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Prevalence of strain (R and C strains) of fall armyworm *Spodoptera frugiperda* in eastern India

SHRAWAN KUMAR SAHANI[1], TAMOGHNA SAHA[2]*, TUSHAR RANJAN[2] and SAILABALA DEI[2]

Bihar Agricultural University, Sabour, Bihar 813 210, India

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Fall armyworm (FAW), *Spodoptera frugiperda* (J E Smith) (Lepidoptera: Noctuidae) is an invasive and phytophagous pest feed on numerous main crops as well as occasionally on other crops which is significant in most countries (Bhavani *et al.* 2019). FAW primarily prefer maize (*Zea mays* L.) because its leaves and cobs are highly nutritional and their soft and tender tassels are used to feed. The young neonate larvae feed by scraping and skeletonizing the upper epidermis of leaves, while the full-grown larvae feed on the developing primordial shoot and tassel, resulting in dead heart symptoms (Shylesha *et al.* 2018), thus causing severe economic losses of 50–70% to the farmer's crop.

The introduction of the fall armyworm in Asia was reported for the first time in India on maize in May 2018 at Karnataka district, Shivamogga (Sharanabasappa *et al.* 2018). Recently noticed genetic variation in the fall armyworm which is morphologically similar but genetically variable strains using mtDNA cytochrome oxidase sequences (Luttrell 2006). There are two sympatric strains, which are morphologically identical but differentiated through biochemical and molecular markers, namely a corn strain (C) that favours corn as a host plant and a rice strain (R) that favours rice and forage grasses (Pashley 1992, Groot *et al.* 2008). Mitochondrial genes-based primers are commonly used to study an animalia group of organisms and their evolutionary relationship. Thus, the *mtCOI* is the "universal" DNA primer for polymerase chain reaction (PCR) amplification (Folmer *et al.* 1994) and is among the most conservative protein-coding genes in the mitochondrial genome. Hence, present studies were carried out to decipher the prevalence and diversity of strains and bring out the host/location-specific variations.

Specimen collection: The neonate larvae collected during 2020–22 from maize fields of Bihar Agricultural University, Sabour, Bihar (Supplementary Fig. 1 and Table 1). The identification of fall armyworm (larvae + adults) was confirmed using morphological keys from Peterson (1962) and Prasanna *et al*. (2018). The eighth (2nd last segment) abdominal segment of the larvae indicated the existence of four pinacula (black spots) forming a square. Larvae were deposited in 70% alcohol at 4°C for DNA isolation and the rest of the samples were treated in the laboratory of the Department of Entomology, Bihar Agricultural University, Sabour, Bihar.

FAW genomic DNA extraction: The DNA was isolated from the neonate larva using a kit, i.e. MN Insect (Germany). The individual larva was homogenised in NucleoSpin**R** Bead Tube Type D (100 µl Elution Buffer BE, 40 µl Lysis Buffer MG, 10 µl of Liquid Proteinase K). The aqueous solution with sample was stirred on Vertex-Genie 2 for approximately 20–25 min. Then it was centrifuged for one min at 11000 to clean the lid. To adjust DNA binding conditions, add MG buffer (600 µl) and vertex mixed 3 to 4 times, then centrifuged to sediment steel bread and cell wreckages. Moreover, fine solution (500–600 µl) was transmitted into the NucleoSpin^{R} the insect column of DNA, placed in a 2 ml collection tube. Furthermore, we washed the column of the silica membrane with both 500 µl buffer BW and buffer B_5 then after silica membrane was dried for 5 min. Finally, the NucleoSpin^{R} DNA insect column was located in a 1.5 ml nuclease free duct and 100 µl of elution buffer BE was added to the column. It was incubated at room temperature for 1 min followed by centrifugation for 1 min at 11000 rpm. The DNA concentration was checked by nano drop followed by gel electrophoresis on 0.8% agarose at 100 V for 20 min.

PCR amplification and cloning: PCR purification of the 5'- fragment (658 bp) of the *mtCOI* gene was done testing the primer pair LCO1490/ HC02198 (5'GGTCAACAAATCATAAAGATATTGG3' and 5'TAAACTTCAGGGTGACCAAAAAATCA-3'). PCR amplification protocol was done according to Sahani *et al.* (2023).

The cloning procedures were done followed by Sambrook and Russell method (2001) in which standard

[1]Lovely Professional University, Phagwara, Punjab; [2]Bihar Agricultural University, Sabour, Bihar. *Corresponding author email: tamoghnasaha1984@gmail.com

Table 1 FAW collection sites their host plants, date of collection with life stages and NCBI accession numbers

Place	Sample code	State	Sample size	Host	Life stage collection	Collected date	Latitude and Longitude	NCBI accession number
Bhojpur	BJP	Bihar	10	Maize	Caterpillars	18-11-20	25.529612 and 84.755923	OP002315
Munger	MNG	Bihar	25	Maize	Caterpillars	25-12-20	24.994836 and 86.601203	OP003883
Bhagalpur	BGP	Bihar	30	Maize	Caterpillars	29-0920	25.23498 and 87.04916	OP002858
Rohtas	RHS	Bihar	15	Maize	Caterpillars	09-08-21	24.95352 and 84.01177	OP003528
Purnia	PRA	Bihar	21	Maize	Caterpillars	07-07-21	25.92444 and 87.56134	OP048108
Katihar	KTR	Bihar	32	Maize	Caterpillars	15-09-21	25.537802 and 87.571083	OP004006
Khagaria	KGR	Bihar	23	Maize	Caterpillars	21-10-21	25.538139 and 86.668059	OP048829
Banka	BNK	Bihar	10	Maize	Caterpillars	30-12-20	24.995806 and 86.990954	OP048830
Birbhum	BRM	West Bengal	7	Maize	Caterpillars	02-03-21	23.666823 and 87.661397	OP048831
Begusarai	BGR	Bihar	28	Maize	Caterpillars	19-10-21	25.388577 and 86.255376	OP048832
Samastipur	SMT	Bihar	17	Maize	Caterpillars	02-02-22	25.938822 and 85.687042	OP056096
Muzaffarpur	MZF	Bihar	12	Maize	Caterpillars	06-02-22	25.98336 and 85.56675	OP056097
Sahebganj	SGJ	Jharkhand	9	Maize	Caterpillars	29-04-22	25.277716 and 87.507027	OP056098
Kaimur	KNG	Bihar	16	Maize	Caterpillars	30-11-20	25.034083 and 83.738675	OP049939
Burdwan	BWN	West Bengal	2	Maize	Caterpillars	14-05-22	23.2325 and 87.8634	OP056099
Coochbehar	CBR	West Bengal	2	Maize	Caterpillars	10-05-22	26.4035 and 89.3850	OP056100
Sambalpur	SMB	Odisha	6	Maize	Caterpillars	05-04-22	21.4669 and 83.9812	OP056101
Kanke	KNK	Ranchi	26	Maize	Caterpillars	17-03-22	23.4345 and 85.3214	OP049940

recombinant DNA techniques were used to clone the amplified PCR product. Cloning was certified by colony PCR, plasmid mobility checks and restriction analysis. Isolated plasmid DNA, from an overnight bacterial culture, was sequenced using M13 sequencing primers.

Sequence analysis: Sequencing results attained from sequencer and subsequently, the unwanted sequences of primers were trimmed out from the original paired-end data, a consensus region sequence was created using Bio Edit version 7.2.5 for window (Hall 1999). Multiple filters were applied to check high quality sequences for submission to NCBI GenBank with the following accession numbers mentioned in (Table 1). The end-to-end alignment was done through clustalW and the dendrogram were made employing MEGA11 software version 11.0.11 with neighbour-joining (NJ) with comprehensive gap finding resampled and the alignment of maximum composite likelihood with 1000 bootstrap replication (Tamura *et al.* 2021).

Molecular characterization and genetic diversification of S. frugiperda: The present study successfully amplifies the expected amplicon size of 658 bp from mitochondrial-COI (5'and 3', respectively) from all the collected samples. The comprehensive Insilco analysis confirms the identity of all the collected isolates as *S. frugiperda*.

The ancestor's relationship was established with representative samples of both the R and C strains of FAW using *mtCOI* gene obtained from the NCBI database. BLAST analysis of the sequences against the NCBI and BOLD databases showed that there were considerable nucleotide variations when compared with the rice strain sequence MH753326 *S. frugiperda*. Nucleotide variations were recorded at the positions 52, 97, 151, 187, 238, 388, 469, 540, 550, 580, 614 and 643. Similarly, variations were also observed in nucleotides when compared with corn strain OP002860 *S. frugiperda* at nucleotide positions 57, 102, 156, 192, 243, 393, 474, 549, 555, 585, 619, 636, 642, 648 and 657 (Table 2).

The present phylogenetic tree (Fig. 1) consisted of 18 *mtCOI* sequences. 31 sequences were retrieved from the NCBI Gene database, which includes sequences from across the world and one sequence of *Spodoptera exigua* with the NCBI accession number GU687828.1, which occupies a separate clade. Intriguingly, two distinct clades were obtained out of which the first clade includes all the 12 sequences of *S. frugiperda* that have been assigned to the 'rice' strain. This clade was geographically diverse, with populations from India, Pakistan, Indonesia, the United States, and the West Indies represented, whereas the other clade 2 included 6 sequences and all the specimens previously assigned to the "corn" strain from South Africa, Indonesia, West Indies, India and USA corn populations. Thus, we corroborated the existence of rice and corn strains in these regions of eastern India, and both of these strains feed on maize as a host. The present findings also confirm the existence of both strains in the eastern parts of India feeding on maize, with a preponderance of the rice strain population feeding primarily on maize as the host plant.

Similar trend of findings reported from Africa, Nigeria and Tanzania have shown the predominance of the "R" strain over the "C" strain and maize is the principal crop of attack (Goergen *et al.* 2016). Mahadevswamy (2018) also concluded his report using *mtCOI* (5') based sequence

analyses on six states of India (Karnataka, Tamil Nadu, Andhra Pradesh, Telangana, Madhya Pradesh and Maharashtra) and witnessed that these populaces from India aligned with the "R" strain with minimal genetic diversity, displaying no host or location-specific variations. Kalleshwarswamy *et al.* (2019), however, reported the occurrence of rice strains in FAW populations from Karnataka state feeding on rice. This requires deploying additional markers to decipher the strain variations and population structures in all the regions of infestation by FAW similar to the studies carried out by Nagoshi *et al.* (2018). Michael *et al.* (2018) was recognized based on the partial *mtCOI* gene sequences. This research analysis provides indication to support *S. frugiperda* as likely consisting of two sympatric sister species known as the corn and rice preferred strains.

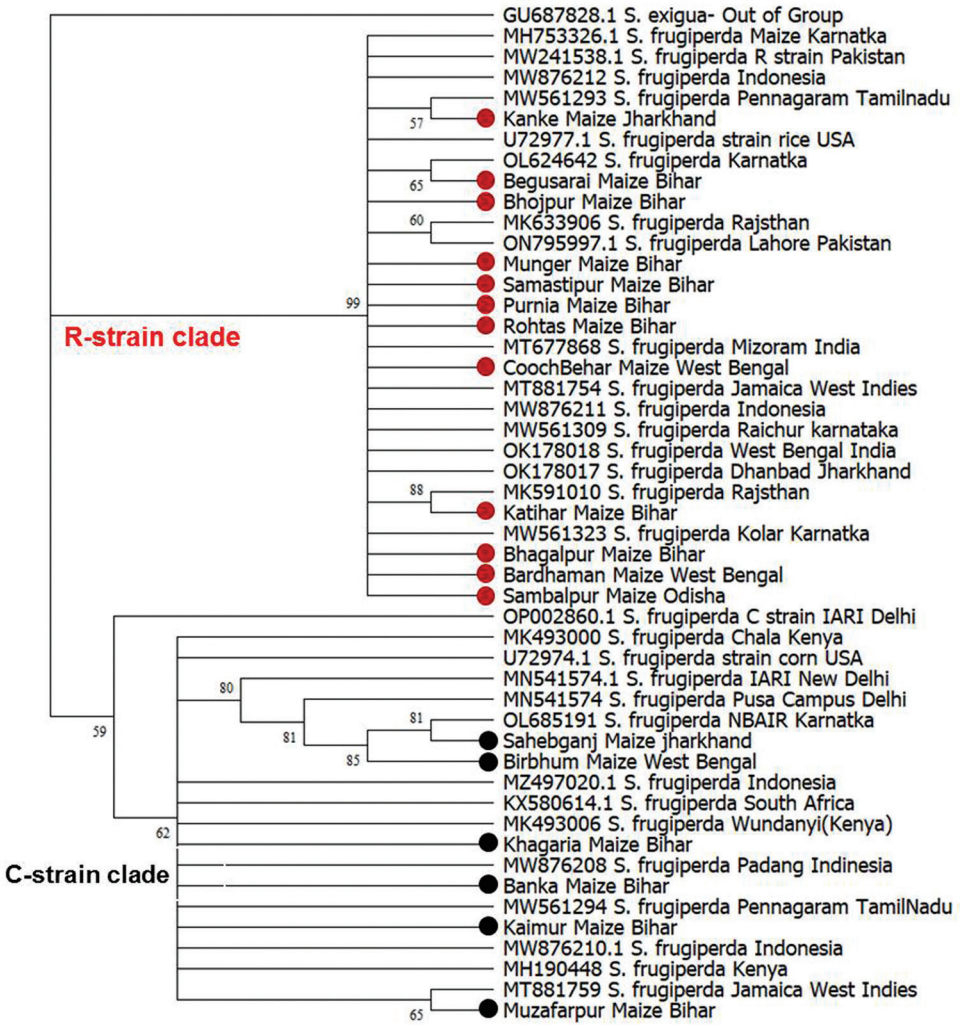

Fig. 1 Neighbor-joining tree of strain specific detection of the samples of *S. frugiperda* from eastern India.

The findings inferred that the strain reported in these regions of India is both the strains i.e. 'R' strain and the 'C' strain are present based on the *mtCOI* gene with the majority of the strain belonging to the rice strain (R strain) in the eastern part of India, and both the strains were found to be highly preferred to feeding on maize.

A like sequence is categorized with their accession's

Table 2 Position wise nucleotide variations detected using mtCOI 5' of FAW eastern Indian populations vs. rice and corn strains

Sample information	Indian FAW population's vs R strain											
	52	97	151	187	238	388	469	544	550	580	614	643
MH753326 *S. frugiperda* **R Strain Maize**	A	A	C	A	T	T	C	C	T	T	C	A
BJP	.	.	.	.	.	.	.	.	.	.	.	.
MNG	.	.	.	.	.	G	.	.	.	.	.	.
BGP	.	.	.	.	.	.	.	.	.	.	.	.
RHS	.	.	.	.	.	.	.	.	.	.	.	.
PRA	.	.	.	.	.	.	.	.	.	.	.	.
KTR	.	.	.	.	.	.	.	.	.	.	.	.
KGR	G	G	T	T	C	.	T	T	C	C	T	T
BNK	G	G	T	T	C	.	T	T	C	C	T	T
BRM	G	G	T	T	C	G	T	T	C	C	T	T
BGR	.	.	.	.	.	.	.	.	.	.	.	.
SMT	.	.	.	.	.	.	.	.	.	.	.	.
MZF	.	G	T	T	C	.	T	T	C	C	T	T
SGJ	G	G	T	T	C	.	T	T	C	C	T	T
KNG	G	G	T	T	C	.	T	T	C	C	T	T
BWN	G	.	.	.	.	.	.	.	.	.	.	.
CBR	.	.	.	.	.	.	.	.	.	.	.	.
SMB	G	.	.	.	C	.	.	.	.	.	.	.
KNK	.	.	.	.	.	.	.	.	.	.	.	.

Sample information	Indian FAW population's vs Corn strain														
	57	102	156	192	243	393	474	549	555	585	619	636	642	648	657
OP002860 *S. frugiperda* **C Strain Maize**	G	G	T	T	C	T	T	T	C	C	T	T	A	A	C
BJP	A	A	C	A	T	.	C	C	T	T	C	C	T	.	T
MNG	A	A	C	A	T	G	C	C	T	T	C	C	T	.	T
BGP	A	A	C	A	T	.	C	C	T	T	C	C	T	.	T
RHS	A	A	C	A	T	.	C	C	T	T	C	C	T	.	T
PRA	A	A	C	A	T	.	C	C	T	T	C	C	T	.	T
KTR	A	A	C	A	T	.	C	C	T	T	C	C	T	.	T
KGR	.	.	.	.	.	.	.	.	.	.	.	C	T	T	T
BNK	.	.	.	.	.	.	.	.	.	.	.	C	T	T	T
BRM	.	.	.	.	.	G	.	.	.	.	.	C	T	T	T
BGR	A	A	C	A	T	.	C	C	T	T	C	C	T	.	T
SMT	A	A	C	A	T	.	C	C	T	T	C	C	T	.	T
MZF	A	.	.	.	.	.	.	.	.	.	.	C	T	T	T
SGJ	.	.	.	.	.	.	.	.	.	.	.	C	T	T	T
KNG	.	.	.	.	.	.	.	.	.	.	.	C	T	T	T
BWN	.	A	C	A	T	.	C	C	C	T	C	C	T	.	T
CBR	A	A	C	A	T	.	C	C	C	T	C	C	T	.	T
SMB	.	A	C	A	.	.	C	C	C	T	C	C	T	.	T
KNK	A	A	C	A	T	.	C	C	C	T	C	C	T	.	T

numeral followed by host, country and strain were reported. Branches with a bootstrap value less than 50 were collapsed. A highlighted sample were reported from eastern part of India falling into different clades. A sequence from a species *S. exigua* is encompassed as an out group.

SUMMARY

An invasive alien pest, fall armyworm has been recently introduced to India. To date, two strains of FAW have been documented, viz. R strain (rice) and C strain (corn) without any clear biological attributes, even though differences are evident. A survey was conducted during 2020–22 over 18 field visits across the eastern part of India (Bihar, West Bengal, Orissa and Jharkhand) to isolate the genetic diversification of FAW populations collected from maize. This investigation was done with a mitochondrial-based 'universal primer' using *mtCOI (mitochondrial cytochrome c oxidase subunit I)* sequence analyses revealed that both strains (R strain and C strain) were present with the FAW. Interestingly, our comprehensive analysis indicates the dominance of the R strain over the C strain in the sample collected from the eastern part of India. This would be the first report from the eastern part of India, mainly the regions of Bihar, West Bengal, Orissa and Jharkhand, where FAW primarily feeds on maize. This study generates an idea about a probable incursion by a genetic asset of FAW in India, which needs dissection of the haplotype.

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