RESEARCH ARTICLE

Genetic Characterization of Fall Armyworm (*Spodoptera frugiperda*, J.E. Smith) Feeding on Pearl Millet (*Pennisetum glaucum* L.R.Br.) in Tamil Nadu

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ABSTRACT

In the present study, molecular characterization was carried out at the Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore to identify the strains of fall armyworm collected from pearl millet crop at four different locations in Tamil Nadu, India. PCR-RFLP profile of mitochondrial *COI* fragment exhibited the presence of both 'C' and 'R' strains of FAW. Mt*COI* region analysis revealed that, thirteen samples showed 'R' strain identity, and others assumed 'C' strain identity. Sequence analyses of mt*COI* region of FAW feeding on pearl millet showed nucleotide variations in 22 positions. The Strain, ON142640 (Pearl millet Vridhachalam India) totally diverged from all populations subjected to phylogenetic analysis in this study and was suspected to be an inter-strain (R/C) hybrid. The per cent identity analysis of *S. frugiperda* ranged from 99% to 100% with previously deposited sequences in the NCBI-GenBank database.

Keywords: *Fall armyworm; Pearl millet; Molecular characterization; Strain; PCR-RFLP; Phylogenetic analysis*

INTRODUCTION

Pearl millet (*Pennisetum glaucum* L.R.Br.), popularly known as "Candle millet" or "Indian millet" grown widely in dry areas of the arid and semi-arid tropics where no other cereal can thrive (Reddy *et al*., 2021). It covers 6.93 million hectares and produces an average of 8.61 million tonnes per year, with productivity of 1,243 kg ha^{-1} (Directorate of Millets Development, 2020). Villupuram, Thoothukudi, Tiruvannamalai, Virudhunagar, Cuddalore, Vellore, and Madurai are the major pearl millet growing districts of Tamil Nadu (Season and Crop Report, 2022).

About 300 insects have been found feeding on pearl millet in various regions of the world (Sharma *et al*., 1981), although the number that can cause major damage is likely less than a dozen, and those that cause serious damage on a continuous annual basis are even fewer (Verma, 1980). Recently, the invasive fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) has been found infesting the pearl millet crop. Apart from maize, sorghum (60.1 per cent) was the most popular millet host, followed by pearl millet (41.4 per cent), barnyard millet (22.9 per cent), and finger millet (10.2 per cent) (ICAR-IIMR, 2019). FAW was reported on maize, sorghum, pearl millet, and finger millet in different districts of Andhra Pradesh from August to November, 2018 (Bhavani *et al*., 2019). This pest's voracious feeding and long-distance flight behavior pose a serious threat to grain production. The pest feeds on more than 350 different plant species (Montezano *et al*., 2018). Though it infests a wide range of plants, it has a clear affinity for graminaceous plants (Casmuz *et al*., 2010).

The races in FAW can only be distinguished molecularly, not morphologically (Prowell *et al*., 2004; Nagoshi *et al*., 2007). FAW is genetically divided into two races: one is the corn-designated 'C' strain, which feeds mostly on maize, sorghum, cotton, and pulses, and the other is the rice-

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designated 'R' strain, which favors rice, sugarcane, and other short grasses (Pashley, 1998; Nagoshi *et al*., 2016). Mitochondrial *COI* markers are frequently employed in insects to identify and determine population structure, and they are known for maternal inheritance, high copy number, conservative nature *etc*. (Mauro *et al*., 2006). Identifying fall armyworm strains is aided by PCR-RFLP investigation of the mitochondrial *COI* gene using restriction enzymes (Meager and Nagoshi, 2003; Nagoshi *et al*., 2018). In this context, the current work used genetic approaches to identify the fall armyworm strain(s) feeding on pearl millet.

MATRIALS AND METHODS

Insect collection and preservation

FAW larvae (5 nos. in each location) were collected from pearl millet crop in four different locations and are listed in table 1. The collected insects were preserved individually in microfuge tubes and stored at -20 ºC until taken for DNA extraction.

DNA preparation and quantification

The genomic DNA of FAW was isolated using the CTAB technique (cetyl trimethylammonium bromide) (Doyle and Doyle, 1990). DNA was extracted from the prolegs of larvae. Tissues were homogenized thoroughly in 200 mL of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4M NaCl, 0.02M EDTA, two per cent CTAB, and 0.2 per cent mercaptoethanol), maintained at 65°C for an hour, then centrifuged for 15 minutes at 12,000 rpm. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and centrifuged at 10,000 rpm for 10 minutes to separate the phases. This process was repeated with the top aqueous phase transferred to a 1.5 µL sterile microfuge tube. About 200 µL of ice-cold isopropanol was added to this aqueous phase to precipitate the DNA, which was then incubated at -20 ºC overnight. After incubation, the DNA pellet was obtained by centrifugation at 10,000 rpm for 10 minutes at 4 ºC. The DNA pellet was washed twice with absolute ethanol and air-dried. The pellet was suspended in 30-50 µL of 1X TE buffer and stored at -20 ºC until used for PCR analysis. The isolated DNA was analyzed quantitatively and qualitatively by Nanodrop Spectrophotometer (Genova, USA) and agarose gel electrophoresis, respectively.

PCR analysis

The PCR amplification of DNA obtained from FAW larvae in various places was done. To run PCR, DNA samples were diluted with 1X TE buffer to obtain a working solution of 50-100 ng/L (working aliquot). The primers were synthesized from Bioserve Biotechnologies Private Limited, Hyderabad, India. The primer pair JM 76F (5'GAGCTGAATTAGG(G/A) ACTCCAGG3') and JM 77R (5'ATCACCTCC(A/T) CCTGCAGGATC3') were used to amplify the Mt*COI* region, yielding a 568-bp fragment. PCR amplification for all segments was carried out with 25 µL reaction mixture (12.5 µL of 2X Thermo Scientific PCR Master Mix, 1 µL forward primer, 1 µL reverse primer, 9.5 µL nuclease-free water and 1 µL DNA template) using Eppendorf thermocycler with ambient thermo-cycling profile at 94 ºC (1 min) followed by 33 cycles of 92 ºC (45 s), 56 ºC (45 s), 72 ºC (1 min) and a final segment of 72 ºC for 3 min. PCR amplified products were fractionated on a 2% PCR grade agarose horizontal gel and documented in the Bio-Rad Gel Doc XR imaging system.

RFLP analysis

To determine strain identity, PCR products of the *COI* region were digested with *Sac*I and *Msp*I restriction enzymes purchased from Thermo Fisher Scientific and New England Biolabs, respectively. A reaction mixture including 10 µL PCR product, 2 µL buffer, 1 µL enzyme, and 17 µL nuclease-free water was prepared, incubated at 37 °C for 2 hours, fractionated on a 2% agarose gel, and documented.

Sequencing and GenBank submissions

For double-pass DNA sequencing, the unpurified PCR amplified product (20 µL) was sent to Bioserve Biotechnologies India Pvt Ltd. in Hyderabad, Telangana, India. Bioedit software (version 7.25) was used to align and edit the raw sequence and construct the sequence identity and nucleotide difference count matrix. A dendrogram was created by aligning the select partial nucleotide sequences of Mt*COI* of *S. frugiperda* using Clustal W, and a phylogenetic tree was constructed using MEGA11 (version 11.0). The evolutionary distance between the sequences was determined using a bootstrap analysis with 1000 replications using the neighbor-joining method (Tamura *et al*., 2021).

RESULTS AND DISCUSSION

Strain identity analysis using PCR-RFLP

The PCR analysis results revealed that the expected fragment size of about ~568 bp from mt*COI* region was amplified in all the tested samples (Fig. 1A). Restriction enzymatic analysis of mt*COI* segments exposed that, *Sac*I enzyme cut the DNA into ~413 bp and ~155 bp fragments in Rice (R) strain whereas DNA remains uncut (~568 bp) in Corn (C) strain (Fig. 1B). Oppositely, *Msp*I enzyme cut the DNA into ~461 bp and ~107 bp fragments in 'C'

Table 1. Details on the FAW collected from pearl millet in different locations of Tamil Nadu

S. No.	Location	District	Life stage	Sample size	Date of collection	GPS information	NCBI Accession number(s) received
1.	New Area, TNAU	Coimbatore	Four Larvae and one pupa	05	05.10.2019	Latitude: 11.07 N Longitude: 76.99 E	OM462844, OM465797, OM462675, OM463624
2.	KVK, Ramanathapuram	Ramanathapuram	Larvae	05	24.09.2020	Latitude: 11.53 N Longitude: 79.34 E	ON114178
3.	Thalavaipuram	Virudhunagar	Larvae	05	24.09.2020	Latitude: 9.39 N Longitude: 77.50 E	ON141589
4.	RRS, Vridhachalam	Cuddalore	Larvae	05	24.09.2020	Latitude: 11.53 N Longitude:79.34 E	ON142640

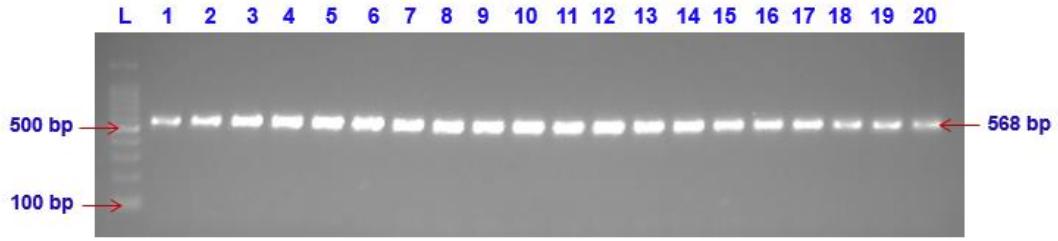

Fig. 1A Agarose gel displaying PCR products of FAW collected from pearl millet

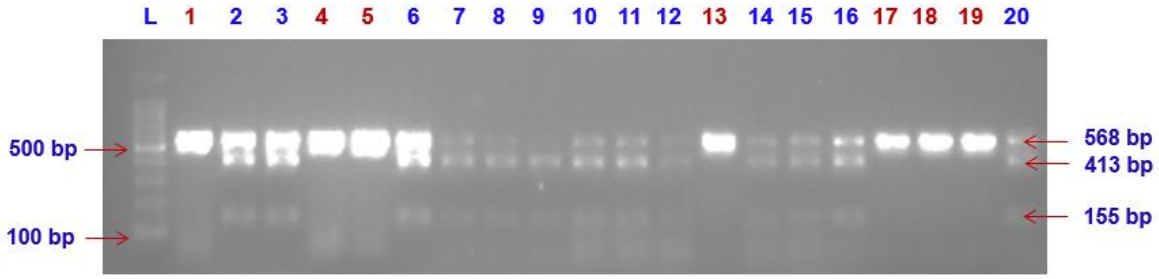

Fig. 1B Strain-specific RFLPs from the mitochondrial *COI* gene *Sac*I digestion

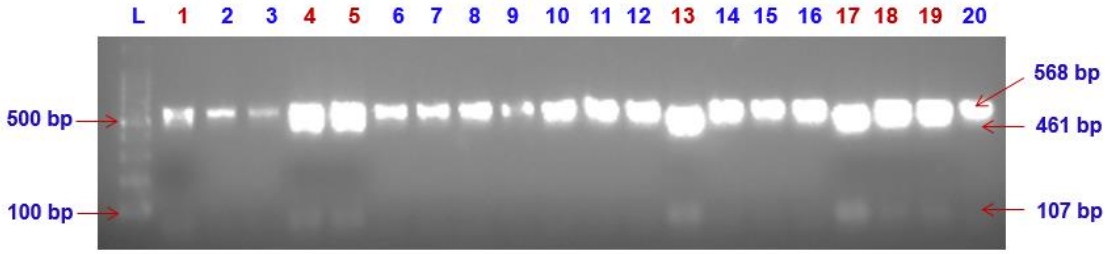

Fig. 1C Strain-specific RFLPs from the mitochondrial *COI* gene *Msp*I digestion
Lane L– Ladder (100 bp); Lanes 1-5 – TNAU, Coimbatore; Lanes 6-10 – KVK, Ramanathapuram; Lanes 11-15 – Thalavaipuram, Virudhunagar; Lanes 16-20 – RRS, Vridhachalam

strain while DNA remains uncut (~568 bp) in 'R' strain (Fig. 1C). Out of 20, 13 samples showed 'R' strain identity, and seven assumed 'C' strain identity. The samples from Coimbatore and Vridhachalam comprised three corn and two rice strain individuals. Samples from Ramanathapuram possessed four rice and one corn strain. All the five samples collected from Thalavaipuram assumed rice strain identity. Studies by Nagoshi *et al*. (2007) and Nagoshi and Meager (2004) implied that the rice strain populations of FAW prefer to feed on millets, grass species *etc*. and corn strain populations prefer corn and sorghum for feeding.

Opposing results to findings were also known to occur. Irrespective of host plants, FAW reported on maize, sweet corn and sorghum assumed 'R' strain identity with minimal genetic diversity exhibiting no host/ location-specific variations (Swamy *et al*., 2018). The presence of 'R' strain in all the FAW populations obtained from maize and sorghum crops in Swaziland was confirmed (Assefa, 2019). The FAW samples collected from Banten, Indonesia were assigned to the 'R' strain (Sartiami *et al*., 2019).

Nucleotide variations

Nucleotide variations were found at different places in the mt*COI* region of FAW populations feeding on pearl millet (Fig. 2). The highest variation in the nucleotides was observed in OM462844 followed by OM465797 and OM463624 collected from pearl millet field at TNAU, Coimbatore, with 22, 21, and 5 bases, respectively. The strain from Vridhachalam (ON142640) possessed variations in 14 places. Nucleotide variations at four (254, 305, 540, 588) and 14 positions (72, 117, 171, 207, 253, 258, 305, 489, 540, 564, 570, 589, 600, 634) when compared with the NCBI accessions U72977 and U72974 for 'C' strain of FAW collected from maize, sweet corn and sorghum crops in India (Swamy *et al*., 2018). In the study, nucleotide variations were observed in 22 (72, 108, 159, 390, 428, 429, 430, 431, 434, 435, 436, 438, 441, 465, 471, 501, 502, 508, 510, 512, 513 and 516) and 21 (72, 108, 159, 390, 428, 429, 430, 431, 434, 435, 436, 438, 441, 465, 471, 501, 502, 510, 512, 513 and 516) positions in strains, OM462844 (Pearl millet 4 TNAU Coimbatore India) and OM465797 (Pearl millet 5 TNAU Coimbatore India) which are suspected to be 'C' strain populations. Though the strain OM463624 (Pearl millet 2 TNAU Coimbatore India) is grouped in 'R' strain populations, nucleotide variations are found to appear in five places *viz*., 34, 36, 61, 69 and 84, and hence more genetic distance was ensured. Also, the strain ON142640 (Pearl millet Vridhachalam India) is found to have nucleotide variations in 12 places *viz*., 72, 108, 159, 381, 390, 445, 459, 465, 468, 486, 487, 512, 513 and 516. The variations in nucleotides may not be due to the host shift but owing to the inter-mating nature of the populations.

Phylogenetic tree analysis

The data matrix included 39 sequences from Mt*COI* genome of two taxa: one taxon includes 38 FAW sequences, of them 13 across the globe and 25 from India including the test sequences (Fig. 3). Another taxon, *S. exigua* served as an out group. The phylogenetic tree built using maximum livelihood (ML) following Tamura 3-parameter model showed that *S. frugiperda* divides into two clear clades in which the strain, ON142640 (Pearl millet Vridhachalam India) totally diverged from all the FAW sequences subjected for analysis. The second clade contained most FAW sequences that were clustered and closely related. The rest of the strains are grouped under this clade and are further divided into two clusters. One cluster contained 'R' strain individuals and another comprised 'C' strain individuals. The strains *viz*. OM462675 (Pearl millet 3 TNAU Coimbatore India), ON114178 (Pearl millet Ramanathapuram India), OM463624 (Pearl millet 2 TNAU Coimbatore India) and ON141589 (Pearl millet Thalavaipuram India) possessed 'R' strain identity whereas, OM462844 (Pearl millet 4 TNAU Coimbatore India) and OM465797 (Pearl millet 5 TNAU Coimbatore India) showed 'C' strain identity. The per cent identity of FAW ranged from 99 to 100. The sequence difference count matrix for the FAW sequences ranged from 0 to 47. The FAW population feeding on sugarcane from Anakapalle formed a separate clade in dendrogram that showed 99.75% resemblance to the Mexico population with 'C' strain identity (Bhavani *et al*., 2019). Similarly, Chormule *et al*. (2019) claimed that the FAW occurs in sugarcane crops from Kolapur, Maharashtra, assumed 'C' strain identity and close resemblance with USA FAW populations (U72974, U72975 and U72976). In the present investigation also, the two strains *viz*. OM462844 (Pearl millet 4 TNAU Coimbatore India) and OM465797 (Pearl millet 5 TNAU Coimbatore India assumed 'C' strain identity and found close resemblance with MT791636 (Panaji Goa India), MT901173 (Kerala India), OM478579 (Ragi 1 Vridhachalam India), OM478578 (Ragi 1 Salem India), MT073266 (Maize Bangladesh), MK318311 (Maize Mexico), KY472248 (Maize Ghana Northern region), KY472251 (Maize Ghana Volta region). The strain, ON142640 (Pearl millet Vridhachalam India) totally diverged from all populations subjected to phylogenetic analysis in this study. This particular sequence has formed a separate clade and could be suspected as an inter-strain hybrid (R/C).

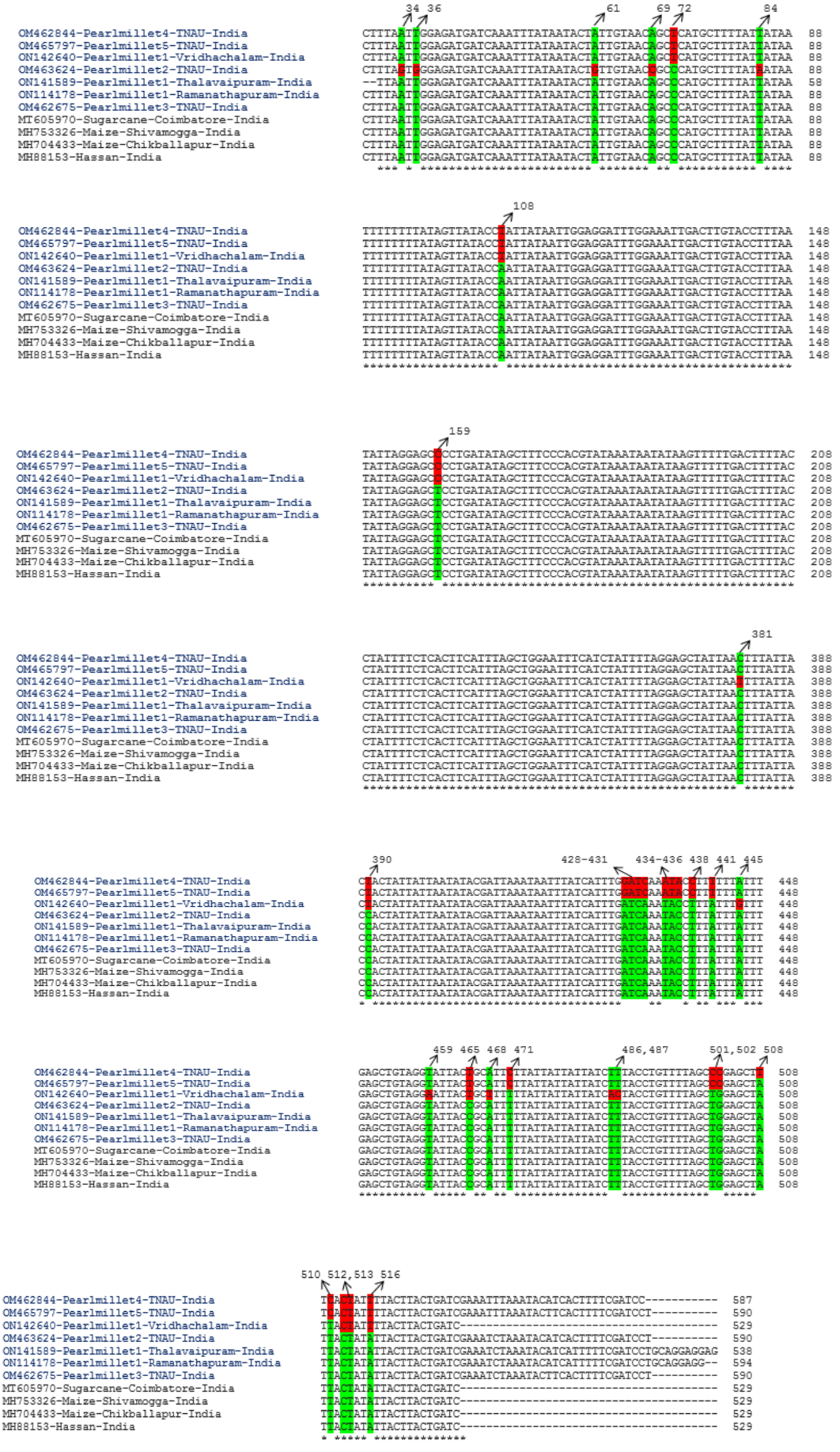

Fig.2. Nucleotide variations in the test and other FAW sequences

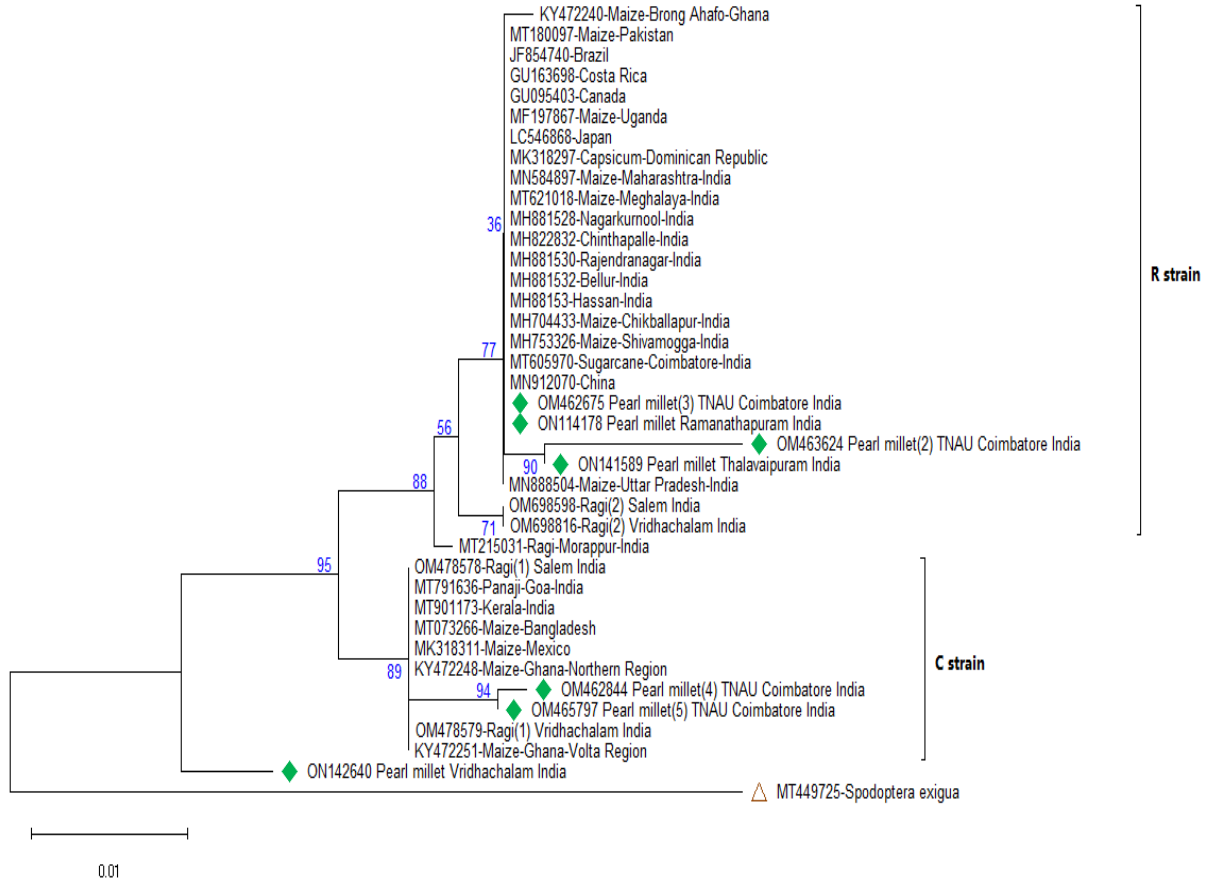

Fig.3 Phylogenetic tree showing the relationships among the FAW strains. Bootstrap support is indicated on the branches

CONCLUSION

The 'R' strain has colonized maize, sorghum, sweet corn, and many other crops in India, while the 'C' strain has begun to adapt sugarcane, finger millet and pearl millet. As a result, both groups migrated to India and colonized a variety of crops. Because both strains are interbreeding populations, hybridized strains (R/C or C/R) could emerge, feeding on a wide range of hosts and causing damage to a variety of agricultural crops.

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Ethics statement

No specific permits were required for the described field studies because no human or animal subjects were involved in this research

Originality and plagiarism

All the data in this manuscript are our original works. This work is not copied from any other article and has not been published anywhere.

Consent for publication

All the authors agreed to publish the content

Competing interests

There was no conflict of interest in the publication of this content

Data availability

All the data of this manuscript are included in the MS.

Author contributions

Idea conceptualization-NS, Experiments- TS, TS, NS, EK, Guidance -NS, SM, VB, EK, JSK, RR, Writing original draft - TS, NS, SM, VB, EK, JSK, RR, TS, Writing- reviewing &editing - TS, NS, SM, VB

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