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# Genetic Diversity Analyses of Key Stored Grain Insect Pests of Rice Collected from the Grain Supply Chains of Tamil Nadu

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**ABSTRACT: The rice weevil, *Sitophilus oryzae,* and the red flour beetle, *Tribolium castaneum* are the two commonly identified stored grain pests worldwide. The development of phosphine resistance and the spread of phosphine resistant alleles poses a serious threat to the grains in storage. Therefore, to develop an effective pest management strategy, it is essential to understand the movement of these insects which helps in preventing the spread of phosphine resistant alleles that is a serious threat to the global food security. Grain samples were collected from the grain supply chains of Theni and Trichy. Storage pets were collected from the grain samples and mass cultured. Genetic diversity analyses were done with both mitochondrial COI and nuclear markers. Genetic diversity analyses with mitochondrial marker revealed a significant gene flow in both populations. A significant negative Tajima's D from both the insect pest species revealed the evidence for population expansion among these pests. Whereas, the genetic diversity analyses using microsatellite markers revealed a low genetic differentiation in both *S. oryzae* and *T. castaneum* populations. Also, a high level of gene flow was observed between both the populations and the genetic structure revealed the existence of admixed populations. These results suggested the need for broad-scale and species-specific management measures to prevent the spread of phosphine-resistant alleles.**

**Keywords:** *Sitophilus oryzae*, *Tribolium castaneum*, mitochondrial COI, microsatellite markers, genetic diversity.

## INTRODUCTION

*Sitophilus oryzae* (Linnaeus) and *Tribolium castaneum* (Herbst) are the cosmopolitan pests that causea serious threat to the grains in storage when left uncontrolled (both qualitatively and quantitatively) in storage (Cotton, 1920). Losses along the grain supply chain account for about 10-15 percent loss in quantity and 25-50 percent loss in quality (Mesterházy *et al.,* 2020). It destroys various food grains such as rice, maize, wheat, oats, and other cereals. Both larvae and adults feed on the grains thereby reducing the quality of the grain leading to economic losses (Park *et al.,* 2003). Excessive use of the fumigant (phosphine) has led to the development of heritable resistance that makes them difficult to control (Champ and Dyte 1976; Daglish *et al.,* 2002) and has increased the possibility of the spread of these resistant alleles. Knowledge of the patterns of insect movement which likely affect the population structure aids in developing an alternative pest management strategy (Kim and Sappington 2013). Investigations of stored grain insect movement using pheromone traps and other techniques are limited to both geographical extent and temporal scale, and rare occurrences of movement may be ecologically relevant yet easily overlooked (Chapman *et al.,* 2003). Hence, analyses of population genetics have been widely employed to investigate patterns and magnitudes of dispersal in both geographic and temporal dimensions. This data is especially important for analysing the spread of pesticide resistance, which is a serious problem in many agricultural systems (Guedes *et al.,* 2019). Understanding the genetic diversity with neutral markers *viz.,* mitochondrial cytochrome oxidase I (COI) has provided information on the movement and the levels of genetic variation among the populations (Avise, 2000). In addition, the ability of population genetics to understand the gene flow has been dependent on the development of suitable molecular markers and population genetics theory for deriving strong inferences from the observed variations in marker loci (Kim and Sappington 2013).

*S. oryzae* has been characterized with limited flight activity (Vásquez-Castro *et al.,* 2009), while *T. castaneum* has been characterized by its ability to fly from the nearest grain storage facility to several kilometers (Ridely *et al.,* 2011; Rajan *et al.,* 2018). In addition, anthropogenic movement during the transport of grain enhances the threat of resistant insect movement. Hence, studying the movement and the genetic diversity of these insect pests is important for

developing an effective pest management strategy. This information helps in monitoring the spread of resistant alleles which is a potent challenge in the post-harvest grain pest management system. In this study, the mitochondrial and nuclear markers have been used to elucidate the movement of the pests across the two grain supply chains in Tamil Nadu by analysing the population structure and genetic differentiation among these pest populations.

## MATERIALS AND METHODS

*A. Insect collection and Mass culturing*

*S. oryzae* and *T. castaneum* populations were collected from the two districts of Tamil Nadu *viz.,* Trichy and Theni belonging to the two major rice-producing agro-climatic zones namely the Cauvery Delta zone and Southern zone respectively. Grain samples were collected from the supply chain covering the bulk grain storage, public distribution systems, processing units, wholesale shops, retail shops, and households. The bulk grain storages are comprised of the central and state warehouses that store and distribute food grains to the consumers through the public distribution system (ration shops) (Pal, 2011). Processing units such as rice mill distribute grains through retail or wholesale shops (Table 1).

**Table 1: Sampling details of *S. oryzae* and *T. castaneum* populations collected from grain supply chains (Trichy and Theni districts), Tamil Nadu.**

| Sr. No. | Cluster of grain supply chain | Location | Code | Commodity | Latitude | Longitude |
|---|---|---|---|---|---|---|
| 1. | Bulk grain storage | Trichy | TRY_CWC | Rice | 10.76 N | 78.69 E |
| 2. | Processing Unit | Trichy | TRY_RM | Rice | 10.77 N | 78.62 E |
| 3. | Wholesale shop | Trichy | TRY_WS | Rice | 10.81 N | 78.69 E |
| 4. | Retail shop | Trichy | TRY_RS | Rice | 10.75 N | 78.60 E |
| 5. | Public Distribution System | Trichy | TRY_PDS | Rice | 10.99 N | 78.32 E |
| 6. | House Hold storage | Trichy | TRY_HH | Rice | 10.99 N | 78.32 E |
| 7. | Processing Unit | Theni | TH_RM | Rice | 10.04 N | 77.50 E |
| 8. | Wholesale shop | Theni | TH_WS | Rice | 10.01N | 77.47 E |
| 9. | Retail shop | Theni | TH_RS | Rice | 10.00 N | 77.48 E |
| 10. | Public Distribution System | Theni | TH_PDS | Rice | 10.00 N | 77.44 E |
| 11. | House Hold storage | Theni | TH_HH | Rice | 10.01 N | 77.48 E |

**Bulk Grain Storage:** Central Warehousing Corporation (CWC); Processing Unit – Rice Mill; Public Distribution System – Ration Shop.

Grain samples were collected from 3 to 5 sites in each cluster through the standard zigzag sampling method (Semple *et al.,* 1992). Samples were drawn from the periphery and sides of the grain stacks in a zigzag pattern. This yielded about 3-5 kg of grains. The collected samples were screened for the presence of insects using a metal brass sieve (1.7–4.0 mm size, IS460 – Gilson Company, Inc., Lewis Center, Ohio, 43035, USA) and mass cultured in culture media (wheat flour for *T. castaneum* and whole wheat grains for *S. oryzae*) under laboratory conditions by following the FAO methodology (1975). Culture media were disinfested by freezing it at -20°C for 24-48 hours to eliminate the existing insect pests. *T. castaneum* and *S. oryzae* insects were released separately in a 2.5 kg plastic container with the respective media and 5.0 percent brewer's yeast and placed for oviposition. Throughout the study period, the cultures were maintained at a temperature of 30±2ºC and relative humidity of 60±5 percent. After oviposition, the emerged grubs were allowed for development into adults and the resulting progenies were used for the study.

*B. DNA isolation, PCR amplification of the mitochondrial gene, and sequencing*

Genomic DNA was isolated from the individual adult of *S. oryzae and T. castaneum* from each node of the grain supply chain by using the HotSHOT method (Montero-Pau *et al.,* 2008). Two buffers were used in this method that comprised of alkaline lysis buffer containing 25mM NaOH and 0.2 mM $Na_2EDTA$, and a neutralizing solution containing 40mM Tris-HCl. The individual insect was homogenized with 100 µl alkaline lysis buffer (pH-8.0) and incubated at 95°C in a hot water bath for 30minutes. After incubation, the samples were allowed to cool at 4°C for 5-10 min. Then 100 µl neutralizing solution (pH-5.0) was added to each tube and vortexed to settle down the debris. The extracted DNA samples were stored at -20°C. The DNA samples were checked both qualitatively and quantitatively using agarose gel electrophoresis (0.8 percent agarose) and nanodrop spectrophotometer respectively.

A fragment of mt-COI region was amplified using primers LCO 1490(5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer *et al.,* 1994). Polymerase chain reactions were performed in 25µl reactions, containing 14.7µl water, 2.5µl of 10X Taq Buffer (TaKaRa™), 2.5µl of 250µM dNTPs, 1.5µl of 10µM forward primer, 1.5µl of 10µM reverse primer, 0.3µl of 5U/µl Taq polymerase (TaKaRa™) and 2µl of template DNA (50 ng/µl). PCR reactions were performed using Mastercycler® Nexus (Eppendorf) 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 and a final extension at 72°C for 10 min. Amplified PCR products were sequenced in both directions at AgriGenome Labs Pvt. Ltd., Kochi, Kerala, India. DNA sequences obtained in this study were identified using the BLASTn algorithm.

*C. Nucleotide sequence analyses*

The 33mt-COI sequences of *S. oryzae and T. castaneum* were trimmed and aligned using Geneious version 11.1.3 (https://www.geneious.com; Kearse *et al.,* 2012). Haplotype (Hd), nucleotide diversity ($\pi$), and genetic flow index (*Nm*) were calculated in DnaSP

version 6.12.03 (Librado and Rozas, 2009). Kimura 2-parameter (K2P) was used to compute the genetic distance (d) between and within the populations using MEGA X Ver. 10.0.5 (Kumar *et al.,* 2018). Pairwise $F_{ST}$ was calculated to analyze the genetic differences between the population pairs using ARLEQUIN version 3.5.1.2 (Excoffier and Lischer 2010). The level of significance was assessed with 10,000 coalescent simulations. Pairwise genetic differentiation and gene flow between the populations were also determined.

*D. Nuclear marker analyses*

DNA from *S. oryzae* and *T. castaenum* collected from different nodes of the grain supply chain were amplified using a set of 11 and 9 microsatellite markers respectively (Thangaraj *et al.,* 2016; Ridely *et al.,* 2011) (Table 2). Polymerase chain reactions (PCR) were carried out in 10 µlof cocktail mixtures containing 6.0 µl of PCR Master Mix (Emerald Amp PCR Master mix- TaKaRa Bio), 1.0 µl of forward primer, 1.0 µlof reverse primer, and 2 µl of template DNA. Reactions were performed using Mastercycler® Nexus (Eppendorf). PCR conditions 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 30s at 56°C, extension for 30 s at 72°C and a final extension at 72°C for 10 min. PCR products were resolved in agarose gel electrophoresis to analyze the banding patterns.

**Table 2: List of microsatellite markers used in this study.**

| Locus Name | Primer Sequences (5' – 3') | Motif | Tm (°C) |
|---|---|---|---|
| ***S. oryzae*** | | | |
| Sit_02 | F: ACTCTTCCTTGCGACCATTG | AAAGAG | 58 |
| | R: GAAACATCGCAGTATCCAGACA | | |
| Sit_12 | F: TGCAAGGCTGAACAGTGCTA | ACT | 56 |
| | R: TCTAGATTACATTCTTCCATTGTATCG | | |
| Sit_08 | F: CAGGGTGAGAAGGAAGGTCA | AAC | 58 |
| | R: CCGGTGAAATGGAAGGGTAT | | |
| Sit_15 | F: GGCTCGACCTGATGGTATGT | ACCGC | 57 |
| | R: CCATTTCCGCGACTGATTT | | |
| Sit_24 | F: CGAAGGTCCTTTGAAGCAGA | ACT | 56 |
| | R: CGGAAGCATTACCCTCATCA | | |
| Sit_26 | F: AGCAGCATTGATCGGATTGT | AACCAG | 56 |
| | R: GTGCATCACCGGGATTTAGT | | |
| Sit_34 | F: AACCAAGGCCAAGACCAAGT | ACAT | 55 |
| | R: GATGGCGTTCGACTTCTTTG | | |
| Sit_40 | F:GAAACACCTAGTTAACAATAGAGACCG | ACCGAG | 56 |
| | R: GCAACTTTCTAGAGCAATTCGT | | |
| Sit_42 | F: CCCTCTTGAATGGATGGATG | AAAG | 57 |
| | R: CAAGGAAGGAGGATGGGATT | | |
| Sit_47 | F: AAATCCGAGGACATCCGTCT | AAACC | 58 |
| | R: TCACACGCTAGTTCGATAGAGG | | |
| Sit_33 | F: GAGAGGAGTGTGGACATGGG | ACCGTC | 57 |
| | R: GCACGCCAATGACACATAAA | | |
| ***T. castaneum*** | | | |
| Tca. 9.8 | F: GTAAAACGACGGCCAGTCTCGGAAAATCCTGACCAT | CTTTC | 55 |
| | R: ACCTTCACCACAAAGTGCAA | | |
| Tca. 2.7 | F: GTAAAACGACGGCCAGCTTACGACGGCTGTGAAACA | TAT | 54 |
| | R: GCCCTCACAGGGATGAAATA | | |
| Tca. 3.1 | F: GTAAAACGACGGCCAGCCAGTTTAATTTTCCACAATCTCC | ATTT | 58 |
| | R: AGTCAACTTTAATTATGTTTTCCGATA | | |
| Tca. 3.11 | F: GTAAAACGACGGCCAGCCTCCTGAAAGGACACAGGA | CAT | 58 |
| | R: GGTGCAACTCGCTTCTTCAT | | |
| Tca. 5.11 | F: GTAAAACGACGGCCAGAAACCACGGCAGTTCTTTCA | ATTT | 56 |
| | R: GTTTCTTAAACAGCAACACCGAAGACA | | |
| Tca. 7.4 | F: GTAAAACGACGGCCAGCGTCTGTCTGGCTGTTTTGA | CAC | 55 |
| | R: CACAATTTAACACTTGGCACGTA | | |
| Tca. 7.9 | F: GTAAAACGACGGCCAGTGACTCAGGGTCCAGTGAAA | TTTTA | 58 |
| | R: GCTCCGTTATTTTTCCGGTTA | | |
| Tca. 7.23 | F: GTAAAACGACGGCCAGGCCCAGTTTTGTCTCAAGCA | AATA | 56 |
| | R: ACAAATAGAAACGCCCATGC | | |
| Tca. 8.6 | F: GTAAAACGACGGCCAGTCCTGGACACAATCTCCCTAA | GGTCA | 55 |
| | R: GCGTGGGTCGGATAGATATG | | |

From the banding patterns of SSR amplification, the fragment lengths of each allele were scored manually using Alpha Ease FC™ software. GenAlex version 6.5 was used to calculate the Analysis of Molecular Variance (AMOVA), genetic distance, and other diversity parameters including the number of alleles (Na) and the number of effective alleles (Ne), Observed Heterozygosity (Ho), and Expected Heterozygosity (He) (Peakall and Smouse 2006). Free NA was used to calculate global *Fst* values using ENA correction (Chapuis and Estoup 2007). Population structure was determined with Principal Component Analysis using the facto extra package in R (Kassambara & Mundt 2017).

In addition, an individual-based Bayesian clustering algorithm, STRUCTURE version 2.3.3 (Pritchard *et al.*, 2000) was used to investigate the population structuring and gene flow. The analysis was carried out under the non-admixture model with alleles correlated. Twenty replicates for each value from K=1 to K=5 were conducted, with an initial burn-in of 100,000 iterations followed by 1,000,000 iterations. The most likely value of K was determined using the web server of STRUCTURE HARVESTER. The clustering results of STRUCTURE were visualized over CLUMPAK (Clustering Markov Packager across K) (http://clumpak.tau.ac.il/index.html), a web server that provides a full pipeline for clustering, summarizing, and visualizing STRUCTURE results.

## RESULTS AND DISCUSSION

Genetic diversity using mitochondrial and microsatellite markers in *S. oryzae* and *T. castaneum* populations collected from two grain supply chains of Tamil Nadu are presented in this study. Mitochondrial COI gene, a neutral genetic marker was used to draw information on the genetic diversity and the movement among the populations. In addition, microsatellite markers were used for population genetic studies because of their ability to detect multiple alleles (Choudhary *et al.*, 2009). *S. oryzae* and *T. castaneum* individuals were genotyped using 11 and 9 microsatellite markers respectively to evaluate the genetic diversity and differentiation among these populations.

*A. Genetic diversity analyses using mitochondrial COI in S. oryzae and T. castaneum populations*

The 623 and 654 bp COI sequences from 33 *S. oryzae* and *T. castaneum* populations were respectively obtained. NCBI-BLAST analysis of these sequences revealed the respective *S. oryzae* and *T. castaneum* populations (E-value=0.0; percentage identity ranging from 99 to 100 percent. Sequencing of *S. oryzae* and *T. castaneum* populations from two grain supply chains revealed 8 and 9 haplotypes respectively. High haplotype and low nucleotide diversity were observed in these sequences. The haplotype and nucleotide diversity in *S. oryzae* was 0.472 ± 0.01 and 0.04 ±0.00 respectively. Whereas, in *T. castaneum*, the haplotype and nucleotide diversity were 0.684 ± 0.00 and 0.09 ±0.00 respectively. The overall average nucleotide differences between the two sequences (K) in *S. oryzae* was 3.12 and *T. castaneum* was 4.27 (Table 3).

**Table 3: Summary statistics for mt-COI.**

| | *S. oryzae* | *T. castaneum* |
|---|---|---|
| Sample Size | 33 | 33 |
| Sequence length | 623 | 654 |
| Parsimony informative site | 7 | 6 |
| Haplotype diversity (d) | 0.472 ± 0.01 | 0.684 ± 0.00 |
| Nucleotide Diversity (π) | 0.04 ±0.00 | 0.09 ±0.00 |
| Number of Alleles/Haplotypes | 8 | 9 |
| Tajima's D | -2.878 (P < 0.01) | -1.12 (P<0.01) |
| Fu's | -5.84 (P<0.02) | -1.95 (P< 0.02) |
| Average number of pairwise differences (k) | 3.12 | 4.27 |

High haplotype diversity in combination with low nucleotide diversity has been associated with population expansion (Grant and Bowen, 1998). In addition, the recent spread of endosymbionts or the emergence of new strains from the existing endosymbionts may be the reason for low genetic diversity within the population (Hurst and Jiggins, 2005). No ambiguous site or stop codon was detected which ensured that these sequences were not nuclear pseudogenes. The distribution of haplotypes among *S. oryzae* and *T. castaneum* is mentioned in Table 4. The overall mean distance between *S. oryzae* and *T. castaneum* was 0.36 ± 0.006. The neutrality test revealed the demographic history of these populations. Tajima's D and Fu's F values were negative and statistically significant suggesting the occurrence of population expansion. AMOVA results of *S. oryzae* populations showed that 98.93 percent of the variation was observed within the populations and 1.07 percent variation was observed among the populations. The molecular variances in *S. oryzae* suggested that this species has a weak and unstable regional genetic structure (Cheng *et al.*, 2011). Whereas in *T. castaneum*, 97.66 percent variation was observed among the populations and 2.34 percent variation was observed within the populations (Table 5a & b). The high flight activity along with the anthropogenic movement during transport might have contributed to the genetic diversification among the populations. The overall $F_{ST}$ was low for *S. oryzae* (0.01; P< 0.05) and high for *T. castaneum* populations (0.97; P<0.05). Also, the gene flow was high in *S. oryzae* populations (Nm=2.62) and lowin *T. castaneum* populations (Nm=0.70).When Nm <1, genetic drift becomes the dominant force causing genetic differences among populations, according to gene flow studies. When Nm >1, gene flow between populations is sufficient to overcome the effects of genetic drift, preventing genetic differentiation (Xu*et al.*, 2019a). The genetic diversity of *S. oryzae* and *T. castaneum* populations was less which probably might be due to the closer geographical distance. This also indicated that the *S. oryzae* and *T. castaneum* are likely to spread to adjacent locations. In addition, based on the genetic differentiation and gene flow between the populations, increased transportation would have facilitated the dispersal of the insect populations. Low genetic differentiation might increase the species' adaptability to environmental change (Xu *et al.*, 2019b).

**Table 4: Distribution of COI haplotypes in *S. oryzae* and *T. castaneum* populations.**

| Haplotypes | Nodes of grain supply chain |
|---|---|
| ***S. oryzae*** | |
| Hap 1 | TRY_WS3 |
| Hap 2 | TRY_WS2, TRY_WS1, TRY_RS3, TRY_RS2, TRY_RS1, TRY_RM2, TRY_RM1, TRY_PDS3, TRY_PDS2, TRY_PDS1, TRY_HH3, TRY_HH2, TRY_HH1, TRY_CWC3, TRY_CWC2, TRY_CWC1, TH_WS3, TH_WS2, TH_WS1, TH_RM3, TH_RM2, TH_PDS2, TH_PDS1, TH_HH1] |
| Hap 3 | TRY_RM3 |
| Hap 4 | TH_RS3 |
| Hap 5 | TH_RS2, TH_RS1, TH_RM1 |
| Hap 6 | TH_PDS3 |
| Hap 7 | TH_HH3 |
| Hap 8 | TH_HH2 |
| ***T. castaneum*** | |
| Hap 1 | TH_HH1 |
| Hap 2 | TH_HH2 |
| Hap 3 | TH_HH3 |
| Hap 4 | TH_PDS1, TH_PDS2, TH_PDS3, TH_RM1, TH_RM2 |
| Hap 5 | TH_RM3, TH_WS1, TH_WS2 |
| Hap 6 | TH_RS1, TH_RS2, TH_RS3, TH_WS3, TRY_CWC3, TRY_HH1, TRY_HH2, TRY_HH3, TRY_PDS1, TRY_PDS2, TRY_RM1, TRY_RM2, TRY_RM3, TRY_RS1,TRY_RS3, TRY_WS1, TRY_WS2, TRY_WS3 |
| Hap 7 | TRY_CWC1, TRY_CWC2 |
| Hap 8 | TRY_PDS3 |
| Hap 9 | TRY_RS2 |

**Table 5a: AMOVA results for each level of variation evaluated among the populations of *S. oryzae.***

| Source of variation | d.f. | Sum of Squares | Variance components | Percentage Variation |
|---|---|---|---|---|
| Among populations | 10 | 15.81 | 0.26 | 25.14 |
| Within populations | 22 | 17.33 | 0.78 | 74.86 |
| Total | 32 | 33.15 | 1.05 | |

**Table 5b: AMOVA results for each level of variation among the populations of *T. castaneum*.**

| Source of variation | d.f. | Sum of Squares | Variance components | Percentage Variation |
|---|---|---|---|---|
| Among populations | 10 | 954.15 | 31.55 | 97.66 |
| Within populations | 22 | 16.67 | 0.75 | 2.34 |
| Total | 32 | 970.81 | 32.31 | |

*B. Genetic diversity characterized with microsatellite markers in S. oryzae and T. castaneum populations*

The genetic diversity and differentiation among the *S. oryzae* and *T. castaneum* populations are tabulated (Table 6). This showed a total of 127 alleles with an average of 11.39 alleles per locus. The effective number of alleles ranged from 7.47 to 10.89 with a mean of 8.58. Whereas in *T. castaneum*, a total of 201 alleles with an average of 18.35 alleles per locus. The effective number of alleles ranged from 10.14 to 14.16 with an average of 12.62 effective alleles per locus. The frequency of null alleles was (<0.15) at all loci except Sit_02which might be due to the differential amplification of size-variant alleles (Wattier *et al.*, 1998).

These results indicated that these markers are effective in differentiating among the *S. oryzae* and *T. castaneum* populations collected from different locations. The expected and observed number of heterozygosity ranged from 0.83 to 0.88 and 0.02 to 0.05 respectively in *S. oryzae*. Whereas in *T. castaneum*, the expected and observed heterozygosity ranged from 0.84 to 0.92 and 0.85 to 0.88 respectively. The mean observed heterozygosity of all loci was much lower than the expected heterozygosity indicating an elevated level of inbreeding (mean *Fs*=0.94). However, these results are found to bein contrast with the previous reports where geographically distinct populations were studied (Thangaraj *et al.*, 2016). In addition, the observed heterozygosity was similar to the expected heterozygosity in all the clusters suggesting that there is no evidence for recent population bottlenecks in *T. castaneum* populations.

**Table 6: Genetic diversity parameters generated by SSR markers in *S. oryzae* and *T. castaneum* populations.**

| Marker | Na | Ne | He | Ho |
|---|---|---|---|---|
| ***S. oryzae*** | | | | |
| TH_HH | 12.54±1.86 | 09.45 ± 1.61 | 0.83 ± 0.04 | 0.05 ± 0.04 |
| TH_RM | 13.54 ± 1.89 | 10.89 ± 1.61 | 0.87 ± 0.02 | 0.05 ± 0.03 |
| TH_RS | 11.00 ± 1.24 | 07.65 ± 1.03 | 0.83 ± 0.02 | 0.04 ± 0.02 |
| TH_PDS | 11.45 ± 1.44 | 08.67 ± 1.38 | 0.85 ± 0.02 | 0.02 ± 0.01 |
| TH_WS | 09.63 ± 1.23 | 07.47 ± 1.08 | 0.84 ± 0.02 | 0.42 ± 0.04 |
| TRY_CWC | 10.54 ± 0.88 | 07.49 ± 0.74 | 0.85 ± 0.01 | 0.04 ± 0.03 |
| TRY_HH | 10.45 ± 1.67 | 07.61 ± 1.56 | 0.81 ± 0.03 | 0.03 ± 0.03 |
| TRY_RM | 10.63 ± 1.70 | 07.96 ± 1.32 | 0.81 ± 0.03 | 0.02 ± 0.02 |
| TRY_RS | 12.18 ± 1.53 | 09.60 ± 1.33 | 0.87 ± 0.01 | 0.03 ± 0.03 |
| TRY_PDS | 13.00 ± 1.11 | 09.87 ± 1.05 | 0.88 ± 0.01 | 0.02 ± 0.02 |
| TRY_WS | 10.36 ± 1.53 | 07.75 ± 1.21 | 0.83 ± 0.02 | 0.03 ± 0.03 |
| Mean | 11.39 ±0.44 | 08.58 ± 0.38 | 0.84 ± 0.00 | 0.04 ± 0.00 |
| ***T. castaneum*** | | | | |
| TH_HH | 18.77 ± 2.33 | 12.35 ± 2.06 | 0.84 ± 0.70 | 0.88 ± 0.11 |
| TH_RM | 18.66 ± 2.50 | 12.39 ± 1.63 | 0.90 ± 0.02 | 0.87 ± 0.10 |
| TH_RS | 19.11 ± 2.59 | 13.54 ± 2.06 | 0.90 ± 0.02 | 0.88 ± 0.11 |
| TH_PDS | 17.55 ± 1.87 | 12.54 ± 1.55 | 0.90 ± 0.02 | 0.88 ± 0.11 |
| TH_WS | 20.11 ± 1.89 | 14.16 ± 2.00 | 0.91 ± 0.01 | 0.87 ± 0.11 |
| TRY_CWC | 20.11 ± 1.95 | 14.06 ± 1.34 | 0.92 ± 0.01 | 0.87 ± 0.11 |
| TRY_HH | 20.44 ± .67 | 13.82 ± 1.31 | 0.92 ± 0.01 | 0.87 ± 0.11 |
| TRY_RM | 15.44 ± 1.76 | 10.14 ± 1.14 | 0.88 ± 0.02 | 0.86 ± 0.10 |
| TRY_RS | 16.44 ± 2.28 | 11.26 ± 1.82 | 0.89 ± 0.01 | 0.85 ± 0.10 |
| TRY_PDS | 19.55 ± 1.52 | 13.86 ± 1.12 | 0.92 ± 0.00 | 0.87 ± 0.11 |
| TRY_WS | 15.66 ± 2.52 | 10.67 ± 1.89 | 0.88 ± 0.02 | 0.86 ± 0.10 |
| Mean | 18.35 ± 0.62 | 12.62 ± 0.49 | 0.89 ± 0.00 | 0.87 ± 0.03 |

These differences in the two insect species revealed the presence of potential genetic differences. The genetic differentiation among the populations was estimated using fixation index and the gene flow at each locus was calculated. Global *Fst* values for *S. oryzae* and *T. castaneum* populations were 0.037 and 0.075 respectively. Pairwise *Fst* values ranged from 0.00 to 0.125 in *S. oryzae* and 0.00 to 0.10 in *T. castaneum* respectively and were found to be significant (Table 7a, 8a). The low level of genetic differentiation might be due to more closely related populations. However, the estimation of gene flow (Nm) was relatively high (Nm > 1) (Table 7b, 8b). It was reported that the migration of *S. oryzae* was relatively low in comparison with *S. zeamais* (Vásquez-Castro *et al.,* 2009). Despite its limited flight activity, anthropogenic movement during the transportation of food grains appeared to be the most likely mode of movement on a large scale. Due to insufficient rice production, Tamil Nadu procured rice from surplus states such as Andhra Pradesh, Chhattisgarh and Orissa on monthly basis (https://fci.gov.in/movements.php). This might be a probable reason for the migration of these insects. Alternatively, *T. castaneum* has a high active (flight) or passive (anthropogenic dispersal capacity (Daglish*et al.,* 2017; Rafter *et al.,* 2019; McCulloch *et al.,* 2021) which might be the possible reason for the high gene flow among these populations. The high level of gene flow characterized by active and passive flight movement suggested a threat in the spread of phosphine-resistant alleles across the grain supply chains. In contrast, the previous studies reported that the increased phosphine resistance reduced the flight activity in these insect pests (Malekpour *et al.,* 2016).

**Table 7a: Estimation of pairwise fixation index ($F_{ST}$) between the populations of *S. oryzae* from two grain supply chains.**

| TH_HH | TH_PDS | TH_RM | TH_RS | TH_WS | TRY_CWC | TRY_HH | TRY_PDS | TRY_RM | TRY_RS | TRY_WS | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.000 | | | | | | | | | | | TH_HH |
| 0.128 | 0.000 | | | | | | | | | | TH_PDS |
| 0.109 | 0.092 | 0.000 | | | | | | | | | TH_RM |
| 0.118 | 0.116 | 0.101 | 0.000 | | | | | | | | TH_RS |
| 0.130 | 0.100 | 0.106 | 0.115 | 0.000 | | | | | | | TH_WS |
| 0.118 | 0.099 | 0.088 | 0.105 | 0.105 | 0.000 | | | | | | TRY_CWC |
| 0.147 | 0.133 | 0.115 | 0.121 | 0.140 | 0.097 | 0.000 | | | | | TRY_HH |
| 0.098 | 0.099 | 0.084 | 0.094 | 0.104 | 0.092 | 0.105 | 0.000 | | | | TRY_PDS |
| 0.124 | 0.129 | 0.104 | 0.111 | 0.134 | 0.111 | 0.122 | 0.103 | 0.000 | | | TRY_RM |
| 0.107 | 0.103 | 0.087 | 0.106 | 0.091 | 0.089 | 0.114 | 0.077 | 0.101 | 0.000 | | TRY_RS |
| 0.120 | 0.109 | 0.099 | 0.118 | 0.119 | 0.097 | 0.120 | 0.100 | 0.123 | 0.091 | 0.000 | TRY_WS |

**Table 7b: Estimation of pairwise gene flow (Nm) between the populations of *S. oryzae* from two grain supply chains.**

| TH_HH | TH_PDS | TH_RM | TH_RS | TH_WS | TRY_CWC | TRY_HH | TRY_PDS | TRY_RM | TRY_RS | TRY_WS | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.000 | | | | | | | | | | | TH_HH |
| 1.707 | 0.000 | | | | | | | | | | TH_PDS |
| 2.038 | 2.457 | 0.000 | | | | | | | | | TH_RM |
| 1.877 | 1.901 | 2.223 | 0.000 | | | | | | | | TH_RS |
| 1.668 | 2.238 | 2.114 | 1.915 | 0.000 | | | | | | | TH_WS |
| 1.873 | 2.270 | 2.591 | 2.126 | 2.141 | 0.000 | | | | | | TRY_CWC |
| 1.447 | 1.628 | 1.931 | 1.808 | 1.541 | 2.316 | 0.000 | | | | | TRY_HH |
| 2.308 | 2.280 | 2.735 | 2.416 | 2.157 | 2.460 | 2.133 | 0.000 | | | | TRY_PDS |
| 1.759 | 1.686 | 2.159 | 2.006 | 1.615 | 2.009 | 1.807 | 2.189 | 0.000 | | | TRY_RM |
| 2.085 | 2.180 | 2.624 | 2.114 | 2.493 | 2.561 | 1.950 | 3.016 | 2.228 | 0.000 | | TRY_RS |
| 1.834 | 2.034 | 2.266 | 1.868 | 1.855 | 2.315 | 1.831 | 2.247 | 1.781 | 2.484 | 0.000 | TRY_WS |

**Table 8a. Estimation of pairwise fixation index ($F_{ST}$) between the populations of *T. castaneum* from two grain supply chains.**

| TH_HH | TH_PDS | TH_RM | TH_RS | TH_WS | TRY_CWC | TRY_HH | TRY_PDS | TRY_RM | TRY_RS | TRY_WS | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.000 | | | | | | | | | | | TH_HH |
| 0.101 | 0.000 | | | | | | | | | | TH_PDS |
| 0.097 | 0.067 | 0.000 | | | | | | | | | TH_RM |
| 0.103 | 0.067 | 0.070 | 0.000 | | | | | | | | TH_RS |
| 0.079 | 0.063 | 0.053 | 0.063 | 0.000 | | | | | | | TH_WS |
| 0.074 | 0.060 | 0.052 | 0.061 | 0.050 | 0.000 | | | | | | TRY_CWC |
| 0.075 | 0.048 | 0.055 | 0.057 | 0.042 | 0.039 | 0.000 | | | | | TRY_HH |
| 0.082 | 0.040 | 0.058 | 0.060 | 0.048 | 0.050 | 0.031 | 0.000 | | | | TRY_PDS |
| 0.107 | 0.074 | 0.057 | 0.080 | 0.068 | 0.061 | 0.049 | 0.071 | 0.000 | | | TRY_RM |
| 0.108 | 0.064 | 0.062 | 0.057 | 0.063 | 0.061 | 0.060 | 0.063 | 0.056 | 0.000 | | TRY_RS |
| 0.108 | 0.071 | 0.071 | 0.065 | 0.046 | 0.070 | 0.067 | 0.065 | 0.086 | 0.043 | 0.000 | TRY_WS |

**Table 8b: Estimation of pairwise gene flow (Nm) between the populations of *T. castaneum* from two grain supply chains.**

| TH_HH | TH_PDS | TH_RM | TH_RS | TH_WS | TRY_CWC | TRY_HH | TRY_PDS | TRY_RM | TRY_RS | TRY_WS | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.000 | | | | | | | | | | | TH_HH |
| 2.229 | 0.000 | | | | | | | | | | TH_PDS |
| 2.326 | 3.458 | 0.000 | | | | | | | | | TH_RM |
| 2.172 | 3.487 | 3.339 | 0.000 | | | | | | | | TH_RS |
| 2.926 | 3.731 | 4.429 | 3.694 | 0.000 | | | | | | | TH_WS |
| 3.148 | 3.928 | 4.564 | 3.880 | 4.783 | 0.000 | | | | | | TRY_CWC |
| 3.068 | 4.917 | 4.324 | 4.101 | 5.692 | 6.180 | 0.000 | | | | | TRY_HH |
| 2.800 | 6.006 | 4.055 | 3.937 | 4.951 | 4.704 | 7.933 | 0.000 | | | | TRY_PDS |
| 2.078 | 3.145 | 4.164 | 2.890 | 3.444 | 3.834 | 4.838 | 3.282 | 0.000 | | | TRY_RM |
| 2.075 | 3.639 | 3.772 | 4.103 | 3.707 | 3.842 | 3.940 | 3.692 | 4.209 | 0.000 | | TRY_RS |
| 2.060 | 3.265 | 3.270 | 3.583 | 5.198 | 3.320 | 3.502 | 3.576 | 2.645 | 5.532 | 0.000 | TRY_WS |

The PCA analysis for *S. oryzae* showed that PC1 had 37.1% variance and PC2 had 27.3% variance and suggested that there was no significant genetic structure in *S. oryzae* populations. Similarly, PCA analysis for *T. castaneum* showed that PC1 had a variance of 34.5% whereas PC2 had a variance of 22.2%. The analysis of principal components showed that there was no significant genetic structure in *T. castaneum* populations (Fig. 1a & b). Significant overlap was found between the populations.

**Fig. 1a.** Principal Component Analysis (PCA) showing overlapping clusters among the *S. oryzae* populations.

**Fig. 1b.** Principal Component Analysis (PCA) among the *T. castaneum* populations.

Several approaches have been used for interpreting the structure data that often appear to be conservative (Earl & von-Holdt, 2012) or liberal (Falush *et al.*, 2003) in assigning the number of genetic clusters. In this study, Earl & von-Holdt's (2012) method indicated K=2 (Fig. 2a & b) showing admixed populations in both *S. oryzae* and *T. castaneum*. The lnP(K) value was -19887.46 and -16125.63 for *S. oryzae* and *T. castaneum* respectively and recorded as a maximum at K=2. The less distinctive genetic structure indicated the occurrence of the high gene flow between the populations (Redlarski *et al.*, 2021).

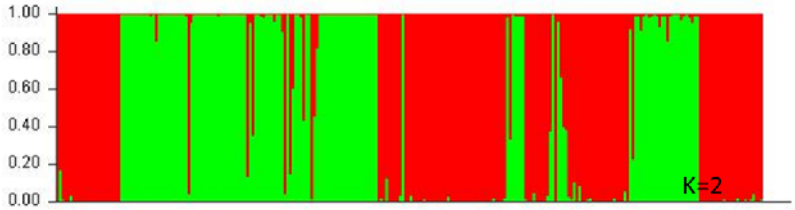


**Fig. 2a.** Population structure of *S. oryzae* populations from two grain supply chains.

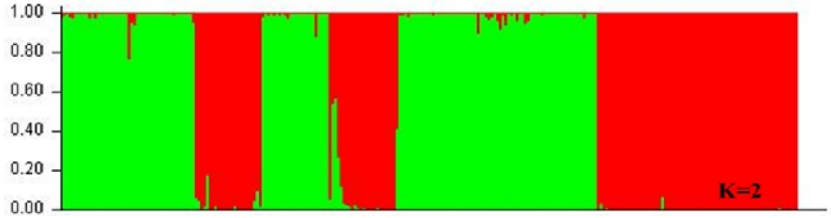


**Fig. 2b.** Population structure of *T. castaneum* populations from two grain supply chains.

## CONCLUSION

Both mitochondrial and microsatellite markers used for the characterization of genetic diversity revealed that there is gene flow between these populations and there is a potential risk in the spread of phosphine resistant alleles. The migration of these insect pests due to transportation and flight activity are the major concerns to be resolved. Also, broad-scale species-specific management measures are required for phosphine resistance management of these key stored grain insect pests.

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