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Unravelling the genetic diversity and population structure of *Sitophilus oryzae* L. in North India using mitochondrial *COI* sequences

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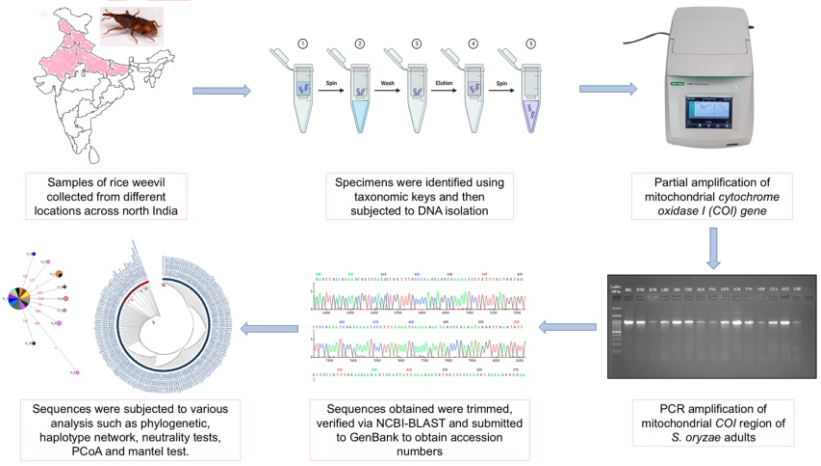
Abstract

Aim: *Sitophilus oryzae* (Linnaeus) (Coleoptera: Curculionidae) is a notorious pest inflicting substantial economic damage to stored grains worldwide. Population genetic studies offer valuable insights into insects' evolutionary and adaptive strategies and demonstrate how environmental factors influence genetic diversity and gene flow among populations.Therefore, the present study was undertaken to study the genetic diversity of *S. oryzae* populations from different geographical regions of North India.



Methodology: In this study, a total of 150 samples of *S. oryzae* were collected from 15 different locations, representing diverse agro-climatic zones of India. The samples were then identified by utilizing taxonomic keys. Thereafter, the specimens were subjected to PCR amplification of mitochondrial cytochrome c oxidase I (*COI*) gene to study their genetic variation and population structure.

Results: The results revealed low values of haplotype diversity (0.178) and nucleotide diversity (0.00032). Significant negative values of neutrality tests suggested a recent demographic expansion of the population. Mantel's test revealed a weak correlation between genetic differentiation and geographical distance. Haplotype network analysis revealed a single major clade, representing the major population assumed to be the ancestral population. Moreover, low-frequency haplotypes with one or two populations suggested ongoing population divergence, possibly influenced by phosphine resistance mechanisms. The phylogenetic tree supported the genetic homogeneity of the population since there was no clustering of the population based on geographical area.

Interpretation: Based on the *COI* markers, it can be concluded that North Indian populations of *S. oryzae* exhibit low genetic diversity, which has implications for pest management strategies primarily involving the use of fumigants.

Key words: *COI*, Genetic homogeneity, Gene flow, Phylogeny, *Sitophilus oryzae*

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Introduction

The global population is projected to reach 9.1 billion by the year 2050, necessitating an approximate 70% increase in the food production to meet future demands (FAO, 2011). Addressing the escalating food requirements driven by this expanding global population presents a substantial challenge. This mandates a dual approach: simultaneously enhancing food production and significantly curtailing post-harvest food losses. Annually, post-harvest operations account for the loss of one-third of global food production, equating to approximately 1.3 billion tons with an estimated value of US $1 trillion (FAO, 2011). Recent UN population estimates highlight India's emergence as the world's most populous nation, forecasting continued growth in its food demand in the foreseeable future. Furthermore, India ranks as the second-largest global producer of food grains, behind only China (APEDA, 2023). Despite this, India confronts considerable difficulties in food grain storage, experiencing estimated annual losses ranging from 12 to 16 million metric tons, valued at approximately ₹7,000 crores, of which insect infestations alone contribute ₹1,300 crores to these losses (IGMRI, 2019).

Rice, serving as a primary dietary staple for a considerable segment of the Indian population, constitutes a significant portion of the country's overall food grain output (Anandhi *et al.,* 2022). With approximately 20% of global rice production attributed to India, its role in maintaining worldwide food security is undeniably critical (Saha *et al.,* 2021). Nevertheless, rice production faces impediments from diverse biotic and abiotic factors, with insect pests notably responsible for considerable annual yield reductions, occasionally reaching up to 30% (Kumar, 2017). Among the various biotic factors contributing to post-harvest grain deterioration, the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae), emerges as a particularly devastating insect pest impacting stored grains across the globe (Devi *et al.,* 2017; Khan *et al.,* 2023). While its origins trace back to India, the substantial increase in the international grain trade has recently enabled the extensive global dissemination of this formerly regionally distributed *S. oryzae,* consequently establishing its status as a cosmopolitan species (Choudhury and Chakraborty, 2014). This pest exhibits a polyphagous feeding habit, consuming a diverse array of grains, including but not limited to wheat, rice, barley, sorghum, oats, and maize (Hasan *et al.,* 2017; Subedi *et al.,* 2009). Functioning as a primary storage pest, adult weevils possess the capability to infest intact grain kernels, while their larvae develop and consume material internally within these kernels (Mason and McDonough, 2012).

The concealed feeding behavior of them renders early detection of these weevils challenging, which subsequently complicates effective control measures. In India, the documented extent of damage caused by this pest varies significantly, ranging between 11-69% (Banerjee and Nazimuddin, 1985; Sittisuang and Imura, 1987; Thakur and Sharma, 1996). Nevertheless, under conditions of prolonged and undisturbed grain storage, this specific pest has the potential to inflict damage exceeding 80% (Park *et al.,* 2004). Consequently, a wide spectrum of control strategies has been extensively implemented to mitigate these considerable losses and manage *S. oryzae* infestations effectively. In stored grain environments, the primary method for controlling *S. oryzae* populations typically involves the application of synthetic chemical insecticides and fumigants (Dent, 2000; Doan *et al.,* 2021). However, the frequent and often indiscriminate use of these synthetic insecticides has led to the emergence of insect resistance and pest resurgence issues (Huang and Subramanyam, 2005). Notably, the rice weevil has evolved resistance against a broad spectrum of over 35 insecticides and fumigants, encompassing phosphine, organophosphorus compounds and pyrethroid insecticides (Nguyen *et al.*, 2016).

Consequently, a thorough understanding of the genetic diversity and migratory patterns of insect populations is indispensable for developing and implementing effective strategies to combat insecticide resistance. Furthermore, comprehensive knowledge concerning biology, ecology, and the morphological, physiological, and genetic mechanisms underpinning resistance development in pest species is paramount. This understanding is particularly critical given that the widespread transportation of food grains facilitates the extensive distribution and genetic intermingling of *S. oryzae* populations, thereby exacerbating the complexities of resistance management efforts. Between the stages of harvest and ultimate consumption, grains undergo storage in warehouses or comparable facilities, and are subsequently transported across diverse regions domestically or internationally as demanded. This anthropogenic movement of food grains represents the primary mechanism facilitating the geographical dispersal of this pest. Such transportation leads to the intermingling of these introduced insects with indigenous populations or species, potentially giving rise to populations characterized by distinct genetic structures and behavioral patterns. Furthermore, the genus *Sitophilus* comprises certain species that present challenges in morphological differentiation.

Reports have additionally indicated that *S. oryzae* populations observed in both India and China demonstrate variable responses to several classes of insecticides (Bansode and Bhattia, 1990; Joia and Kumar, 1996; Nguyen *et al.*, 2016; Rajan *et al.,* 2017; Singh *et al.,* 2007; Visalakshi *et al.,* 2005). The capacity of a population to flourish in heterogeneous environments is contingent upon the extent of genetic variability inherent within it (Sambathkumar *et al.,* 2017). Consequently, it is plausible that *S. oryzae* populations residing in various agro-climatic zones across India also exhibit genetic diversity. Population genetics offer invaluable insights into the spatial structure and movement dynamics of insect pests over both short and long distances (Kim and Sappington, 2013). To elucidate these complexities and acquire a more profound understanding of genetic composition and dispersal of *S. oryzae* populations, molecular approaches have emerged as indispensable methodological tools. Molecular approaches represent valuable tools for investigating inter-population genetic differences, owing

to their practical, rapid, and precise methodologies (Sun *et al.,* 2011). Among these, the mitochondrial cytochrome oxidase subunit I (*COI*) gene is recognized as a premier tool in insect molecular systematics. This is attributed to its rapid evolutionary rate, maternal inheritance, high nucleotide substitution rate, and ease of amplification, making it ideal for assessing intraspecific diversity and distinguishing cryptic species (Mandal *et al.,* 2014; Nachimuthu *et al.,* 2015; Shashank *et al.,* 2018). Prior research has indicated limited mitochondrial diversity within *S. oryzae* populations both in India and on a global scale. Notwithstanding these existing findings, a thorough comprehension of intraspecific genetic variation and differentiation of *S. oryzae* has been notably absent in key regions of North India.

Therefore, to understand population genetics and provide new insights for controlling this important pest, *S. oryzae* samples were collected from 15 geographical locations covering the five agro-climatic zones of India and examined for their population genetic structure on the basis of concatenated sequences of the mitochondrial *COI* gene.

Materials and Methods

Sample collection: Sampling of *S. oryzae* populations was carried out between February 2021 and March 2022 from two hosts, wheat and rice, from 15 different geographical locations across northern parts of India falling under five agro-climatic zones (Fig. 1; Table 1). Identification of *S. oryzae* samples was carried out through morphological analysis, employing taxonomic keys and examining their genitalia via dissection (Halstead, 1963). Each insect specimen was then preserved in 95% ethanol and stored at -20 °C for subsequent DNA isolation. The geographical coordinates of the collection sites were documented using a GPS device.

DNA isolation, amplification and sequencing of *COI* gene fragment: DNA extraction was performed separately from ten individuals of adult *S. oryzae* from each location using the Qiagen DNeasy® Blood & Tissue Kit following the manufacturer's protocol. The quality and quantity of the extracted DNA samples were evaluated using a 0.8% agarose gel and a NanoDrop

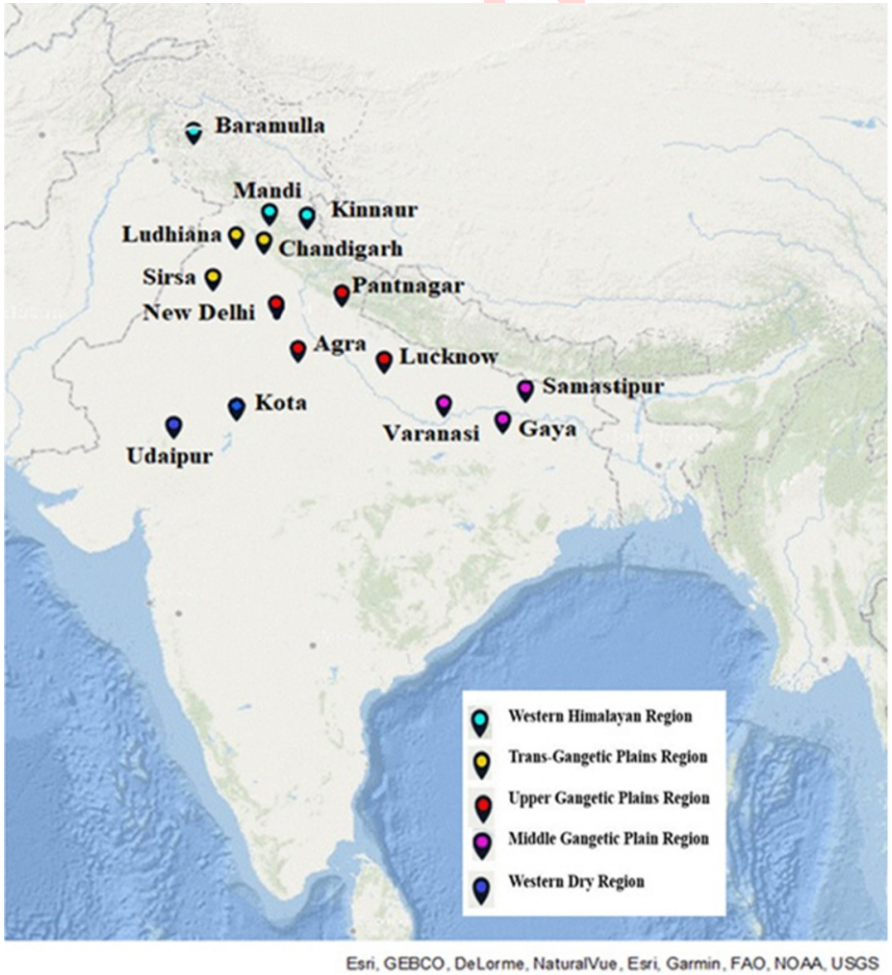

Fig. 1: Sampling locations of *S. oryzae* populations across India.

Table 1: Details of the *S. oryzae* populations sampled from different agro-climatic zones of North India

Agro-climatic zone	Location (State)	Location Code	Latitude (°N)	Longitude (°E)	Host (Grains)	Sampling Period	Accession Number
WHR	Baramulla (Jammu & Kashmir)	BRL	34°12' N	74°20' E	Wheat	May, 2022	OP618395- OP618404
(n=30)	Mandi (Himachal Pradesh)	MND	31°73' N	76°93' E	Rice	Oct., 2020	OP618405- OP618414
	Kinnaur (Himachal Pradesh)	KNR	31°52' N	78°24' E	Wheat	Feb., 2022	Op618415- Op618424
TGPR	Ludhiana (Punjab)	LDH	30°54' N	75°48' E	Rice	April, 2021	OP618425- OP618434
(n=30)	Sirsa (Haryana)	SRS	29°53' N	75°03' E	Wheat	June, 2021	OP618435- OP618444
	Chandigarh	CHD	30°73' N	76°78' E	Wheat	Mar., 2022	OP618445- OP618454
UGPR	New Delhi	DLH	28°61' N	77°21' E	Wheat	June, 2021	OP618455- OP618464
(n=40)	Pantnagar (Uttarakhand)	PNG	29°02' N	79°49' E	Rice	Feb., 2022	OP618465- OP618474
	Lucknow (Uttar Pradesh)	LKW	26°85' N	80°95' E	Wheat	April, 2022	OP618475- OP618484
	Agra (Uttar Pradesh)	AGR	27°18' N	78°01' E	Wheat	Oct., 2021	OP618485- OP618494
MGPR	Varanasi (Uttar Pradesh)	VNS	25°15' N	82°59' E	Rice	Sept., 2020	OP618495- OP618504
(n=30)	Samastipur (Bihar)	SMP	25°86' N	85°79' E	Wheat	Mar., 2021	OP618505- OP618514
	Gaya (Bihar)	GYA	24°79' N	85°00' E	Rice	June, 2021	OP618515- OP618524
WDR	Kota (Rajasthan)	KOT	25°07' N	75°56' E	Wheat	June, 2021	OP618525- OP618534
(n=20)	Udaipur (Rajasthan)	UDP	24°34' N	73°42' E	Wheat	April, 2022	OP618535- OP618544

n, number of individuals; WHR, Western Himalayan Region; TGPR, Trans-Gangetic Plain Region; UGPR, Upper Gangetic Plain Region; MGPR, Middle Gangetic Plain Region; WDR, Western Dry Region

Spectrophotometer ND-1000 (Nano Drop products, Wilmington, DE, USA), respectively. Subsequently, partial amplification of 5' terminus *COI* gene was done using species-specific primers for *S. oryzae* (5'-AGTTTGCTAATTCGGGCAGA-3' and 5'-ACTCCGGTTAATCCTCCAAT-3') reported by Correa *et al.* (2013). The PCR reaction of 25 µl, composed of 2 µl of DNA template (100 ng), 2.5 µl of PCR buffer (10×), 1 µl of dNTP mix (2 mM), 0.2 µl of Taq polymerase (1U, GeNei™), 1µl of $MgCl_2$ (2 mM), 0.5 µl of 10 pmol of each primer (forward and reverse), and 17.3 µl of nuclease-free water. Thereafter, the polymerase chain reaction was performed in a Bio-Rad T100 thermal cycler (Bio-Rad, Hercules, CA, USA). The amplification process of PCR was done with an initial denaturation phase at 94°C for 4 min. This was succeeded by 34 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 52°C for 45 sec, and extension at 72°C for 1 min. A final extension step was then executed for 7 min at 72°C. After amplification, the amplicons were separated using 1% agarose gel electrophoresis, purified, and then subjected to Sanger sequencing with the forward primer on an automated DNA analyzer at M/s Eurofins Analytical Services India Pvt. Ltd., Bengaluru.

Sequence analysis: The *COI* sequences obtained in the study were aligned and trimmed using Clustal W with default parameters in MEGA 11 (Kumar *et al.,* 2018). The trimmed sequences were verified via NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and submitted to GenBank to obtain accession numbers (Table 1). The analysis was performed on the basis of 619 bp of the amplified region across different populations. The DnaSP software v5.10.1 (Librado and Rozas, 2009) was utilized to infer various diversity indices including the number of haplotypes, haplotype diversity, nucleotide diversity, number of segregating sites, mean number of pairwise differences, neutrality tests (Tajima's D, Fu's Fs, Fu and Li's F, Fu and Li's D), and mismatch distribution analysis (Rogers and Harpending, 1992). The Kimura 2-parameter distance model (Kimura, 1980) was employed to estimate the average sequence divergence of *S. oryzae* populations. The phylogenetic analysis was done by constructing a neighbour-joining (NJ) tree with 1000 bootstrap replicates utilizing MEGA 11. MEGA 11 was also used to determine the mean nucleotide base composition and the overall genetic distance of the sequences obtained. To understand the genetic structure, an analysis of molecular variance (AMOVA) and pairwise F_{ST} values were computed using Arlequin v3.5.2.2 (Excoffier and Lischer, 2010), and the level of significance was determined with 1000 permutation replicates. Principal coordinate analysis (PCoA) based on the genetic distance matrix and the Mantel test (Mantel, 1967) using pairwise geographical distance (Ln km) against pairwise linearized genetic distance among populations were performed in GenAlEx v 6.503, with 1000 random permutations (Peakall and Smouse, 2012). The haplotype network was constructed utilizing the median-joining algorithm in Network 5.1 (Bandelt *et al.,* 1999).

Results and Discussion

The study generated 150 *COI* gene sequences from *S. oryzae,* which exhibited high similarity (98-100% identity with 100% query coverage) to existing sequences in the NCBI public database via a BLAST search. The *COI* sequences showed high similarity in terms of nucleotide composition across all populations, with a relatively higher content of AT (64.95%) than that of GC (35.05%) (Table 2). The sequences included 10 polymorphic sites, with 7 variable singleton sites and 3 parsimony variable sites. Overall, *S. oryzae* showed 10 haplotypes, ranging from 1 to 5, that were sampled in different zones (Table 2). The

Table 2: Genetic diversity indices and neutrality tests based on *COI* gene sequences of *S. oryzae* populations across different agro-climatic zones of North India

Index	*S. oryzae* Population					
	WHR	**TGPR**	**UGPR**	**MGPR**	**WDR**	**Pooled**
N	30	30	40	30	20	150
S	3	1	5	-	1	10
H	4	2	5	1	2	10
Hd	0.251	0.239	0.236	-	0.100	0.178
K	0.262	0.239	0.297	-	0.100	0.199
Π	0.00042	0.00039	0.00048	-	0.00016	0.00032
Nucleotide Composition (%)						
A (%)	30.87	30.86	30.84	30.86	30.86	30.85
C (%)	19.70	19.71	19.71	19.71	19.70	19.71
G (%)	15.35	15.33	15.36	15.35	15.35	15.35
T (%)	34.08	34.11	34.09	34.09	34.09	34.09
A + T (%)	64.95	64.97	64.93	64.94	64.95	64.95
G + C (%)	35.05	35.04	35.07	35.06	35.05	35.05
Neutrality tests						
Tajima's D	-1.53889	-0.08237	-1.90075*	N/A	-1.16439	-2.15007*
Fu and Li's D	-1.47512	0.59448	-2.58871*	N/A	-1.53959	-3.88965*
Fu and Li's F	-1.72941	0.46901	-2.77678*	N/A	-1.64766	-3.89515*
Fu's Fs	-2.716	0.388	-3.748	N/A	-0.879	-14.413

n, number of sequences; H, number of haplotypes; Hd, haplotype diversity; Π, nucleotide diversity; k, average number of nucleotide differences (genetic differences) among haplotypes; S, number of polymorphic (segregating) sites; A, adenine; C, cytosine; G, guanine; T, thymine. *Significant at 0.01< p <0.005, while other values are non- significant (p > 0.10) for neutrality tests; Pooled, the combined set of populations from the five major agro-climatic zones of India; N/A, not applicable

Table 3: Analysis of Molecular Variance of the *COI* sequences of *S. oryzae* populations from different locations across North India

Source of Variation	Df	Sum of Squares	Variance Components	Percentage of Variance	Fixation Index
Among groups	4	0.778	0.00143 Va	1.43	F_{CT}: 0.01434
Among populations within groups	10	1.517	0.00591 Vb	5.91	F_{SC}: 0.05997
Within Populations	135	12.500	0.09259 Vc	92.66	F_{ST}: 0.07345
Total	149	14.793	0.09993		

Df, degrees of freedom; F_{CT}, fixation index between groups; F_{SC}, fixation index among populations within groups; F_{ST}, fixation index within populations

populations from the Western Himalayan Region displayed notably higher levels of haplotype (0.251) and nucleotide (0.00042) diversity. Contrary to this, the population of the Middle Gangetic Plain Region had only single haplotype. For the pooled population, the nucleotide and haplotype diversities were 0.00032 and 0.178, respectively.

Demographic population history was assessed independently using neutrality tests for all populations of *S. oryzae* (Table 2). Statistically insignificant and negative values of Tajima's D test in all the zones, except in the overall population and Upper Gangetic Plain Region indicate the presence of low-frequency polymorphisms. Additionally, a negative value of Fu's Fs (based on the distribution of haplotypes) in most populations provided evidence for a probable expansion of this pest species. Populations collected from the Western Himalayan Region and Western Dry Region displayed non-significant negative values for Fu's Fs, Fu and Li's F, and Fu and Li's D tests whereas significant negative values were observed from the Upper Gangetic Plain Region and overall populations. Additionally, the negative deviations from zero observed in all the populations suggested that an excess of rare mutations favoured population expansion or growth after a bottleneck or selective sweep. The population expansion was also shown by the unimodal mismatch distribution curve, suggesting a demographic growth in the past (Fig. 2).

The analysis of molecular variance (AMOVA) revealed relatively little differentiation among the groups (1.43%), followed by populations within groups (5.91%) and within populations (92.66%) (Table 3). The overall fixation index (F_{ST}) observed for all

Table 4: Pairwise fixation index amongst different populations of *S. oryzae* populations in North India based on mitochondrial *COI* gene sequences

Population code	BRL	MND	KNR	LDH	SRS	CHD	DLH	PNG	LKW	AGR	VNS	SMP	GYA	KOT	UDP
BRL	0.000														
MND	0.074	0.000													
KNR	0.056	0.000	0.000												
LDH	0.148	0.222	0.167	0.000											
SRS	0.056	0.000	0.000	0.020	0.000										
CHD	0.074	0.000	0.000	0.222	0.000	0.000									
DLH	0.056	0.044	0.037	0.111	0.037	0.044	0.000								
PNG	0.074	0.000	0.000	0.222	0.000	0.000	0.044	0.000							
LKW	0.056	0.000	0.000	0.167	0.000	0.000	0.037	0.000	0.000						
AGR	0.074	0.000	0.000	0.222	0.000	0.000	0.044	0.000	0.000	0.000					
VNS	0.074	0.000	0.000	0.222	0.000	0.000	0.044	0.000	0.000	0.000	0.000				
SMP	0.074	0.000	0.000	0.222	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000			
GYA	0.074	0.000	0.000	0.222	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000	0.000		
KOT	0.074	0.000	0.000	0.222	0.000	0.000	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
UDP	0.056	0.000	0.000	0.167	0.000	0.000	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

(BRL: Baramulla, MND: Mandi, KNR: Kinnaur, LDH: Ludhiana, SRS: Sirsa, CHD: Chandigarh, DLH: New Delhi, PNG: Pantnagar, LKW: Lucknow, AGR: Agra, VNS: Varanasi, SMP: Samastipur, GYA: Gaya, KOT: Kota, UDP: Udaipur

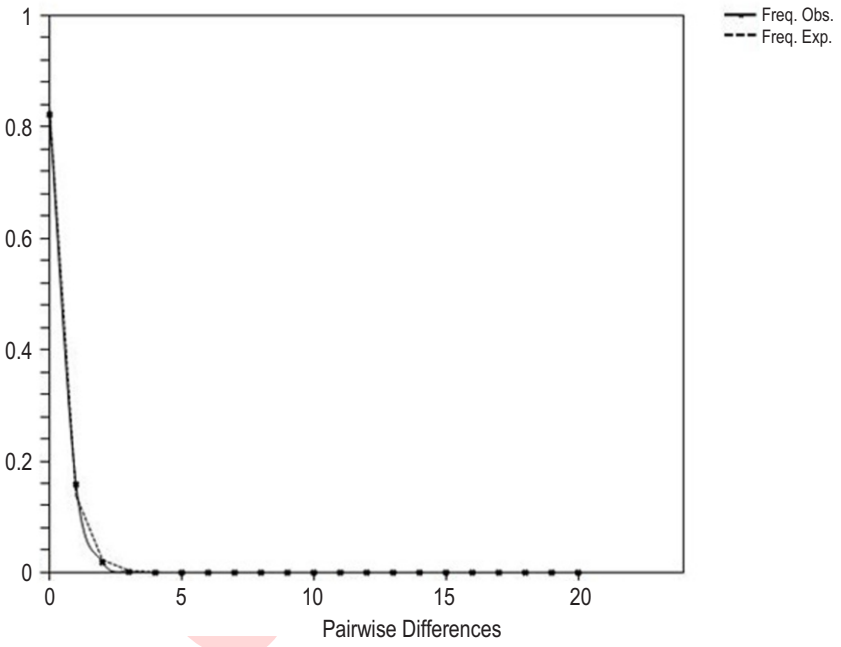

Fig. 2: Frequencies of the observed and expected mismatch distribution of *S. oryzae* populations in North India. X-axis, number of pairwise nucleotide differences; Y-axis, frequency of mismatches; Freq. Obs, frequency observed (solid line); and Freq. Exp., frequency expected (dashed line).

the populations (0.07345) indicated a moderate level of genetic differentiation among the populations collected across the zones (Table 3). The genetic differentiation estimates of F_{ST} between geographically distinct locations ranged from 0 to 0.222, indicating varying degrees of genetic differentiation across different populations, ranging from low to high levels (Table 4). The pairwise F_{ST} analysis indicated that the lowest value (0.00) was recorded across nearly all the locations, with the exceptions being Baramulla, Delhi, and Ludhiana. Whereas, the highest value (0.222) was observed between the Ludhiana population with Chandigarh, Pantnagar, Agra, Varanasi, Samastipur, Gaya and Kota populations.

The results of the principal coordinate analysis (PCoA) revealed that the first and second principal coordinates contributed 74.90% and 17.36% of the total variation,

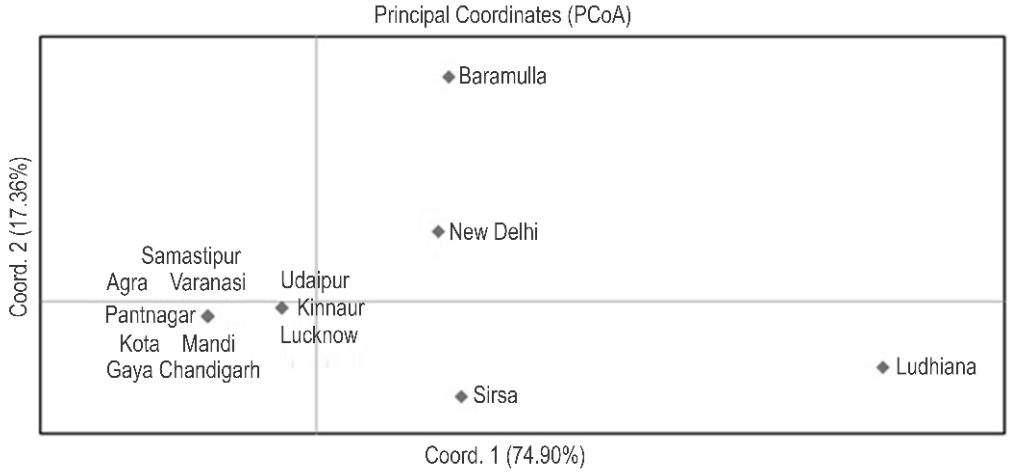

Fig. 3: Principal coordinate analysis (PCoA) score plot of *S. oryzae* populations from 15 different locations across North India based on 619 bp of mitochondrial *COI* gene.

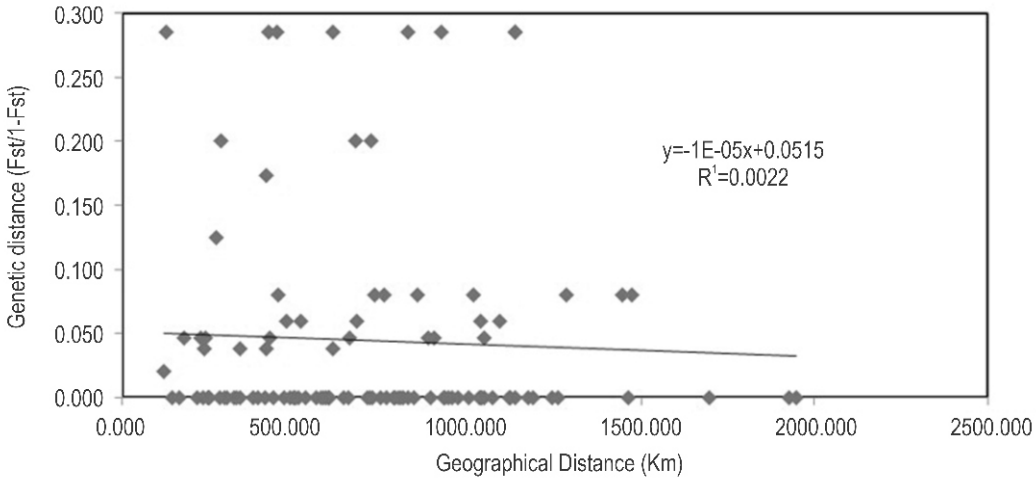

Fig. 4: Relationship between pairwise genetic differentiation (F_{ST}/1- F_{ST}) using *COI* sequences and geographical distances among different *S. oryzae* populations across North India.

respectively, with a cumulative variation of 92.26% explained by the first two axes (Fig. 3). It provided a rough division of populations into three main groups: the first group consisted of populations from the Varanasi, Samastipur, Agra, Pantnagar, Lucknow, Mandi, Kinnaur, Chandigarh, Kota, Gaya and Udaipur populations; the second group from the Baramulla and New Delhi populations and the third group consisted of the Sirsa and Ludhiana populations. Furthermore, the Mantel test results (Fig. 4) confirmed this pattern, as there was no delineation of populations on the basis of their geographic locations. The results revealed a very weak, non-significant correlation coefficient (R^2=0.0022) between the pairwise genetic distance matrix of all *S. oryzae* populations and their corresponding geographic (Ln km) matrix across the study area, suggesting that no IBD effect was present in fifteen populations.

A total of 10 *COI* haplotypes were identified among 150 individuals (Fig. 5). The haplotype network exhibited complexity, with no distinct geographical pattern in their distribution. Haplotype 1, containing 136 *S. oryzae* individuals, was the most frequently found haplotype in all the locations. This haplotype seems to be ancestral, as it occupies a central position in the network, with all other lineages emerging from it. Following this, Haplotype 5 included only 4 individuals: 3 from Ludhiana (LDH1, LDH7, LDH10) and 1 from Sirsa (SRS6). Haplotypes 2 and 6 included two individuals from Baramulla (BRL2, BRL6) and New Delhi (DLH1, DLH3), respectively. The remaining haplotypes were specific to a single location [H3 (BRL4), H4 (KNR4), H7

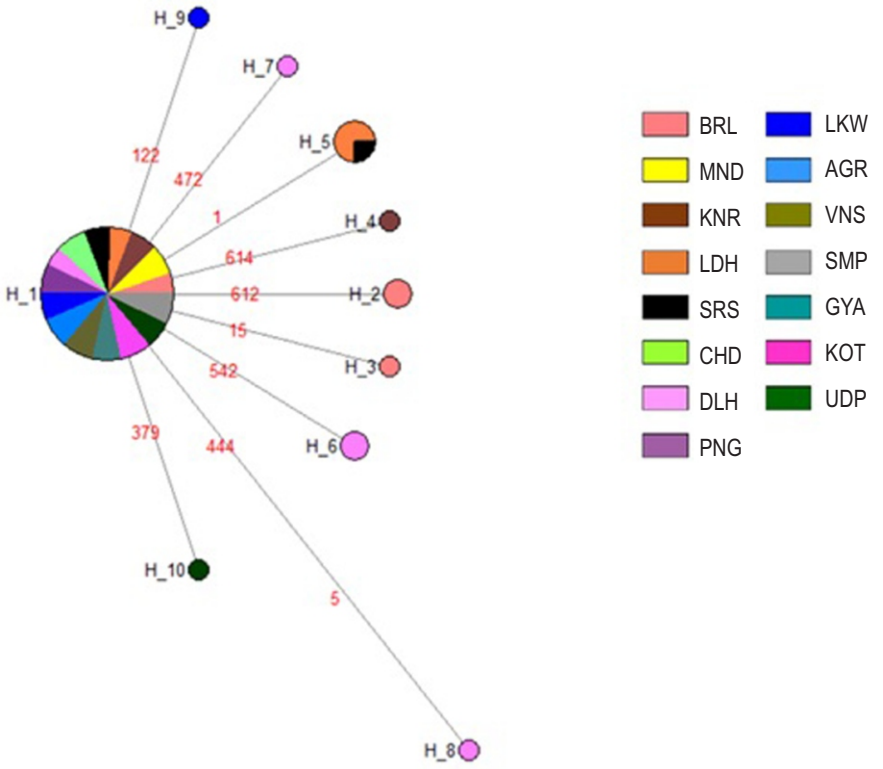

Fig. 5: Median-joining haplotype network of *S. oryzae* based on 619 bp fragment of mitochondria *COI* gene. The area of circles indicate the proportion of haplotypes frequencies, while the color portions indicate the proportions of same haplotype occurring in each geographical region.

(DLH4), H8 (DLH5), H9 (LKW6) and H10 (UDP8)]. This high degree of genetic admixture observed among different populations is further substantiated by the neighbor-joining phylogenetic tree, which did not reveal distinct clustering of individuals into groups on the basis of their geographic locations (Fig. 6). In the phylogenetic analysis comparing Indian populations with global genetic assemblages, an additional 13 *COI* gene sequences were retrieved from the NCBI database. These sequences correspond to populations from 13 countries outside India, reflecting the dispersal of *S. oryzae*.

All Indian populations of *S. oryzae* clustered within a single major clade in the neighbour-joining tree. The analysis revealed predominantly monophyletic groupings, along with instances of paraphyly, but no evidence of polyphyly. The *COI* gene sequences from Indian populations were highly similar to sequences of *S. oryzae* from other countries available in the GenBank database, indicating close genetic relatedness across geographic regions. The mitochondrial *COI* gene is widely recognized as an optimal molecular marker for the identification of cryptic species and invasive pest detection (Sethy *et al.,* 2023), alongside its utility in assessing population genetic structure (Chakravarty *et al.,* 2021; Mahalle *et al.,* 2022; Nachimuthu *et al.,* 2015). Moreover, the *COI* gene plays a significant role in phylogenetic analyses, species identification, and comprehensive biodiversity assessments. Consequently, leveraging its extensive applicability and informative characteristics in population genetic investigations, our study explored the genetic variability of *S. oryzae* populations across northern India, employing the mitochondrial *COI* gene marker to elucidate their population genetic structure and patterns of mobility. The identity of *S. oryzae* specimens, collected from diverse geographical sites throughout northern India, was unequivocally confirmed by homology search results of their *COI* sequences against existing sequences in the NCBI database, utilizing the BLASTN algorithm. Analysis of the *COI* sequences indicated a mean nucleotide composition of 30.85% for Adenine (A), 34.09% for Thymine (T), 15.35% for Guanine (G), and 19.71% for Cytosine (C). Furthermore, the mean AT and GC contents were determined to be 64.95% and 35.05%, respectively. This observed pattern is consistent with the characteristic AT-rich composition of the *COI* gene found across various arthropod species, including *S. oryzae* (Ndiaye *et al.,* 2014; Ndong 2015; Ojo *et al.,* 2016; Aslam *et al.,* 2019). The low haplotype diversity (Hd) and nucleotide diversity (π) for all the zones and overall populations indicated that the entire population may have undergone recent expansion after a period of low effective population size (Grant and Bowen, 1998). Our study is in accordance with the previous study reporting that the Indian population of *S. oryzae* exhibits no geographic differentiation owing to significant gene flow among the population (Thangaraj *et al.,* 2019). Similar results regarding the low nucleotide diversity of

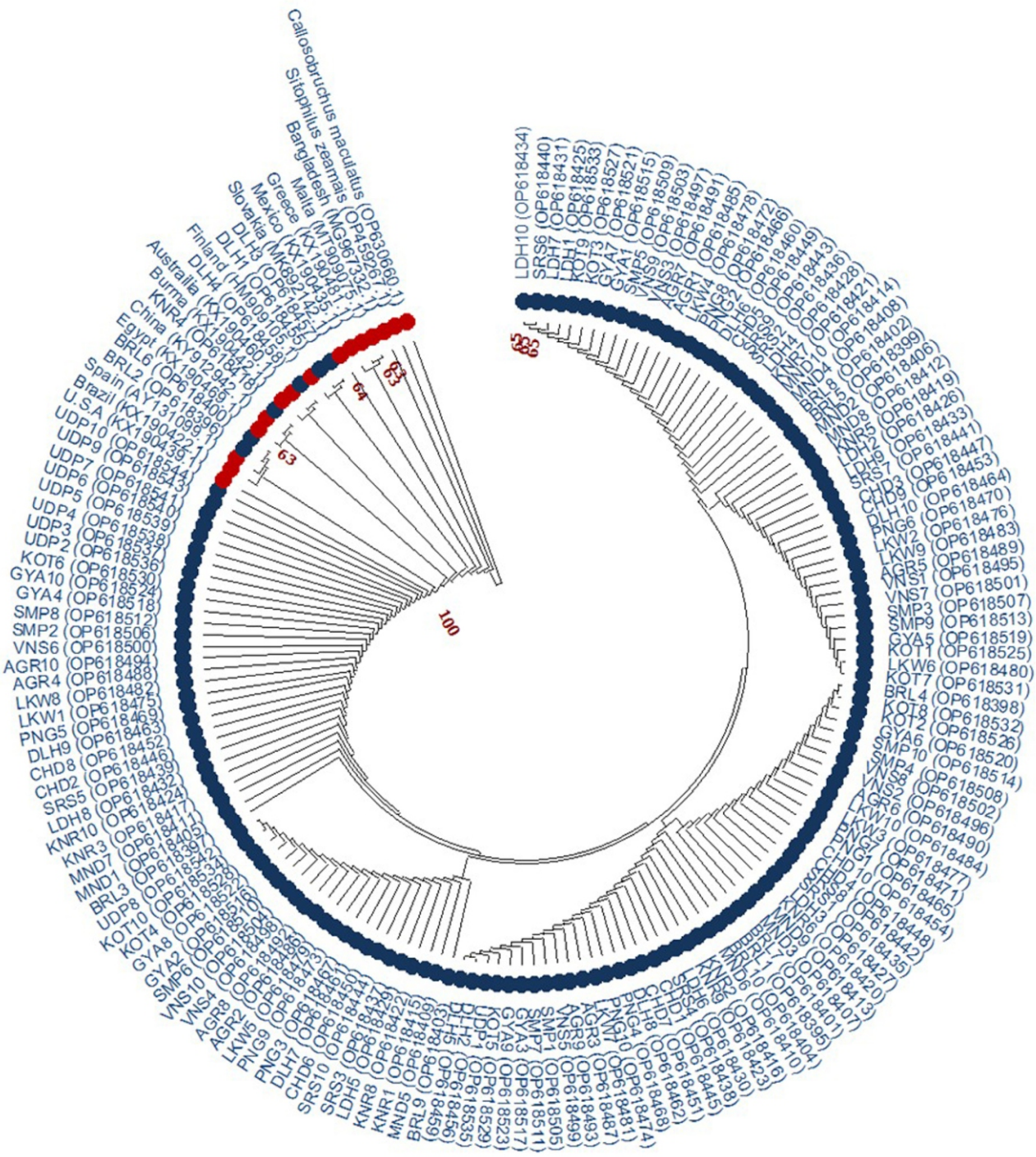

Fig. 6: Neighbour-joining tree of *S. oryzae* based on Kimura-2-Parameter distances based on the *COI* gene sequences. Numbers at branch point indicate 1000 bootstrap value. *Sitophilus zeamais* (accession- OP459267.1) and *Callosobruchus maculatus* (OP630660.1) as outlier sequences.

S. oryzae were also exhibited among the Indian populations collected from Tamil Nadu, India (Upasna and Mohankumar, 2022). The mean genetic distance of *S. oryzae* in North India was exceptionally low (0.00032), providing strong evidence for its single-species status. Among the 10 identified haplotypes, haplotype 1 was the most prevalent, comprising 136 samples distributed in all populations across 15 locations, suggesting that it may be the ancestral haplotype with high gene flow or a

common origin Haplotype 5 also contained 4 individuals from two locations. On the other hand, the remaining peripheral haplotypes exhibited a limited geographical distribution, implying minimal sequence divergence and a high occurrence of unique mutations, which are indicative of rapid population expansion.

The high number of mutational steps separating some haplotypes may be due to accumulated mutations in small, isolated subpopulations over time. Haplotype 8 was genetically distinct and connected to haplotype 1 by five mutational steps, indicating possible geographic or reproductive isolation. These findings are consistent with earlier studies indicating that longer branch lengths in haplotype networks are associated with greater isolation and evolutionary divergence (Dash *et al.,* 2025). Similarly, in a study undertaken in Senegal and The Republic of Guinea, 16 haplotypes were identified in 24 specimens of *S. oryzae* (Ndiaye *et al.,* 2014). Wu and Yan (2018) also documented 6 haplotypes among populations of *S. oryzae* collected from China, Australia, and South India. In India, 8 haplotypes were identified from 143 individuals collected from 37 different locations (Thangaraj *et al.,* 2019), and 8 haplotypes were identified from 33 individuals from two districts of Tamil Nadu (Upasna and Mohankumar, 2022). The majority of *S. oryzae* individuals formed a large monophyletic cluster (blue nodes) with high bootstrap support (≥85), indicating strong genetic similarity and low divergence among most populations, consistent with the haplotype network and low pairwise values. The mixing of Indian and non-Indian sequences within the main clade indicates extensive gene flow at global scale. This pattern likely results from extensive grain trade and storage movements, which homogenize genetic variation across regions (Drury *et al.,* 2009).

A smaller, distinct subclade (red nodes) emerged within the *S. oryzae* cluster, supported by moderate to high bootstrap values (≥65). The presence of a smaller subclade within *S. oryzae* suggests the beginning stages of lineage diversification, potentially due to geographic isolation or local adaptation in the Udaipur, Kota, and Gaya populations. Although this group is monophyletic within itself, its separation from the main clade indicates reduced historical connectivity. One Ludhiana individual (LDH10) branched closer to the smaller subclade rather than clustering with the remaining Ludhiana samples. This branching pattern is indicative of paraphyly within the Ludhiana population, where not all members share the same most recent common ancestor with the exclusion of others. These patterns highlight the complexity of the population structure of pest species that experience both natural dispersal and human-mediated movement (Drury *et al.,* 2009). No polyphyletic grouping was evident for *S. oryzae,* as all sequences ultimately clustered within a single overarching lineage. Neutrality tests, specifically Fu and Li's D, Fu and Li's F, Fu's Fs test and Tajima's D, are important in analyzing the demographic history of an organism (Fu, 1997; Tajima, 1989). In this context, negative values are typically associated with phenomena such as selective sweeps or population expansion subsequent to a recent sharp decline whereas positive values commonly signify that populations are subdivided and have reached equilibrium. In the present study, Tajima's D test yielded statistically insignificant value across all analyzed zones, with the exception of the Upper Gangetic Plain region and the overall populations, thereby implying a low-frequency polymorphism within these specific populations. Similarly, the observation of negative values for Fu's F is generally indicative of an excess of single nucleotide polymorphisms, which is characteristic of population expansion events (Fu, 1995; Fu, 1997). The consistently negative values observed overall suggest that the *S. oryzae* population investigated herein has recently undergone a period of population expansion or growth following either a bottleneck event or a selective sweep, leading to an abundance of rare mutations and a low-frequency polymorphism. These findings from the current investigation are consistent with previous research (Thangaraj *et al.,* 2019; Upasna and Mohankumar, 2022), which also reported negative and statistically insignificant values for Tajima's D in Indian *S. oryzae* populations.

Genetic variation observed within populations accounted for 92.66%, with an additional 5.91% of genetic variation attributed to differences among populations within groups, and merely 1.43% among the groups themselves. These findings collectively imply that the principal determinant of variability is likely not geographical barriers demarcating regions, but rather intrinsic differences among the populations. Furthermore, an F_{ST} value of 0.07345 signified considerable moderate genetic differentiation among the populations sampled across the distinct geographical zones. Moreover, no significant correlation was established between the observed genetic variance among the populations and their corresponding geographical locations. This observation supports earlier works, which similarly indicate that stored grain pests, including *S. oryzae,* typically do not manifest a pattern of isolation by distance (Bas *et al.,* 2000; Drury *et al.,* 2009; Thangaraj *et al.,* 2016).

The intermingling of geographically distant populations in the present investigations indicated the lack of genetic differentiation and structure which could be explained in two ways: the migration of *S. oryzae* in different parts of the country and a recent population expansion. The movement of *S. oryzae* on a large scale is probably due to the anthropogenic transportation of food grains within the country which may lead to the intermixing of populations as it has limited flight activity (Throne and Cline, 1991; Drury *et al.,* 2009; Thangaraj *et al.,* 2019). Therefore, the probable reason for low genetic variation among various populations of *S. oryzae* is their passive migration by the transportation of grains, similar to other storage pests, which prevent an increase in the genetic diversity by breeding with geographically distinct populations (McCulloch *et al.,* 2019; Ndiyae and Sembene, 2018). The pairwise F_{ST} values among various populations varied from 0.000 to 0.222.The majority of comparisons indicated no genetic differentiation between populations, supporting high connectivity inferred from the haplotype network. Notably, the Ludhiana population (LDH) was highly differentiation from several other populations, suggesting genetic distinctness possibly due to geographic isolation, unique

storage practices, or limited insect dispersal from this location. These results also partly explain the high adaptive capacity of *S. oryzae*, which is evident from its wide geographical distribution and ability to infest a diverse range of commodities (Hasan *et al.,* 2017; Subedi *et al.,* 2009).

This study revealed the homogenous genetic structure of *S. oryzae* in Northern India based on *COI* gene, possibly due to stable inherited gene flow among different populations. The relatively high *COI* haplotype diversity confirms the hypothesis that *S. oryzae* may have originated in India. The high level of genetic diversity in some populations may be attributed to the rapid development of resistance in *S. oryzae* populations to different insecticides, such as phosphine, and other forms of local adaptation. Although some populations are genetically structured, the overall genetic distance indicates that they belong to same species. The species dispersion helps to maintain variability, which in turn provides protection against various insecticides used for its control. Tracking information on the anthropogenic movement of this weevil across the country is also helpful in understanding its dispersion. However, further studies that incorporate simple sequence repeats (SSR) markers in combination with mitochondrial *COI* genes, and use a larger sample size from diverse geographical locations and different hosts with known histories, are needed to comprehensively understand the population structure of *S. oryzae* in India. This study provides a base for investigating the dispersal of *S. oryzae,* its implications for the spread of phosphine resistance, and the design of effective control measures.

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