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First report of a new invasive pest fall army worm, *Spodoptera frugiperda* (J.E. Smith) in maize crop at Pantnagar, Uttarakhand

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Abstract
Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is a noxious lepidopterous pest of maize crop. Northern region of India was devoid from incidences of *S. frugiperda* until now in 2019 it has been observed in Uttarakhand state for the first time at N.E.B. Corp Research Center, GBPUA&T Pantnagar, Udham Singh Nagar during the routine survey in the maize field. The morphological and molecular tools were used for the identification of the Pantnagar pest sample. The *mtCOI* gene was used as molecular marker and it was found that the Pantnagar sample sequence showed 100% similarity after BLAST with the sequence accession No. GU095403.1 (658 bp) and MH704433 (658bp) of *S. frugiperda* from Canada and Karnataka (India), respectively. The *mtCOI* consensus sequences generated for Pantnagar sample of 630 bp has been deposited in National Centre for Biotechnology Information GenBank databases as *S. frugiperda* sequence of Pantnagar sample. Morphological and molecular characterization of Pantnagar pest sample marked the conformity of the pest being *S. frugiperda* (J.E. Smith) which is reported for the first time from Uttarakhand state.

Keywords: Invasive pest, maize, morphological and molecular characterization, spodoptera frugiperda

1. Introduction
The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) is a notorious, destructive pest which is native to the tropical region of the western United States of America. Thereafter, its population began to spread from United States to nearby continents [2]. For the first time in 2016, it was reported in Africa, causing significant damages to maize crop [12]. On the Indian subcontinent, the first record of *S. frugiperda* was observed in 2018 from maize fields at University of Agricultural and Horticultural Sciences, Karnataka [15]. Based on morphological and molecular investigations by the scientists of National Bureau of Agricultural Insect Resources (NBAIR), presence of *S. frugiperda* was confirmed in India [19]. Fall army worm has been reported to damage more than 80 types of plants species including maize, sorghum, sugarcane, cotton, soybean, millets and a number of vegetable crops [4, 16, 19]. Due to broad host range, *S. frugiperda* has the potential to cause severe yield losses in many economically important crops, especially maize. The pest is most destructive in larval stage. Yield reductions in maize due to feeding of the fall armyworm have been reported as high as 34% [3, 6, 7, 24, 25]. With short time span *S. frugiperda* has managed to spread over a wide range of area around the world. In India, states like Tamil Nadu, Chhattisgarh, Gujarat, Bihar and Maharashtra have reported the incidences of *S. frugiperda* [22, 8, 21, 1, 5]. The present investigation discussed about the occurrence and identification of the noxious pest in maize crop at Pantnagar, Uttarakhand state.

2. Materials and Methods
On the basis of morphological and molecular attributes, the pest sample from Pantnagar was characterized. For this, the pest was collected manually through hand picking method and reared under laboratory condition in Biological Control Laboratory of Department of Entomology, College of Agriculture, GBPUA&T, Pantnagar, U.S. Nagar, Uttarakhand.

i. Collection and culture maintenance
The pest samples of were collected from maize crop at N.E.B. Crop Research Centre, Pantnagar (Fig.1). The field collected larval samples were placed into different plastic

containers (20×15×8 cm) lined with blotting paper at bottom with moist cotton swabs to maintain the humidity. For suitable aeration in the boxes, fine mesh was fitted at the center of m the lid. Larvae were reared on fresh maize leaves which were properly washed before providing as feed to remove any chances of contamination. Pupa were kept inside insect rearing glass jar (15×15×25cm) and cotton balls dipped in honey solution was provided as feed for emerging adults. Throughout the rearing period all containers and rearing jars were kept under a photoperiod (16h light: 8h darkness (LD 16:8) at 30 ± 2 °C temperature with 75 ± 5% relative humidity. Daily fresh maize leaves were provided to the larvae. All developmental stages *viz*., egg, larva, pupa and adult were critically examined for distinct morphological traits for identification of the pest.

ii. Morphological identification

The Pantnagar pest sample was identified based on morphological characteristics of larva and adult which were further studied in laboratory and compared with the original available identification characters specific to *Spodoptera* spp. [9] [10] [11] [16].

iii. Molecular characterization

To further confirm the pest species, samples were analyzed using molecular diagnostic tools. The standard protocol of DNA extraction, amplification of DNA, purification of amplified product and sequencing of both forward and reverse sequence.

A. DNA extraction

For DNA extraction, the sample larvae were starved for 48 hours before the extraction to avoid chances of DNA contaimination in the sample. An hour before dissection, larva was anesthetized by using chloroform in cotton and stored at -20^0 C for 15 minitue. Through pointed surgical blade larvae was dissested and 50 mg of sample containing soft tissues from individual larvae was used.

DNA isolation was performed using HipurA™ Insect DNA Purification Kit (Himedia) based on HiElute miniprep spin column format which allows rapid processing of DNA isolation. The ratio of $OD_{260/280}$ [18] was maintained to check the impurity (RNA or protein) and concentration (µg/µl) was calculated for further PCR amplification. Mitochondrial cytochrome oxidase (*mtCOI*) primer (subunitI) sequence, Forward5'- GGTCAACAAATCATAAAGATATTGG-3'; Reverse5' TAAACTTCAGGGTGACCAAAAAATCA-3' [19] [4] was used for DNA amplification which was synthesized by Chromous Biotech Bangalore, India.

B. PCR Amplification and Sequencing

DNA amplification was performed with primer of COI sequence in thermo-cycler (Wee 32 Himedia). During amplification 15 µl reaction mixture was prepared in PCR tubes. The mixture contained 50ng template DNA (2µl), 10pmol Primer: Forward (0.6µl), Reverse (0.6 µl), 10X PCR Buffer (1.5µl), 10Mm dNTP mix (0.9 µl), 5U/µl *Taq* DNA polymerase (0.2µl) with molecular biology grade water (9.1µl). The mixture was amplified through PCR machine at: Initial denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 45 second and annealing at 54 ^{0}C for 45 second. The initial extension at 72 °C for 2 minutes and final extension at 72 °C for 5 minutes. The amplified PCR products were employed to electrophoresis on 2.5% agarose gel at constant voltage of 70V with 100 bp ladder for DNA separation. Amplified product was sequenced from Chromous Biotech Pvt. Ltd. Bangalore.

iv. Data analysis

Sequencing of the selected primer was performed by Chromous Biotech Pvt. Ltd. Bangalore, India. The sequenced fragment was compared with *mtCO*I gene sequences of *S. frugiperda* from National Centre for Biotechnology Information (NCBI) databases using BLASTn (Basic Local Alignment Search Tool) and ORF finder. The sequences were aligned using CLC workbench7.7.2 program. Nucleotide distance was measure through Jukes-Cantor parameter and phylogenetic tree was constructed using the Neighbour-joining algorithm (NJ) [17]. The *mtCOI* generated consensus sequence of Pantnagar sample was submitted in NCBI GenBank databases as *Spodoptera frugiperda* sequence (Pantnagar) for acquirement of the accession number.

3. Results

Maize fields at N.E.B. Crop Research Center, GBPUA&T, Pantnagar, were observed with distinguishing damage symptoms on leaves. The damaged maize plants showed characteristic circular hole with scrapped leaf symptoms. Developing cobs were also damaged. Larva along with feacal matter was observed on developing cobs and also inside the plant whorls. As the larvae and its damages observed were different from true army worm, therefore, it was felt necessary to go for identification of this pest.

i. Morphological characterization of Pantnagar Population of *S. frugiperda*

Eggs observed were creamy white in color and covered with greyish-brown scales. Initial larval instars were pale green with distinct black head capsule. In later stages, larval body colour varied from greenish to dark brown and even black. Small, circular, sclerotized black spot (pinacula) was observed on dorsal abdominal segments of larva. In each dorsal abdominal segment, four pinacula were arranged in trapezoidal shape (Fig. 2B). These pinacula were distinctly observed in fully matured larva. Distinguishing morphological characteristic features in in sample larvae were yellow colored inverted 'Y' shaped marking on head capsule (Fig. 2A). and presence of dorsal pinacula on 8th abdominal segment arranged in square pattern (Fig. 2C). Pupae were reddish brown in colour with cremaster having two spines (Fig. 3). Adult male moths were greyish-brown. Forewings have transverse contrasting lines, indistinct reniform oblique orbicular spot on forewing and white irregular patch at the anterior apical region of forewings (Fig. 4). Forewings of adult female lack distinct markings and dark grey coloured orbicular elongate spots were observed on the outer margins of forewing. The comparison of Pantnagar pest sample with the characteristics of *S. frugiperda* (J.E. Smith) [20] [21] indicated that the pest observed at Pantnagar, Uttarakhand was *S. frugiperda*.

ii. Molecular characterization of Pantnagar Population of *S. frugiperda*

Molecular characterization of Pantnagar sample was done using primer *mtCOI* sequence (658bp consensus sequence) for amplification of DNA. The amplified sample was sequenced and both forward and reverse sequences obtained were aligned.

For molecular identification, Pantnagar sample was compared with available database of *S. frugiperda* of *mtCOI* sequence present in NCBI. The Pantnagar sample showed 100% similarity after BLAST with the sequence accession No. GU095403.1 (658 bp) of *S. frugiperda* from Canada and MH704433 (658bp) sequence of *S. frugiperda* from Karnataka, India. Phylogenetic assessment based on *mtCOI* gene sequence (Fig. 5) was also performed with 23 different species of *Spodoptera* genus available from Genbank NCBI database which includes sequences across the world i.e. (*GU095403.1:S.frugiperda; EU812749.1:S.exigua; GU090195.1:S.ornithogalli;HQ567862.1:S.dolichos;KJ634312.1:S.triturata;KJ634310.1:S.pulchella;KJ634308.1:S.ochrea;KJ634306.1:S.mauritia;KJ634304.1:S.litura; KJ634300.1:S.littoralis; KJ634289.1:S.eridania;KJ634282.1:S.androgea;KJ634281.1:S.albula;JN261912.1:S.deprivata;KJ392514.1:S.praefica;AB735237.1:S.cilium;HQ950503.1:S.mauritia;JN262118.1:S.hipparis;GU660526.1:S.latifascia;KX860418.1:S.pecten;HM893111.1:S.exempta; HQ950415.1:S.apertura; HQ950412.1: S. picta*) along with Pantnagar sample sequence through Neighbour-Joining tree. The results showed that the Pantnagar sample had 0.00 nucleotide distances with *S. frugiperda* sequence. The polymorphic site observation through sequence alignment (Fig. 6) also indicated that there was no nucleotide polymorphism found in 630bp gene sequence of Pantnagar sample and *S. frugiperda* sequence. The similarity pattern between *S. frugiperda* sequence (NCBI) and Pantnagar sample was identified again through SIAS (Sequence identities and similarities) which too resulted that Pantnagar sample had maximum 100% similarity with *S. frugiperda* sequence (Table 1). The *mtCOI* generated consensus sequence of Pantnagar sample of 630bp was submitted in NCBI GenBank databases as *Spodoptera frugiperda* sequence (Pantnagar) and got the accession No. MN630563 with total 630 codon in which 186 Adenine (A), 256 Thymine (T), 96 Cytosine (C), 92 Guanine (G) were present and the % G~C content was found to be 29.8%.

***S. Frugiperda mtCOI* gene sequence after BLAST-(Pantnagar sample Accession no. MN630563)**

AACATTATATTTTATTTTTGGAATTTGAGCAGGAATAGTAGGTACTTCTTTAAGTTTA-
TTAATTCGAGCTGAATTAGGAACTCCAGGATCTTTAATTGGAGATGATCAAATTTAT-
AATACTATTGTAACAGCCCATGCTTTTATTATAATTTTTTTTATAGTTATACCAATTA-
TAATTGGAGGATTTGGAAATTGACTTGTACCTTTAATATTAGGAGCTCCTGATATAG-
CTTTCCCACGTATAAATAATATAAGTTTTTGACTTTTACCCCCATCTTTAACTTTATTA-
ATTTCTAGTAGCATTGTAGAAAATGGAGCAGGAACTGGATGAACAGTTTACCCCCC-
CCTCTCCTCTAATATTGCTCATGGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTT-
CATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTAACTTTATTACCACTATTATTA-
ATATACGATTAAATAATTTATCATTTGATCAAATACCTTTATTTATTTGAGCTGTAGG-
TATTACCGCATTTTTATTATTATTATCTTTACCTGTTTTAGCTGGAGCTATTACTATAT-
TACTTACTGATCGAAATCTAAATACATCATTTTTCGATCCTGCAGGAGGAGG-

4. Discussion

In India, *Spodoptera frugiperda* was first time reported in 2018, as an invasive pest of maize crop in Karnataka [19] and identified through molecular marker *mtCOI* gene. Later from other states of India like Tamil Nadu, Chhattisgarh, Gujarat, Bihar and Maharashtra incidences of *S. frugiperda* were reported for the first time [22, 8, 21, 1, 5]. The findings of present investigation are in close conformity with workdone under identification of *S. frugiperda* [13, 23, 1, 20, 10] which reported inverted 'Y' shaped yellow colored marking on *S. frugiperda* head and four black dots in square pattern on eighth abdominal segment. Also, previous researchers [19] [8] have reported the characteristics of male adult forewings with contrasting markings, oblique spots and white patch at the apical end of forewing. These results were in close conformity with the characterization of male adult in the present study. This confirmed that the pest sample from Pantnagar was *S. frugiperda* on the basis of morphological identification.

Also, based on molecular studies it was confirmed that the molecularly analyzed Pantnagar sample belong to *S. frugiperda* species. The *mtCOI* gene analysis of Pantnagar population showed 100% similarity with gene sequence of *S. frugiperda* of Canada (NCBI accession no. GU095403.1 [13]. Thus the present study confirmed the presence of *S.* frugiperda through identification of pest on the basis of both morphological and molecular characters and reported S. frugiperda, in maize crop as a new invasive pest of Uttarakhand state.

5. Conclusion

Both morphological and molecular studies confirmed that the pest sample from Pantnagar is of *S.* frugiperda (J.E. Smith). Hence, in this regard, the present document is the first report on the presence of S. frugiperda in maize crop at Pantnagar in Uttarakhand. As it has been observed that S. frugiperda have devastating effect on field crops, it is thus a potential threat for farmers. Therefore, management of this pest, assessment of its impact on maize yield, early warning symptoms, IPM - led approaches are very important to be studied further. Also, identification of potential natural enemies for biological control of *S. frugiperda* need to be explored and implemented as soon as possible before the pest spread all over the India.

Fig 1: Spodoptera frugiperda (J.E. Smith) infesting maize crop at N.E.B. CRC, Pantnagar

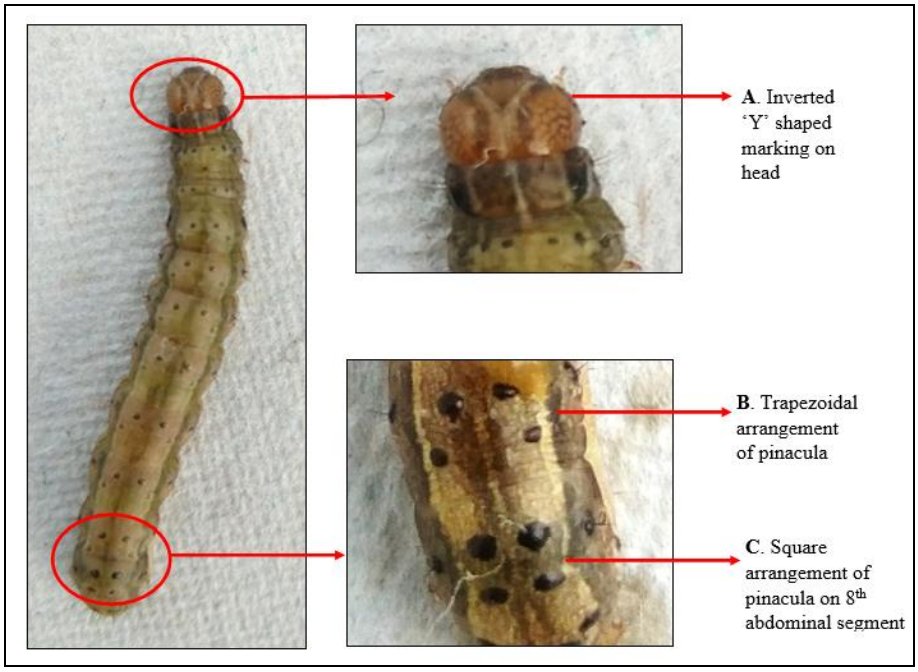

Fig 2: Arrangement of dorsal Pinacula on Spodoptera frugiperda larva.

Fig 3: Pupal Cremaster with two spines

Fig 4: Male adult with white apical patch in forewing

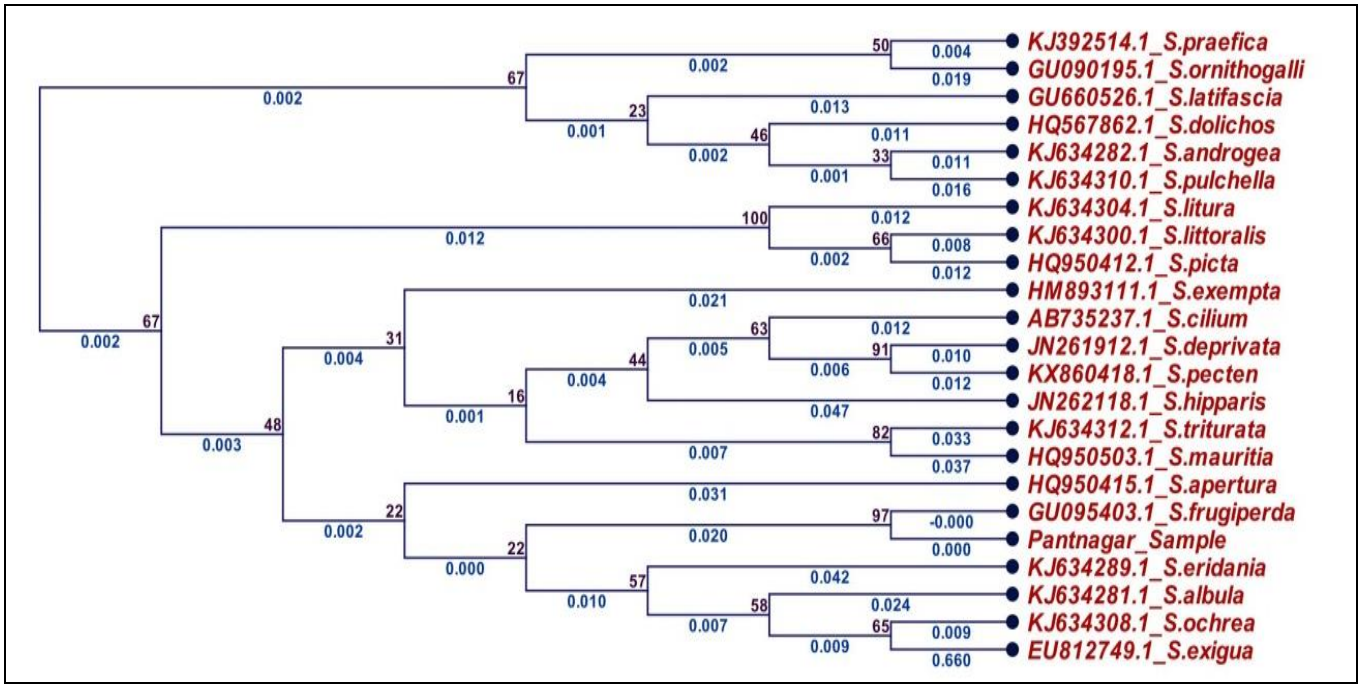

Fig 5: Neighbour-joining tree of phylogenetic relation among the 23 species of *Spodoptera* genus along with Pantnagar pest sample on the basis of *mtCOI* gene. Bootstrap values given at the nodes

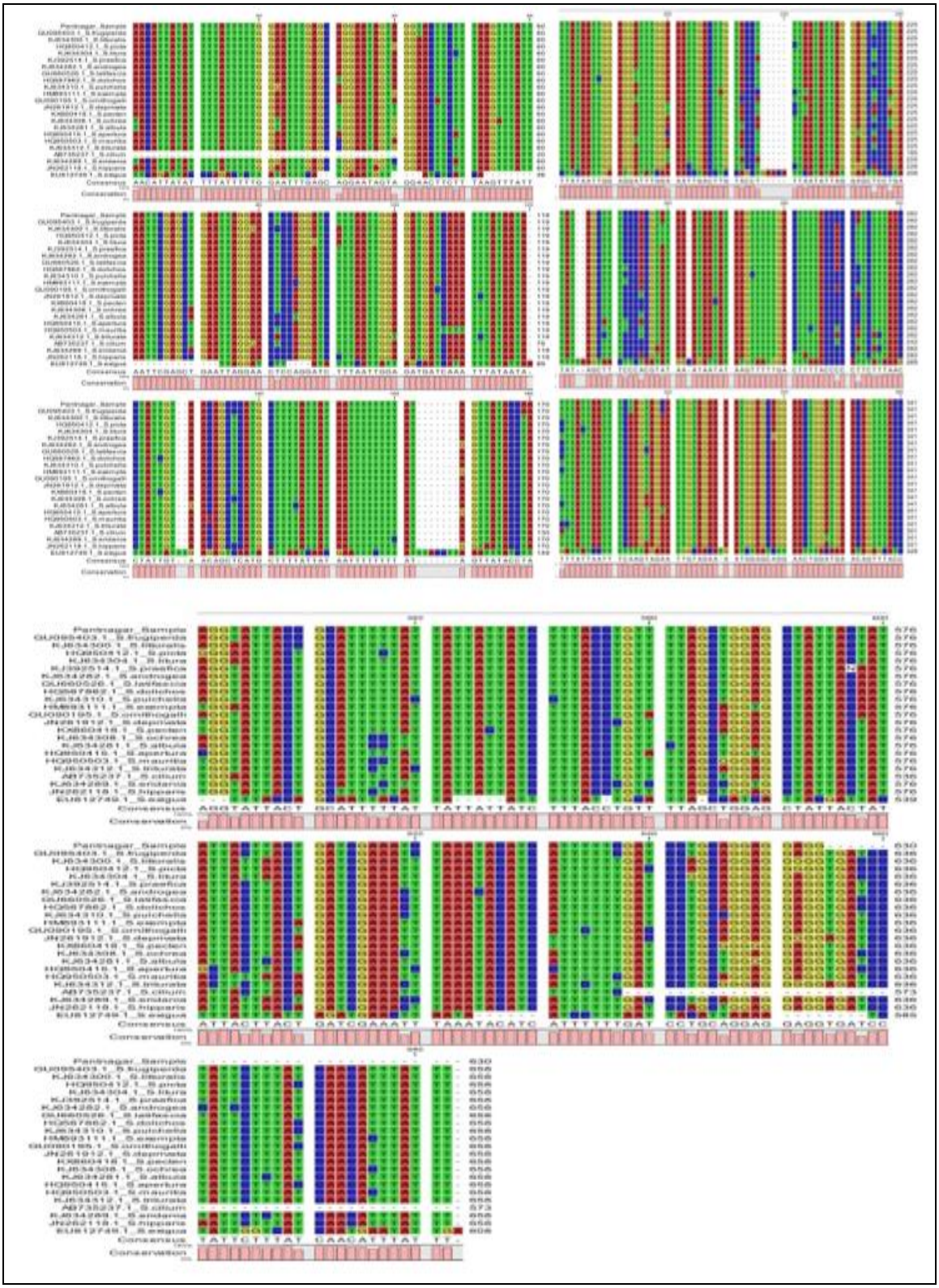

Fig 6: Nucleotides polymorphism in mtCOI gene sequence on the basis of alignment among the 23 species of Spodoptera genus along with Pantnagar pest sample

Table 1: Similarities Matrix (%) between 23 species of *Spodoptera* genus along with Pantnagar pest sample through SIAS (Sequence identities and similarities)

Pantnagar Sample	**100%**				
GU095403.1_S.frugiperda	100%	100%			
EU812749.1_S.exigua	31.57%	31.57%	100%		
GU090195.1_S.ornithogalli	95.55%	95.59%	32.89%	100%	
HQ567862.1_S.dolichos	95.71%	95.59%	31.57%	96.5%	100%
KJ634312.1_S.triturata	93.01%	93.16%	30.09%	93%	93.61%
KJ634310.1_S.pulchella	95.55%	95.59%	32.07%	95.89%	97.26%

KJ634308.1_S.ochrea	94.76%	94.68%	31.08%	93.76%	94.98%
KJ634304.1_S.litura	95.55%	95.59%	31.57%	95.13%	96.04%
KJ634300.1_S.littoralis	95.55%	95.59%	32.4%	95.13%	96.04%
KJ634289.1_S.eridania	93.17%	93.16%	32.23%	92.24%	92.55%
KJ634282.1_S.androgea	95.87%	95.74%	31.9%	96.2%	97.56%
KJ634281.1_S.albula	93.65%	93.76%	31.25%	93.31%	94.22%
JN261912.1_S.deprivata	94.6%	94.68%	32.23%	94.52%	94.98%
KJ392514.1_S.praefica	96.66%	96.8%	32.23%	97.56%	97.56%
AB735237.1_S.cilium	26.52%	26.52%	29.84%	26.52%	26.35%
HQ950503.1_S.mauritia	93.17%	93.31%	31.9%	93.16%	93.61%
JN262118.1_S.hipparis	91.9%	91.94%	32.07%	91.94%	92.4%
GU660526.1_S.latifascia	95.87%	95.89%	32.23%	96.65%	97.56%
KX860418.1_S.pecten	94.12%	94.37%	31.74%	94.07%	95.28%
HM893111.1_S.exempta	94.76%	94.83%	31.9%	94.98%	95.74%
HQ950415.1_S.apertura	95.07%	94.98%	32.07%	94.22%	95.28%
HQ950412.1_S.picta	94.6%	94.52%	32.56%	94.98%	95.89%
	Pantnagar_Sample	GU095403.1_S.frugiperda	EU812749.1_S.exigua	GU090195.1_S.ornithogalli	HQ567862.1_S.dolichos

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