97±0.005)^[22]. High haplotype diversity in *C. maculatus* is ambiguous considering low haplotype diversity typical to most storage pests^[23-25]. In general, storage pests exhibit low haplotype diversity due to rigorous management practices in bulk storage godowns that select for one or two major common haplotypes of the pest. Thus, it is evident that no stringent management measures had been deployed against our populations of pulse beetle. The high haplotype diversity and haplotype number can be explained by large population size, small generation time and multiple introductions of the pest^[21,26]. Insignificant values of Fu's F test (0.5234) and Tajima's D test (0.8807) also showed that there was constant population in the recent past, accepting the null hypothesis in favour of neutral theory of molecular evolution. The high genetic diversity in natural populations of *C. maculatus* might be due to Negative Frequency Dependent Selection (NFDS) as environmental heterogeneity, genotype-by-environment interaction and competition for resources occur in the natural environment. It could be that NFDS favoured the maintenance of high polymorphism levels in ecologically relevant gene like COI subunit that is significant in energy metabolism^[27, 28].

The high global fixation index (Fst value) of COI sequences (0.8683) implied that there has significant percent variation and genetic structuration among population, which is in accordance with the results of Analysis of Molecular Variance (AMOVA – Table 4). Results of AMOVA with controlled geographical locations showed that percentage variation among population is 86.6%. The phylogenetic analysis using Maximum Likelihood showed all the samples of Salem, Sivagangai, Namakkal, Erode and Junagadh were monophyletic under the same cluster (Cluster A) for all 1000 iterations (Fig. 2). The second cluster (Cluster B) included samples from Tiruppur, Coimbatore, Karur, Pudhukottai, Thanjavur, Trichy, Ariyalur, Tumkur and New Delhi. When the tree was constructed again for a total of 66 sequences (42 our own, 24 GenBank retrieved), the same pattern was observed with one additional sequence (Bengaluru) joining Cluster A and all others joining Cluster B (Fig. 3). This clustering pattern does not depend on geographical distance as also testified by nonsignificant Mantel's test value (r=0.05206) and could be due to unrestricted movement of grains within the country. We presume that the movement of pest is closely

associated with seeds, as admixture of populations due to pest movement over long distances is rare, owing to poor flight capacity and sterility observed in flight forms of Indian strains [28]. India has been importing chickpea, black gram, green gram, pigeon pea and pea from Myanmar, Tanzania,

Australia, Russia, USA, Canada, Ukraine, Uzbekistan to meet out the production gap [1]. The two clades may correspond to two different strains, one common to India and another widely distributed strain adapted to different countries widespread due to global trade of pulse seeds in the past years.

Table 1: Location and host details of *C. maculatus* samples used in the study.

S.no	Location	State	Lat long	Host
1.	Coimbatore	Tamil Nadu	11.0152 N, 76.9326 E	Cow pea
2.	Tiruppur	Tamil Nadu	11.1937 N, 77.2686 E	Cow pea
3.	Erode	Tamil Nadu	11.4259 N, 77.1496 E	Green gram
4.	Salem	Tamil Nadu	11.6653 N, 78.2806 E	Bengal gram
5.	Namakkal	Tamil Nadu	11.3871 N, 78.1607 E	Cow pea
6.	Karur	Tamil Nadu	10.8202 N, 78.2708 E	Cow pea
7.	Trichy	Tamil Nadu	10.7556 N, 78.6009 E	Green Gram
8.	Ariyalur	Tamil Nadu	11.1401 N, 79.0786 E	Cow pea
9.	Thanjavur	Tamil Nadu	10.6173 N, 79.2562 E	Cow pea
10.	Pudhukottai	Tamil Nadu	10.3833 N, 78.8001 E	Cow pea
11.	Sivagangai	Tamil Nadu	9.8433 N, 78.4809 E	Butter bean
12.	Tumkur	Karnataka	13.5279 N, 77.0939 E	Cow pea
13.	Junagadh	Gujarat	21.5022 N, 70.4261 E	Cow pea
14.	New Delhi	Delhi	28.35 N, 77.18 E	Black gram

Table 2: Genetic variation statistics observed in different *C. maculatus* populations and their GenBank accession numbers.

	State	GenBank Accession Number	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Ariyalur	Tamil Nadu	MW041912 - MW041914	3	1.000 ± 0.272	0.01432 ± 0.00397
Coimbatore	Tamil Nadu	MT422543 - MT422545	1	0.00 ± 0.00	0.00 ± 0.00
Erode	Tamil Nadu	MT422546 - MT422548	1	0.00 ± 0.00	0.00 ± 0.00
Karur	Tamil Nadu	MT422549 - MT422551	3	1.000 ± 0.272	0.0122 ± 0.0037
Namakkal	Tamil Nadu	MT422552 - MT422555	3	1.000 ± 0.272	0.01223 ± 0.0037
Pudhukottai	Tamil Nadu	MT422555 - MT422557	1	0.00 ± 0.00	0.00 ± 0.00
Salem	Tamil Nadu	MT422558 - MT422560	3	1.000 ± 0.272	0.0071 ± 0.0019
Sivagangai	Tamil Nadu	MW041921 - MW041923	3	1.000 ± 0.272	0.0078 ± 0.0028
Thanjavur	Tamil Nadu	MT422561 - MT422563	3	1.000 ± 0.272	0.0344 ± 0.0127
Tiruppur	Tamil Nadu	MT422564 - MT422566	3	1.000 ± 0.272	0.0053 ± 0.0018
Trichy	Tamil Nadu	MT422567 - MT422569	3	1.000 ± 0.272	0.0375 ± 0.0112
New Delhi	Delhi	MW041915 - MW041917	2	0.667 ± 0.314	0.0220 ± 0.0100
Junagadh	Gujarat	MW041918 - MW041920	3	1.000 ± 0.272	0.0031 ± 0.0011
Tumkur	Karnataka	MT422540 - MT422542	3	1.000 ± 0.272	0.0035 ± 0.0012

Table 3: Analysis of Molecular Variance for 14 *C. maculatus* populations as computed by Arlequin for a total of 42 sequences.

AMOVA results for 42 samples			
Source of variation	Sum of squares	Variance components	Percentage variation
Among populations	717.54	17.51	86.83
Within populations	74.33	2.65	13.16
Total	791.88	20.16	

Table 4: Summary statistics for 42 and 66 mt-COI sequences of *C. maculatus*.

Summary statistics	42 sequences	42 + 24 (retrieved from GenBank)
Sample size	42	66
Sequence length	601	419
Number of variable sites	117	76
Haplotype diversity	0.978 ± 0.011	0.974 ± 0.008
Nucleotide diversity	0.09804 ± 0.00498	0.09428 ± 0.01372
Number of alleles/haplotypes	30	38

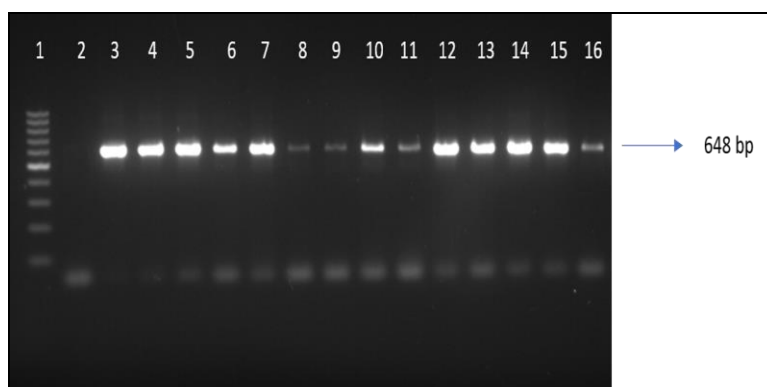


Fig 1: Amplification of mt-COI fragment of *C. maculatus* collected from different locations Lane 1 - 100 bp ladder Lane 2 – Negative control. Lane 3 to 16 – 648 bp COI fragment.

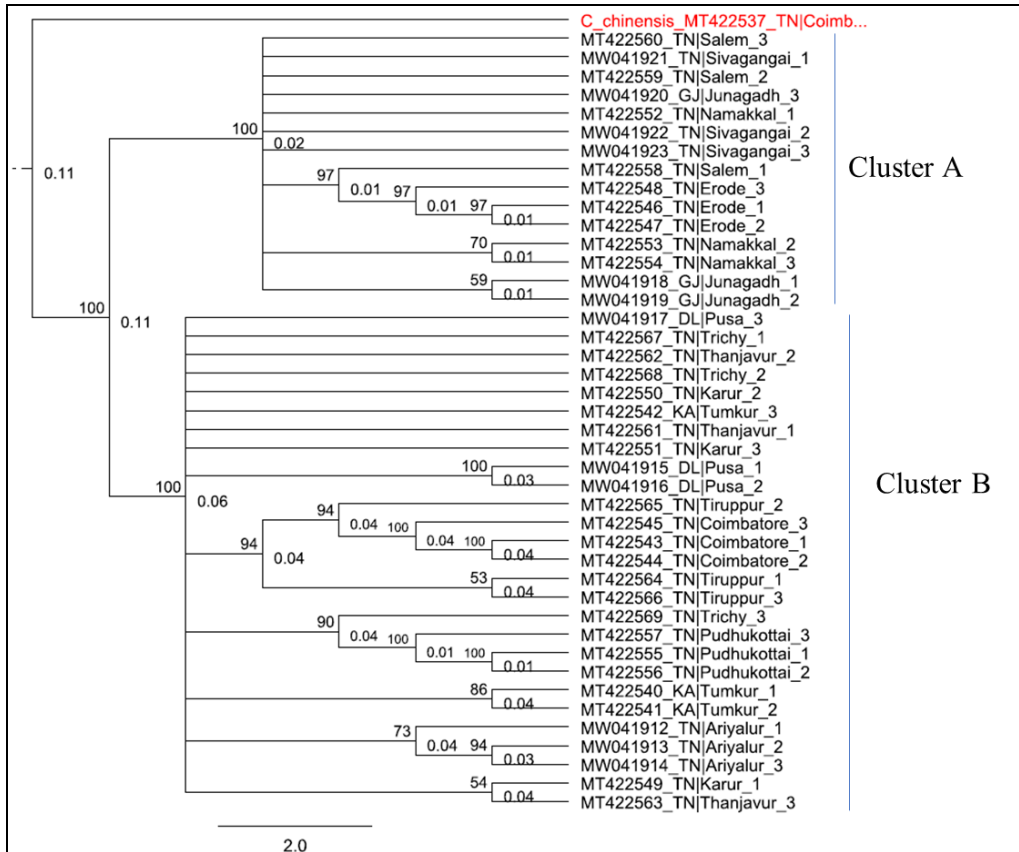


Fig 2: NJ tree computed using Geneious.v.11.1.3. for 42 sequences of *C. maculatus* populations.

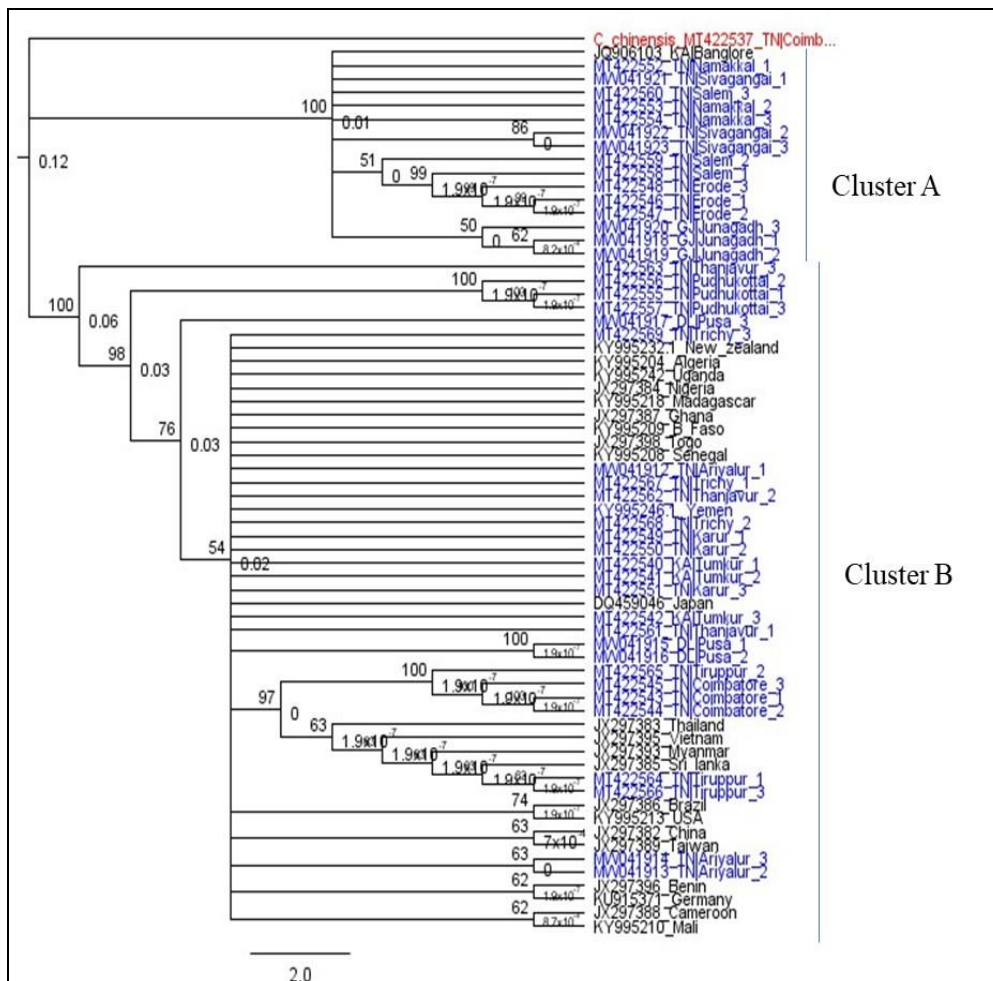


Fig 3: NJ tree computed for 66 sequences of *C. maculatus* populations (42 sequences of this study and 24 other sequences retrieved from GenBank representing 24 countries).

Conclusion

The study revealed high haplotype diversity among our studied populations. This study was done purely on mitochondrial sequences, as the sequences revealed sufficient genetic variation to study the populations. Further studies are needed with nuclear markers like simple sequence repeats with larger sample size from different geographical locations, imported pulse seeds, and different hosts of known history be done to get better understanding of the population structure of *C. maculatus* in India.

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