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Genetic characterization of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) infesting maize in middle Gujarat

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Abstract
The destructive and invasive fall armyworm (FAW) has evoked heavy damage to maize crops throughout the world and also planted its roots in almost every Indian state. Considering the importance of FAW and in order to infer the genetic characterization of *Spodoptera frugiperda* infesting maize, a survey was conducted and samples from 9 districts of middle Gujarat region *viz*., Panchmahal, Mahisagar, Dahod, Anand, Kheda, Chhotaudepur, Vadodara, Ahmedabad and Botad were collected. The characterization of fall armyworm infesting maize in nine districts of middle Gujarat *viz*., Panchmahal (OR144565), Mahisagar (OR145093), Dahod (OR145095), Anand (OR225961), Kheda (OR145135), Vadodara (OR145136), Chhotaudepur (OR145138), Ahmedabad (OR145322) and Botad (OR145139) based on mitochondrial *COX1* gene sequences revealed the categorization under the Corn-strain of *S. frugiperda*. The size of consensus sequences was varied from 656 to 672 base pairs. Subsequent phylogeny analysis indicated prevalence of two divergent population of C-strain of *S. frugiperda* in middle Gujarat. The fall armyworm specimens collected from Chhotaudepur, Vadodara and Dahod were characterized in clade 1, whereas the specimens collected from Panchmahal, Mahisagar, Anand, Kheda, Ahmedabad and Botad were characterized in clade 2. Both the clades exhibited close similarity with geographically isolated locations indicating possible source of introduction as 2 haplotypes were prevalent in fields of maize from middle Gujarat.

Keywords: Fall armyworm, *S. frugiperda*, genetic characterization, phylogeny, C-strain

1. Introduction
Zea mays Linnaeus (maize) is amongst the invaluable staple food alongside its utilization as straw for cattle's and versatile utilities as firewood and for structuring purposes (Abebe and Feyisa, 2017 [1]; Mohapatra and Sisodiya, 2022) [13]. Maize belongs to the Poaceae family, and is extensively cultivated throughout tropic and temperate zones. And faces vagaries of about 130 species of insect-pests in India alone (Atwal and Dhaliwal, 2002) [4]. One such important invasive pest is the fall armyworm (FAW), *S. frugiperda* (Lepidoptera: Noctuidae), which was reported with a mild to severe infestation in West and Central Africa in early 2016 (Goergen *et al*., 2016) [6]. Consequently, its first report in India was published during June, 2018 by Sharanabasappa *et al*. (2018) [20] on maize from Karnataka while, in Gujarat the first occurrence was reported Sisodiya *et al.* (2018) [22] on sweet corn from Anklav taluka of Anand district and now, it has infested every maize field in the state.
FAW is a highly polyphagous pest that feeds insatiably on maize leaves and survives on more than 76 plant families and 353 crop plants (Montezano *et al*., 2018) [15] belonging to cereals (maize, wheat, sorghum, millet, rice) and pasture grasses as well as cotton, sugarcane, sweet potato, potato, ginger, tomato, chrysanthemum, tobacco, crucifers, spinach, cucumber, cucurbits, cowpea, soybean, common bean, groundnut, banana *etc* (Anonymous, 2018) [2] and many such insects have also started making havoc in Gujarat like the recent introduction of black thrips (Lodaya *et al*., 2022) [10]. Notably, two strains of the fall armyworm occur, the 'rice strain' or R-strain (R=rice) and the 'maize strain' or C-strain (C=corn). C-strain is more prevalent that feeds on maize leaves and stem. Since, R-stain and C-strain do not differ morphologically, molecular characterization is the last resort to distinguish the strains (Gurjar *et al*., 2023) [7]. Thus, in order to characterize the FAW prevalent in Gujarat, a field study was carried out for scouting, collecting and genetically identifying with the following methodology.

2. Materials and Methods

2.1 Sample Collection

Maize fields infested by fall armyworm were surveyed and fall armyworm larvae were collected from maize growing districts *viz.,* Panchmahal, Mahisagar, Dahod, Anand, Kheda, Vadodara, Chhotaudepur, Ahmedabad and Botad of middle Gujarat at peak infestation level in *kharif*, 2021 and *rabi*, 2021-22 (Plate 1). The larvae of fall armyworm were collected in plastic vial (5 cm height and 4 cm diameter) with pin holes on the vial cap and were labelled.

2.2 Genomic DNA Isolation

Mature well grown larvae were collected randomly from 10 fields of each district and preserved in 70 per cent alcohol and geographical location *viz.* longitude and latitude were recorded (Table 1). These larvae were stored under - 20 °C until further DNA. For isolation of total DNA, larva was dissected and the contents of gut were removed completely to avoid any DNA contamination owing to gut food. The resulting skin and legs were utilised to isolate total DNA following the described procedure of Doyle and Doyle (1990) [5] with minor modifications as detailed in Table 2.

Table 1: Details of maize fields surveyed in districts of middle Gujarat during July, 2021 to January, 2022 (*Kharif*, 2021 to *Rabi*, 2021-22)

Sr. No.	District	Taluka (No. of samples collected)	Latitude	Longitude
1.	Panchmahal	Ghoghamba (1)	22°57'21"N	73°64'07"E
		Godhra (2)	22°79'65"N	73°71'42"E
			22°77'59"N	73°62'25"E
		Halol (2)	22°49'57"N	73°48'72"E
			22°53'39"N	73°46'90"E
		Jambughoda (1)	22°35'84"N	73°73'59"E
		Kalol (1)	23°25'45"N	72°51'91"E
		Morwa (1)	22°92'65"N	73°84'37"E
		Shahera (2)	23°00'71"N	73°62'12"E
			22°91'48"N	73°64'69"E
2.	Mahisagar	Balasinor (2)	22°95'00"N	73°34'23"E
			22°96'70"N	73°33'89"E
		Kadana (2)	23°28'96N	73°83'82"E
			23°27'45N	73°80'23"E
		Khanpur (1)	23°28'78"N	73°68'25"E
		Lunawada (2)	23°11'40"N	73°59'99"E
			23°06'89"N	73°60'36"E
		Virpur (1)	23°19'27"N	73°48'40"E
		Santrampur (2)	23°15'41"N	73°96'10"E
			23°19'21"N	73°90'04"E
3.	Dahod	Dahod (2)	22°84'42"N	74°17'26"E
			22°93'78"N	74°32'36"E
		Dhanpur (1)	22°63'92"N	74°08'84"E
		Devgadhbaria (1)	22°81'90"N	73°91'10"E
		Fatepur (1)	23°14'70"N	74°07'41"E
		Garbada (1)	22°68'70"N	74°30'96"E
		Jhalod (2)	23°03'72"N	74°13'28"E
			23°00'88"N	74°15'59"E
		Limkheda (2)	22°83'58"N	73°99'03"E
			22°83'44"N	74°04'76"E
4.	Anand	Anand (2)	22°29'54"N	72°57'20"E
			22°36'11"N	72°59'15"E
		Anklav (2)	22°18'46"N	72°58'39"E
			22°23'06"N	73°00'16"E
		Borsad (1)	22°21'23"N	72°53'49"E
		Khambhat (1)	22°23'25"N	72°39'14"E
		Petlad (1)	22°25'08"N	72°47'55"E
		Sojitra (1)	22°30'00"N	72°43'26"E
		Tarapur (1)	22°28'57"N	72°39'26"E
		Umreth (1)	22°41'46"N	73°01'59"E
5.	Kheda	Kapadvanj (2)	23°01'90"N	73°06'49"E
			23°03'94"N	73°10'56"E
		Kathlal (1)	22°87'28"N	72°98'07"E
		Kheda (2)	22°75'48"N	72°68'99"E
			22°74'41"N	72°68'17"E
		Mahudha (1)	22°81'53"N	72°93'65"E
		Matar (1)	22°70'69"N	72°66'64"E
		Mehmedabad (1)	22°60'18"N	72°55'48"E
		Nadiad (1)	22°70'80 "N	72°84'68"E
		Thasra (1)	22°80'73"N	73°21'69"E
6.	Vadodara	Dabhoi (2)	22°14'47"N	73°42'52"E
			22°12'35"N	73°41'34"E
		Karjan (1)	22°05'10"N	73°10'96"E

		Padra (2)	22°24'10"N	73°07'34"E
			22°24'56"N	73°09'21"E
		Savli (2)	22°56'31"N	73°21'87"E
			22°56'98"N	73°22'43"E
		Sinor (1)	21°91'38"N	73°33'51"E
		Vaghodia (1)	22°30'83"N	73°40'40"E
		Vadodara (1)	22°33'45"N	73°13'65"E
7.	Chhotaudepur	Bodeli (2)	22°29'44"N	73°77'60"E
			22°31'72"N	73°80'81"E
		Chhotaudepur (2)	22°31'21"N	74°00'73"E
			22°29'70"N	74°01'72"E
		Jetpur pavi (2)	22°20'17"N	73°90'84"E
			22°50'23"N	73°79'80"E
		Kavant (1)	22°09'56"N	74°05'63"E
		Naswadi (1)	22°04'41"N	73°73'99"E
		Sankheda (2)	22°17'28"N	73°57'60"E
			22°16'87"N	73°58'23"E
8.	Ahmedabad	Ahmedabad (1)	22°94'90"N	72°54'21"E
		Bavla (1)	22°83'30"N	72°38'28"E
		Daskroi (1)	23°14'67"N	72°50'95"E
		Detroj (1)	23°33'80"N	72°18'60"E
		Dhandhuka (1)	22°38'66"N	71°98'87"E
		Dholera (1)	22°25'44"N	72°19'42"E
		Dholka (1)	22°75'10"N	72°45'19"E
		Mandal (1)	23°29'27"N	71°91'91"E
		Sanand (1)	22°99'48"N	72°37'02"E
		Viramgam (1)	23°13'80"N	72°05'31"E
9.	Botad	Barwala (3)	22°15'83"N	71°88'37"E
			22°15'07"N	71°89'87"E
			22°16'23"N	71°89'59"E
		Botad (2)	22°17'33"N	71°65'03"E
			22.19'39"N	71°66'65"E
		Gadhada (3)	21°95'66"N	71°57'30"E
			21°95'80"N	71°58'29"E
			21°97'29"N	71°58'69"E
		Ranpur (2)	22°35'85"N	71°72'42"E
			22°34'45"N	71°70'71"E

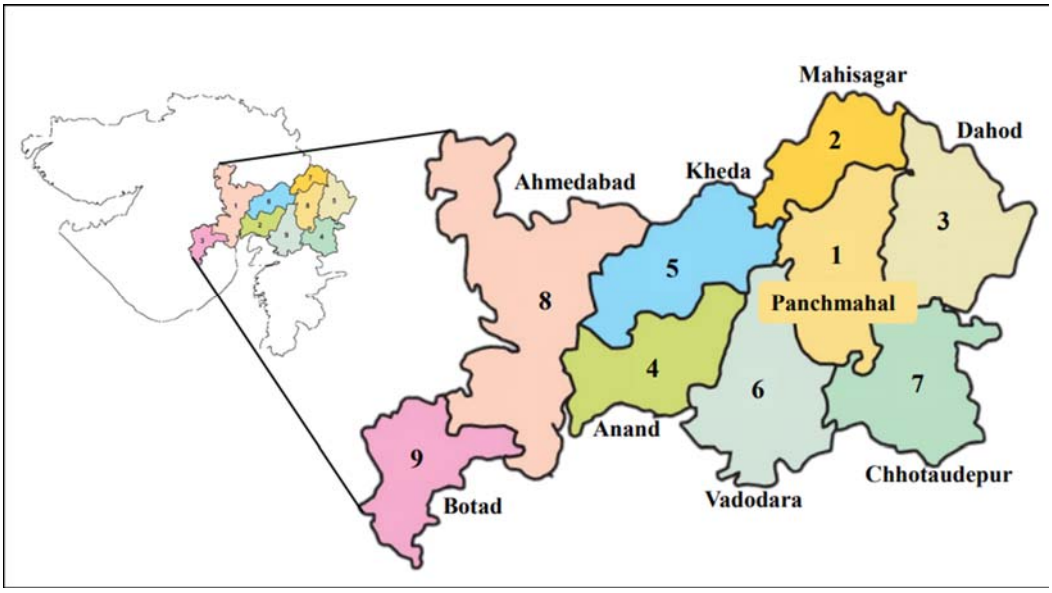

Plate 1: Map of the districts of middle Gujarat surveyed for the collection of fall armyworm, *Spodoptera frugiperda*

Table 2: List and preparation of chemicals used for DNA isolation

Sr. No.	Solution	Method of preparation
1	1 M Tris HCl (pH 8.0), 100 ml	80 cc of distilled water was used to dissolve 12.11 g of Tris base (Himedia). Concentrated HCl was used to get the pH down to 8.0. After adjusting the final volume to 100 ml, the reagent bottle was filled, and autoclaving was used to sterilize it.
2	0.5 M EDTA (pH 8.0), 100 ml	In 80 milliliters of distilled water, 18.60 g of EDTA di sodium salt (Himedia) was dissolved. NaOH pellets were added to bring the pH down to 8.0, and after adjusting the final volume to 100 ml, the reagent container was filled and autoclaved to sterilize it.
3	5 M NaCl, 100 ml	A beaker containing 29.22 g of NaCl (Himedia) was filled, then 50 ml of distilled water was added and thoroughly mixed. Following the full dissolution of the salts, the final volume was changed to 100 milliliters. It was put into a reagent vial and autoclaved to sterilize it.
4	80% Ethanol, 100 ml	After thoroughly mixing 80 milliliters of ethanol with 20 milliliters of distilled water, the mixture was poured into a reagent bottle and kept at 4 °C.
5	Chloroform: Isoamyl alcohol (24:1), 100 ml prepared freshly	Measured and thoroughly mixed, 96 milliliters of chloroform (Qualigens) and 4 milliliters of isoamyl alcohol (Qualigens) were kept at room temperature in a reagent container.
6	Ethidium Bromide (10 mg/l), 1 ml	To make sure the dye had completely dissolved, 10 mg of Ethidium Bromide (Himedia) was added to 1.0 ml of distilled water and the mixture was stirred with a magnetic stirrer. It was put into an eppendorf tube that was amber in color and kept at 40 degrees Celsius.
7	Absolute alcohol + Sodium Acetate (Prepared freshly)	To 10 milliliters of Absolute Alcohol, add 1 milliliter of sodium acetate.
8	1x TE buffer (0.1 mM), 100 ml 10 mM Tris HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)	200 µl of EDTA (0.5 M) and 1.0 ml of Tris HCl (1 M) were taken, and distilled water was added to bring the final volume down to 100 ml. Everything was well mixed, autoclaved, and kept at room temperature.
9	TBE buffer 5x (1 liter) pH 8.0	In addition to 20 milliliters of 0.5 M EDTA (pH 8.0), 54.5 grams of Tris base and 27.5 grams of boric acid (Qualigens) were added. Distilled water was added to the final volume of one liter, and the pH was adjusted to 8.0.
10	10% CTAB, 100 ml	To make the final volume of 100 ml, 10 g of CTAB (Himedia) powder was taken and added to boiling water to dissolve it completely.
11	3 M Sodium acetate, 20 ml pH 7.0	8.16 g of Sodium acetate trihydrate salt (Merck) were dissolved in 20 ml of distilled water in the end. ApH of 7.0 was achieved.

2.3 Equipments and materials

1. Mortar and Pestle
2. Centrifuge tubes (50 ml)
3. Micro centrifuge tubes (1.5 ml)
4. Thermostat
5. Water bath
6. Freezer (-20 °C)
7. Refrigerated centrifuges (Beckman GPR)

2.4 Genomic DNA Extraction Protocol

After being kept in 70% alcohol, the larva (e) were taken out and given a minute to rest on tissue paper so that the excess alcohol would either dry out or absorb. After being submerged in liquid nitrogen, each larva was moved to a 15 ml centrifuge tube and ground with a micro pestle. It was then moved to a 1 ml micro centrifuge tube containing preheated CTAB extraction buffer (Table 3), and it was incubated for 30 to 45 minutes at 65 °C with periodic mixing. Following the incubation period, the tubes were allowed to reach room temperature before adding the phenol, chloroform, and isoamyl alcohol mixture (25:24:1) and mixing by inversion for a duration of 15 minutes. The suspension was centrifuged for 30 minutes at 4 °C at 8000 rpm. After transferring the supernatant to a new tube, the same volume of ice-cold isopropanol was added, gently mixed by inversion, and the mixture was left in the freezer for the next day at a temperature of -20 °C to precipitate DNA. After transferring the transparent aqueous phase to a fresh micro centrifuge tube, the DNA pellet was air-dried after being briefly centrifuged to separate it from the aqueous phase. After that, 100–150 TE (10 mM Tris 1 mM EDTA) buffer was used to dissolve the DNA.

Table 3: Preparation of extraction buffers for DNA isolation

Sr. No.	Buffer	Mode of Preparation
1	CTAB extraction buffer (3%), 10 ml	In a flask, ingredients were combined thoroughly: 1.0 ml of 1 M Tris HCl (pH 8.0), 3.0 ml of 5 M NaCl, 0.8 ml of 0.5 M EDTA (pH 8.0), 0.3 gm of CTAB (w/w), 0.1 g of PVP (w/w), and 5.1 ml of distilled water. The buffer was incubated at 65 °C to facilitate the dissolution of the PVP and CTAB. Just before using, 0.1 ml (1%) of β-mercaptoethanol (Qualigens) was added to the mixture.
2	1X TE buffer, 100 ml 10 mM Tris HCl (pH 8.0) 0.1 mM EDTA (pH 8.0)	200 µl of EDTA (0.5 M) and 1.0 ml of Tris HCl (1 M) were taken, and distilled water was added to bring the final volume down to 100 ml. Everything was well mixed, autoclaved, and kept at room temperature.
3	TBE buffer 5X (1 litre) pH 8.0	Twenty milliliters of 0.5 M EDTA (pH 8.0) were added to 54.5 grams of Tris base and 27.5 grams of Boric acid (Hi media). Distilled water was added to bring the final volume to one liter, the pH was adjusted to 8.0, and the container was kept at room temperature.

2.5 Purification of Genomic DNA

Purification of the isolated genomic DNA was carried out in accordance with the Maniatis *et al*. (1982) [11] protocol. In order to remove any RNA contamination, RNAse (10 µl / 100

µl) was mixed thoroughly by inversions and added to DNA. The mixture was then incubated at 37 °C for 30 minutes. After centrifuging the mixture for 10 minutes at 4 °C at 8000 rpm, the aqueous phase was moved to a second micro centrifuge, to which two volumes of absolute alcohol were added, and it was kept in the freezer for the entire night at -20 °C. A quick centrifugation was used to pellet the DNA, and the supernatant was thrown away. After washing the pellet with 70% ethanol and centrifuging it for five minutes at 8000 rpm and 4 °C, the alcohol was thrown away and the DNA pellet was allowed to air dry entirely. 50 µl of Tris-EDTA was used to dissolve DNA, which was then kept at 4 °C. Nine bulked DNA samples were used for PCR analysis after 10 larvae, each representing a location, were combined to create a better representation of each location. A PCR reaction was started with 40 ng/µl of bulk DNA. The concentration of DNA was measured using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality was checked by 0.8 per cent agarose gel electrophoresis before being used as a template in PCR.

2.6 Quality and Quantity of Genomic DNA

DNA quantification is required for PCR-based analysis to be performed. DNA concentration was measured by spectrophotometry at absorbance ratio 260/280 nm, and Nanodrop N.D.1000 (Software V.3.3.0) was used to analyze the results. Using a 1.5% agarose gel for electrophoresis, the quality of the isolated genomic DNA was assessed. Good DNA demonstrated a sharp, compact band in gel electrophoresis, while poor DNA looked smeared.

2.7 PCR Amplification

The diversity analysis of *COXI* gene (Nagoshi *et al*., 2008) [16] from 9 populations of *S. frugiperda* was carried out using RAPD (Random Amplified Polymorphic DNA) primer as described below:

Forward primer (LCO 1490 5'-GGTCAACAAATCATAAAGATATTGG-3')

Reverse primer (HCO 2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3')

Reactions of 30 µl total volume was prepared as described in Table 4 following standard conditions as described in Table 5.

Table 4: Components used for PCR amplification of *COXI* gene

PCR component	Volume
A. Master Mix	
1 mM KCl 50	-
10 mM Tris-HCl, pH 8.3	-
1.5 mM $MgCl_2$	-
200 mol dNTP	-
1 U Taq DNA polymerase	-
Sub total	12.5 µl
B. Deionized water	7.5 µl
C. Primers	
Forward primer (0.2 mol)	1.0 µl
Reverse primer (0.2 mol)	1.0 µl
Sub total	2.0 µl
D. Genomic DNA (50 ng)	8.0 µl
Total	30 µl

Table 5: RAPD – PCR conditions

Sr. No.	Step	Temperature (ºC)	Duration (Min.)
1	Initial denaturation	94	3.0 min.
2	Denaturation	94	30 sec
3	Annealing	36	45 sec
4	Extension	72	45 sec
5	Final Extension	72	7.0 min
30 repeats from step- 2 to step- 4			
6	Hold	4	∞

2.8 Electrophoresis

The amplicon products were measured using a gel documentation unit, and the PCR products were electrophoresed on a 1.5% agarose gel with ethidium bromide solution (10 mg/ml) and the appropriate base pair of the DNA ladder.

2.9 Sequencing

The amplified DNA PCR products were utilised further for sequence generation. For sequencing, the PCR products were provided to Hi-Gx360®, HiMedia Laboratories Private Limited, 23, Vadhani Industrial Estate, Ghatkopar West, Mumbai – 400 086, Maharashtra, India. The putative sequences were established by comparison with the DNA barcode sequence repository of the Barcode of Life Database (BOLD) (Ratnasingham and Hebert, 2007) [19] and also in National Centre for Biotechnology Information (NCBI), Maryland, United States database. Further the sequences were aligned using Clustal W (Thompson *et al*., 1994) [24] with default settings in MEGA X (Kumar *et al*., 2018) [9]. Moreover, sequences were analysed by Neighbour Joining (NJ) based on Kimura 2 parameter model (Kimura, 1980) [8] with complete gap deletion and resampled with 600 bootstrap replications for phylogenetic analysis and subsequent

construction of phylogenetic tree. The aligned sequences were compared with each other as well as its first report from Gujarat by Sisodiya *et al*. (2018) [22] and diversity sequences from India and other countries to draw valid conclusions.

3. Results and Discussion

3.1 Genomic DNA Isolation, Extraction and Quantification

Fall armyworm larvae were collected from nine districts of middle Gujarat *viz*., Panchmahal, Mahisagar, Dahod, Anand, Kheda, Vadodara, Chhotaudepur, Ahmedabad and Botad (Table 6). Genomic DNA was isolated from ten larvae and equimolar concentrations of isolated DNA larvae from nine districts were pooled and such samples were secluded and tagged. Further, quantification of isolated DNA was carried out using Nanodrop N.D.1000 (Software V.3.3.0). The concentration of DNA ranged from 50-120 ng/µl and based on the absorbance ratio 260/280 nm (1.80 to 1.85), good quality genomic DNA free from protein/RNA contamination was used for gel electrophoresis, sequence generation and further downstream analysis.

3.2 Polymerase Chain Reaction (PCR) for amplification of *COXI* gene

The genomic DNA extracted from the nine population specimens were subjected to PCR amplification of *COXI* gene. Primers used for amplification of *COXI* gene were forward primer (LCO 1490 5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse primer (HCO 2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). The standard PCR protocol mentioned in materials and methods was followed and the amplified fragments of *COXI* gene were utilized for gel electrophoresis.

Table 6: Serial and sample codes alongwith accession number of fall armyworm specimens obtained from nine districts of middle Gujarat

Sr. No.	District	Serial code (Sample code)	Accession No.
1.	Panchmahal	AAU – BACA – FAW – 1 (AAUSF1)	OR144565
2.	Mahisagar	AAU – BACA – FAW – 2 (AAUSF2)	OR145093
3.	Dahod	AAU – BACA – FAW – 3 (AAUSF3)	OR145095
4.	Anand	AAU – BACA – FAW – 4 (AAUSF4)	OR225961
5.	Kheda	AAU – BACA – FAW – 5 (AAUSF5)	OR145135
6.	Vadodara	AAU – BACA – FAW – 6 (AAUSF6)	OR145136
7.	Chhotaudepur	AAU – BACA – FAW – 7 (AAUSF7)	OR145138
8.	Ahmedabad	AAU – BACA – FAW – 8 (AAUSF8)	OR145322
9.	Botad	AAU – BACA – FAW – 9 (AAUSF9)	OR145139

3.3 Electrophoresis

The nine DNA samples each denoting a single district were subjected to gel electrophoresis. For this purpose, 1.5 per cent agarose gel containing ethidium bromide solution (10 mg/ml) along with appropriate base pair of DNA ladder followed by nine population samples of each district (amplicon products) were measured using gel documentation unit. The amplicon of *COXI* gene of size 650-700 bp was visualized for the analysis (Plate 2). After amplification, the PCR products were utilized for sequence generation.

3.4 Sequencing

The amplified PCR products were utilised further for sequence generation by Sanger sequencing method. All the nine population pooled sample sequences were obtained based on *COXI* gene partial sequence and submitted to National Centre for Biotechnology Information (NCBI), Maryland, United States. The nine populations were assigned serial codes of AAU – BACA – FAW – 1, 2, 3, 4, 5, 6, 7, 8 and 9 prior to sequencing and post sequencing were labelled as AAUSF1, 2, 3, 4, 5, 6, 7, 8 and 9 for submission to the NCBI GeneBank database. GeneBank accession number for the corresponding consensus *COXI* gene of fall armyworm samples were obtained. The sequences with sample code AAUSF1, 2, 3, 4, 5, 6, 7, 8 and 9 consisted of 660, 658, 662, 656, 662, 666, 672, 660 and 670 base pairs, respectively. Moreover, DNA barcoding of the nine sequences each from denoting a district of middle Gujarat were also carried out to serve as an identification tool in DNA taxonomic studies (Plate 3).

3.5 Mitochondrial *COXI* Gene Sequences of Fall Armyworm, *S. frugiperda* Isolated from Nine Districts of Middle Gujarat

1. Panchmahal (660 bp) AAU – BACA – FAW – 1 (AAUSF1) OR144565

>AAUSF1 Panchmahal Gujarat

TGGAACATTATATTTTATTTTTGGAATTTGAGCAGGG
ATAGTAGGTACTTCTTTAAGTTTATTAATTCGAGCTG
AATTAGGGACTCCAGGATCTTTAATTGGAGATGATC
AAATTTATAATACTATTGTAACAGCTCATGCTTTTAT
TATAATTTTTTTTATAGTTATACCTATTATAATTGGA
GGATTTGGAAATTGACTTGTACCTTTAATATTAGGAG
CCCCTGATATAGCTTTCCCACGTATAAATAATATAAG
TTTTTGACTTTTACCCCCATCTTTAACTTTATTAATTT
CTAGTAGCATTGTAGAAAATGGAGCAGGAACTGGAT
GAACAGTTTACCCCCCCCTCTCCTCTAATATTGCTCA
TGGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTT
CATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTA
ACTTTATTACTACTATTATTAATATACGATTAAATAA
TTTATCATTTGATCAAATACCTTTATTTATTTGAGCT
GTAGGTATTACTGCATTCTTATTATTATTATCTTTACC
TGTTTTAGCCGGAGCTATTACTATATTACTTACTGAT
CGAAATTTAAATACATCATTTTTCGATCCTGCAGGTG
GAGGTGATCCTATTCTTTATCAACATTTATTT

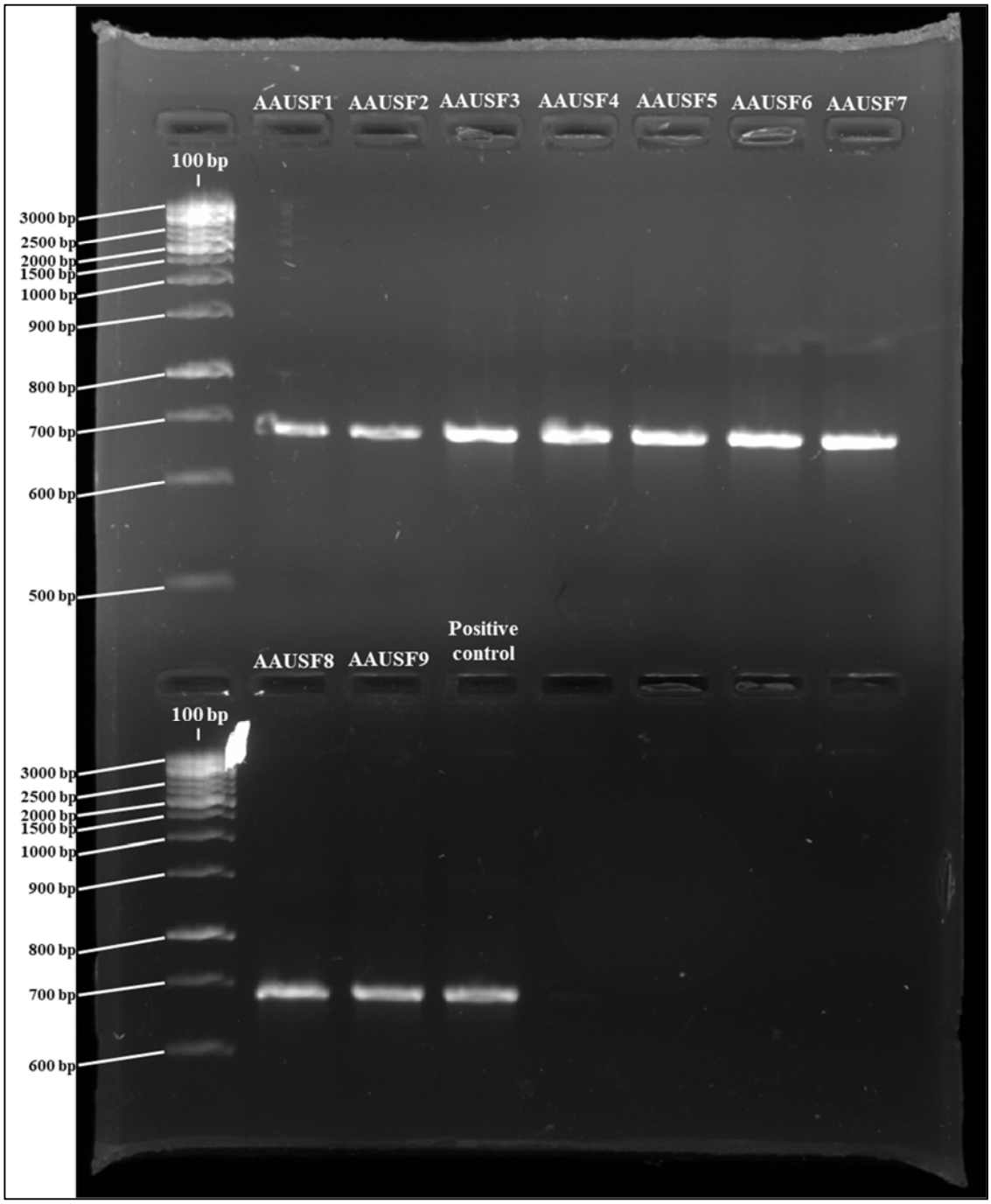

Plate 2: Amplification of *COXI* gene DNA of *S. frugiperda* specimens collected from nine districts of middle Gujarat

2. Mahisagar (658 bp) AAU – BACA – FAW – 2 (AAUSF2) OR145093

>AAUSF2 Mahisagar Gujarat

GGAACATTATATTTTATTTTTGGAATTTGAGCAGGGA
TAGTAGGTACTTCTTTAAGTTTATTAATTCGAGCTGA
ATTAGGGACTCCAGGATCTTTAATTGGAGATGATCA
AATTTATAATACTATTGTAACAGCTCATGCTTTTATT
ATAATTTTTTTTATAGTTATACCTATTATAATTGGAG
GATTTGGAAATTGACTTGTACCTTTAATATTAGGAGC
CCCTGATATAGCTTTCCCACGTATAAATAATATAAGT
TTTTGACTTTTACCCCCATCTTTAACTTTATTAATTTC
TAGTAGCATTGTAGAAAATGGAGCAGGAACTGGATG
AACAGTTTACCCCCCCCTCTCCTCTAATATTGCTCAT
GGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTTC
ATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTAA
CTTTATTACTACTATTATTAATATACGATTAAATAAT
TTATCATTTGATCAAATACCTTTATTTATTTGAGCTG
TAGGTATTACTGCATTCTTATTATTATTATCTTTACCT
GTTTTAGCCGGAGCTATTACTATATTACTTACTGATC
GAAATTTAAATACATCATTTTTCGATCCTGCAGGTGG
AGGTGATCCTATTCTTTATCAACATTTATT

3. Dahod (662 bp) AAU – BACA – FAW – 3 (AAUSF3) OR145095

>AAUSF3 Dahod Gujarat

GGAACATTATATTTTATTTTTGGAATTTGAGCAGGAA
TAGTAGGTACTTCTTTAAGTTTATTAATTCGAGCTGA
ATTAGGAACTCCAGGATCTTTAATTGGAGATGATCA

AATTTATAATACTATTGTAACAGCCCATGCTTTTATT
ATAATTTTTTTTATAGTTATACCAATTATAATTGGAG
GATTTGGAAATTGACTTGTACCTTTAATATTAGGAGC
TCCTGATATAGCTTTCCCACGTATAAATAATATAAGT
TTTTGACTTTTACCCCCATCTTTAACTTTATTAATTTC
TAGTAGCATTGTAGAAAATGGAGCAGGAACTGGATG
AACAGTTTACCCCCCCCCTCTCCTCTAATATTGCTCAT
GGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTTC
ATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTAA
CTTTATTACCACTATTATTAATATACGATTAAATAAT
TTATCATTTGATCAAATACCTTTATTTATTTGAGCTG
TAGGTATTACCGCATTTTTATTATTATTATCTTTACCT
GTTTTAGCTGGAGCTATTACTATATTACTTACTGATC
GAAATCTAAATACATCATTTTTCGATCCTGCAGGAG
GAGGTGATCCTATTCTTTATCAACATTTATTTTGA

4. Anand (656) AAU – BACA – FAW – 4 (AAUSF4) OR225961

>AAUSF4 Anand Gujarat

AACATTATATTTTATTTTTGGCATTTGAGCAGGGATA
GTAGGTACTTCTTTAAGTTTATTAATTCGAGCTGAAT
TAGGGACTCCAGGATCTTTAATTGGAGATGATCAAA
TTTATAATACTATTGTAACAGCTCATGCTTTTATTAT
AATTTTTTTTATAGTTATACCTATTATAATTGGAGGA
TTTGGAAATTGACTTGTACCTTTAATATTAGGAGCCC
CTGATATAGCTTTCCCACGTATAAATAATATAAGTTT
TTGACTTTTACCCCCATCTTTAACTTTATTAATTTCTA
GTAGCATTGTAGAAAATGGAGCAGGAACTGGATGA
ACAGTTTACCCCCCCCCTCTCCTCTAATATTGCTCATG
GTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTTCA
TTTAGCTGGAATTTCATCTATTTTAGGAGCTATTAAC
TTTATTACTACTATTATTAATATACGATTAAATAATT
TATCATTTGATCAAATACCTTTATTTATTTGAGCTGT
AGGTATTACTGCATTCTTATTATTATTATCTTTACCTG
TTTTAGCCGGAGCTATTACTATATTACTTACTGATCG
AAATTTAAATACATCATTTTTCGATCCTGCAGGTGGG
GGTGATCCTATTCTTTATCAACATTTATT

5. Kheda (662) AAU – BACA – FAW – 5 (AAUSF5) OR145135

>AAUSF5 Kheda Gujarat

GGAACATTATATTTTATTTTTGGAATTTGAGCAGGGA
TAGTAGGTACTTCTTTAAGTTTATTAATTCGAGCTGA
ATTAGGGACTCCAGGATCTTTAATTGGAGATGATCA
AATTTATAATACTATTGTAACAGCTCATGCTTTTATT
ATAATTTTTTTTATAGTTATACCTATTATAATTGGAG
GATTTGGAAATTGACTTGTACCTTTAATATTAGGAGC
CCCTGATATAGCTTTCCCACGTATAAATAATATAAGT
TTTTGACTTTTACCCCCATCTTTAACTTTATTAATTTC
TAGTAGCATTGTAGAAAATGGAGCAGGAACTGGATG
AACAGTTTACCCCCCCCCTCTCCTCTAATATTGCTCAT
GGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTTC
ATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTAA
CTTTATTACTACTATTATTAATATACGATTAAATAAT
TTATCATTTGATCAAATACCTTTATTTATTTGAGCTG
TAGGTATTACTGCATTCTTATTATTATTATCTTTACCT
GTTTTAGCCGGAGCTATTACTATATTACTTACTGATC
GAAATTTAAATACATCATTTTTCGATCCTGCAGGTGG
AGGTGATCCTATTCTTTATCAACATTTATTTTGA

6. Vadodara (666) AAU – BACA – FAW – 6 (AAUSF6) OR145136

>AAUSF6 Vadodara Gujarat

TATTGGAACATTATATTTTATTTTTGGAATTTGAGCA
GGAATAGTAGGTACTTCTTTAAGTTTATTAATTCGAG
CTGAATTAGGAACTCCAGGATCTTTAATTGGAGATG
ATCAAATTTATAATACTATTGTAACAGCCCATGCTTT
TATTATAATTTTTTTTATAGTTATACCAATTATAATTG
GAGGATTTGGAAATTGACTTGTACCTTTAATATTAGG
AGCTCCTGATATAGCTTTCCCACGTATAAATAATATA
AGTTTTTGACTTTTACCCCCATCTTTAACTTTATTAAT
TTCTAGTAGCATTGTAGAAAATGGAGCAGGAACTGG
ATGAACAGTTTACCCCCCCCCTCTCCTCTAATATTGCT
CATGGTGGTAGTTCAGTAGATTTAGCTATTTTCTCAC
TTCATTTAGCTGGAATTTCATCTATTTTAGGAGCTAT
TAACTTTATTACCACTATTATTAATATACGATTAAAT
AATTTATCATTTGATCAAATACCTTTATTTATTTGAG
CTGTAGGTATTACCGCATTTTTATTATTATTATCTTTA
CCTGTTTTAGCTGGAGCTATTACTATATTACTTACTG
ATCGAAATCTAAATACATCATTTTTCGATCCTGCAGG
AGGAGGTGATCCTATTCTTTATCAACATTTATTTTGA

7. Chhotaudepur (672) AAU – BACA – FAW – 7 (AAUSF7) OR145138

>AAUSF7 Chhotaudepur Gujarat

GATATTGGAACATTATATTTTATTTTTGGAATTTGAG
CAGGAATAGTAGGTACTTCTTTAAGTTTATTAATTCG
AGCTGAATTAGGAACTCCAGGATCTTTAATTGGAGA
TGATCAAATTTATAATACTATTGTAACAGCCCATGCT
TTTATTATAATTTTTTTTATAGTTATACCAATTATAAT
TGGAGGATTTGGAAATTGACTTGTACCTTTAATATTA
GGAGCTCCTGATATAGCTTTCCCACGTATAAATAAT
ATAAGTTTTTGACTTTTACCCCCATCTTTAACTTTATT
AATTTCTAGTAGCATTGTAGAAAATGGAGCAGGAAC
TGGATGAACAGTTTACCCCCCCCCTCTCCTCTAATATT
GCTCATGGTGGTAGTTCAGTAGATTTAGCTATTTTCT
CACTTCATTTAGCTGGAATTTCATCTATTTTAGGAGC
TATTAACTTTATTACCACTATTATTAATATACGATTA
AATAATTTATCATTTGATCAAATACCTTTATTTATTT
GAGCTGTAGGTATTACCGCATTTTTATTATTATTATC
TTTACCTGTTTTAGCTGGAGCTATTACTATATTACTT
ACTGATCGAAATCTAAATACATCATTTTTCGATCCTG
CAGGAGGAGGTGATCCTATTCTTTATCAACATTTATT
TTGATTTT

8. Ahmedabad (660) AAU – BACA – FAW – 8 (AAUSF8) OR145322

>AAUSF8 Ahmedabad Gujarat

TGGAACATTATATTTTATTTTTGGAATTTGAGCAGGG
ATAGTAGGTACTTCTTTAAGTTTATTAATTCGAGCTG
AATTAGGGACTCCAGGATCTTTAATTGGAGATGATC
AAATTTATAATACTATTGTAACAGCTCATGCTTTTAT
TATAATTTTTTTTATAGTTATACCTATTATAATTGGA
GGATTTGGAAATTGACTTGTACCTTTAATATTAGGAG
CCCCTGATATAGCTTTCCCACGTATAAATAATATAAG
TTTTTGACTTTTACCCCCATCTTTAACTTTATTAATTT
CTAGTAGCATTGTAGAAAATGGAGCAGGAACTGGAT
GAACAGTTTACCCCCCCCCTCTCCTCTAATATTGCTCA
TGGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTT
CATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTA
ACTTTATTACTACTATTATTAATATACGATTAAATAA
TTTATCATTTGATCAAATACCTTTATTTATTTGAGCT
GTAGGTATTACTGCATTCTTATTATTATTATCTTTACC
TGTTTTAGCCGGAGCTATTACTATATTACTTACTGAT
CGAAATTTAAATACATCATTTTTCGATCCTGCAGGTG
GAGGTGATCCTATTCTTTATCAACATTTATTT

9. Botad(670) AAU – BACA – FAW – 9 (AAUSF9) OR145139

>AAUSF9 Botad Gujarat

TGGAACATTATATTTTATTTTTGGAATTTGAGCAGGG
ATAGTAGGTACTTCTTTAAGTTTATTAATTCGAGCTG
AATTAGGGACTCCAGGATCTTTAATTGGAGATGATC
AAATTTATAATACTATTGTAACAGCTCATGCTTTTAT
TATAATTTTTTTTATAGTTATACCTATTATAATTGGA
GGATTTGGAAATTGACTTGTACCTTTAATATTAGGAG
CCCCTGATATAGCTTTCCCACGTATAAATAATATAAG
TTTTTGACTTTTACCCCCATCTTTAACTTTATTAATTT
CTAGTAGCATTGTAGAAAATGGAGCAGGAACTGGAT
GAACAGTTTACCCCCCCCTCTCCTCTAATATTGCTCA
TGGTGGTAGTTCAGTAGATTTAGCTATTTTCTCACTT
CATTTAGCTGGAATTTCATCTATTTTAGGAGCTATTA
ACTTTATTACTACTATTATTAATATACGATTAAATAA
TTTATCATTTGATCAAATACCTTTATTTATTTGAGCT
GTAGGTATTACTGCATTCTTATTATTATTATCTTTACC
TGTTTTAGCCGGAGCTATTACTATATTACTTACTGAT
CGAAATTTAAATACATCATTTTTCGATCCTGCAGGTG
GAGGTGATCCTATTCTTTATCAACATTTATTTTGATT
TTTTG

3.6 Sequence Analysis, Retrieval from NCBI Database, Phylogeny Analysis and Characterization

3.6.1 Sequence analysis and retrieval from NCBI database

The obtained sequences of *COXI* gene from nine samples under study were aligned using Clustal W function in Mega X software and aligned sequences were utilized for phylogenetic analysis. The consensus sequences of *COXI* gene of fall armyworm specimens from nine districts of middle Gujarat were plaid for homology assessment post alignment using NCBI-BLAST finder. The *COXI* gene sequence of *S. frugiperda* specimens from all the nine districts of middle Gujarat exhibited 100 per cent identity alongside 100 per cent query coverage with multiple sequences of *S. frugiperda* Corn-strain available in the NCBI database.

3.6.2 Phylogeny analysis and characterization

Further, the phylogeny analysis of *S. frugiperda* aligned sequences obtained in the present study was carried out in MEGA X software with 2000 bootstrap support (replications). The sequences of *S. frugiperda* exhibiting similarity with current specimens were obtained from NCBI GenBank database for phylogenic analysis alongside *S. frugiperda* sequence from Gujarat as reported by Sisodiya *et al*. (2018) [22]. The phylogenetic tree was constructed by Neighbour-Joining (NJ) method. In order to construct evolutionary tree of specimen sequences and other reports from India as well as abroad, *Spodoptera litura* Fabricius genomic sequence (NC_022676) was utilised to serve as an outgroup sequence. The phylogenetic tree constructed truncated the distribution into three clades. The two former clades consisted of consensus sequences from *S. frugiperda* population characterized under Corn-strain, while the third clade consisted of the outgroup (Plate 4).

The consensus sequences of *S. frugiperda* population from districts of middle Gujarat *viz*., Chhotaudepur (OR145138), Vadodara (OR145136) and Dahod (OR145095) exhibited distribution under clade 1 alongwith NCBI database isolates *viz*., Anand, Gujarat (MK034861); Coimbatore, Tamil nadu (MH753328); Bhadravati, Karnataka (MH753330); Haniyuru, Karnataka (MH753333); Shivamogga, Karnataka (MH753326); Umiam, Meghalaya (MN640598); Faisalabad, Pakistan (MT180097); Lalitpur, Nepal (MN011579); Wuhan, China (MN068212); Nairobi, Kenya (MH190446) and Oyo, Nigeria (KX580616). While, the population of *S. frugiperda* from Panchmahal (OR144565), Mahisagar (OR145093), Anand (OR225961), Kheda (OR145135), Ahmedabad (OR145322) and Botad (OR145139) were distributed under clade 2 which consisted of *S. frugiperda* sequences from New Delhi, India (MN541474); Yunan, China (MK860937); Nairobi, Kenya (MK492942 AND MK492971); Volta, Ghana (KY472255); Sulawesi Utara, Indonesia (OQ891323);

Table 7: Details of fall armyworm, *S. frugiperda* sequences recorded in India and abroad used for the phylogeny analysis

Sr. No.	Place/ Location	Description	Accession No.
1.	Anklav, Anand, Gujarat, India	*Spodoptera frugiperda* isolate Gujarat cytochrome oxidase subunit I gene, partial cds; mitochondrial	MK034861
2.	Coimbatore, Tamil Nadu, India	*Spodoptera frugiperda* voucher TNAU_Maize cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial	MH753328
3.	Oyo, Nigeria	*Spodoptera frugiperda* isolate 3L-LepS8 cytochrome oxidase subunit 1 (*COI*) gene, partial cds; mitochondrial	KX580616
4.	Indian Institute of Maize Research, Ludhiana, Punjab, India	*Spodoptera frugiperda* cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial	MN541574
5.	Yunnan, China	*Spodoptera frugiperda* isolate TS-1 cytochrome oxidase subunit 1 (*COI*) gene, partial cds; mitochondrial	MK860937
6.	Nairobi, Kenya	Spodoptera frugiperda voucher 43_ShimbaHills cytochrome c oxidase subunit 1 (*COI*) gene, partial cds; mitochondrial	MK492971
7.	ICAR – Research complex for North Eastern Hill Region, Umiam, Meghalaya, India	*Spodoptera frugiperda* isolate GTB404DN3012 cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial	MN640598
8.	Faisalabad, Pakistan	*Spodoptera frugiperda* isolate FS-SF03 cytochrome c oxidase subunit I (*COX1*) gene, partial cds; mitochondrial	MT180097
9.	Wuhan, China	*Spodoptera frugiperda* NADH dehydrogenase subunit 2 gene, partial cds	MN068212
10.	Sulawesi Utara, Indonesia	*Spodoptera frugiperda* voucher JMEM-Ton cytochrome c oxidase subunit I (*COX1*) gene, partial cds; mitochondrial	OQ891323
11.	Nairobi, Kenya	*Spodoptera frugiperda* voucher 14_Ngeria cytochrome c oxidase subunit 1 (*COI*) gene, partial cds; mitochondrial	MK492942
12.	Vancouver, Canada	*Spodoptera frugiperda* voucher BIOUG<CAN>:2005-ONT-2131 cytochrome oxidase subunit 1 (*COI*) gene, partial cds; mitochondrial	GU090723
13.	Coimbatore, Tamil Nadu, India	*Spodoptera frugiperda* voucher TNAU_Maize cytochrome oxidase	MH753328

		subunit I (*COI*) gene, partial cds; mitochondrial	
14.	Bhadravati, Karnataka, India	*Spodoptera frugiperda* voucher Badravathi_Maize cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial	MH753330
15.	Haniyur, Karnataka, India	*Spodoptera frugiperda* voucher Haniyuru_Maize cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial.	MH753333
16	Lalitpur, Nepal	*Spodoptera frugiperda* isolate N4 cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial	MN011579
17.	Volta, Ghana	*Spodoptera frugiperda* isolate CABI-AWV06 cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial	KY472255
18.	Oyo, Nigeria	*Spodoptera frugiperda* isolate 3L-LepS8 cytochrome oxidase subunit 1 (*COI*) gene, partial cds; mitochondrial	KJ634299
19.	Wageningen, Netherlands	*Spodoptera frugiperda* isolate ENT1216 cytochrome oxidase subunit 1 (*COI*) gene, partial cds; mitochondrial	MF197868
20.	Shivamogga, Karnataka, India	*Spodoptera frugiperda* voucher Shivamogga_Maize cytochrome oxidase subunit I (*COI*) gene, partial cds; mitochondrial	MH753326
21.	Maryland, USA	*Spodoptera litura* mitochondrion, complete genome	NC_022676

Source: Anonymous, 2023[3]

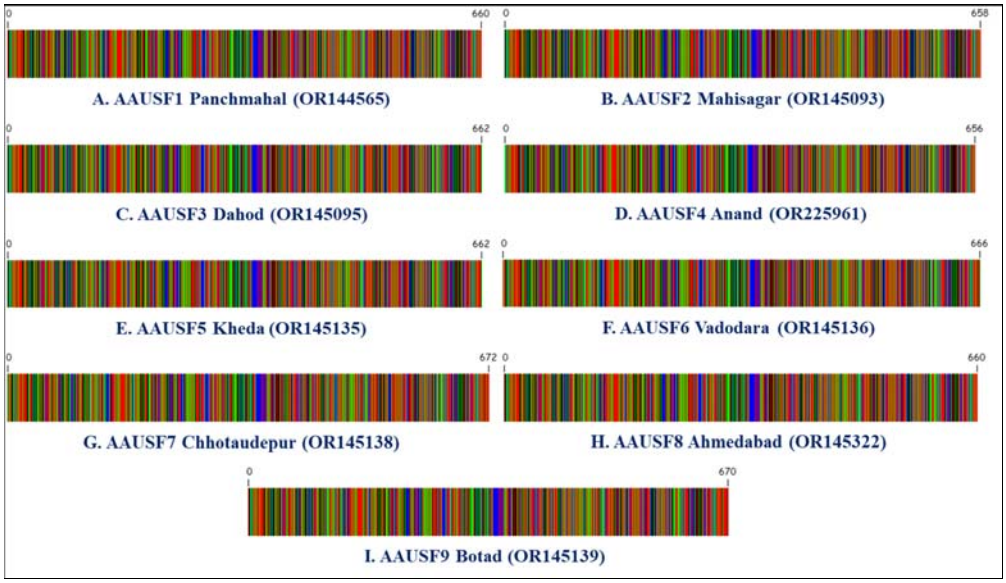

Plate 3: DNA barcodes of *S. frugiperda* specimens collected from nine districts of middle Gujarat

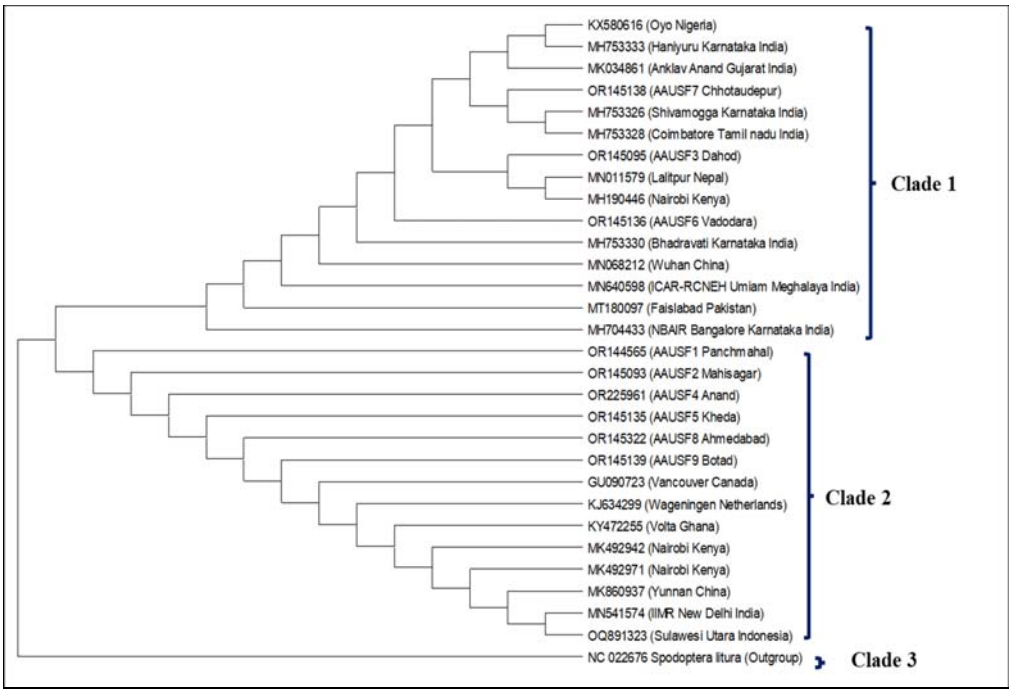

Plate 4: Phylogenetic tree inferred using the Neighbour Joining method of the Mt*COI* region of *S. frugiperda* sequences specimens obtained from nine districts of middle Gujarat

Wageningen, Netherlands (KJ634299) and Vancouver, Canada (GU090723). The phylogenic similarity of sequences indicated that though the specimens were collected from geographically nearer districts, it portrayed higher similarity with geographically distant populations of FAW C-strain. *Spodoptera frugiperda* population from Chhotaudepur, Vadodara and Dahod were similar and followed similar clade distribution as Anklav, Anand population (Sisodiya *et al*., 2018) [22] and shared more similarity with diverge populations of FAW geographically distant as compared to geographically proximal locations of Panchmahal, Mahisagar, Anand, Kheda, Ahmedabad as well as Botad and *vice versa*. Notably, the Anand population of *S. frugiperda* sampled under current study and the first report by Sisodiya *et al*. (2018) [22] from Anklav, Anand did not characterize under the similar clade. This indicates prevalence of inter-strain and inter-specific divergence as well as the results profoundly depict presence of two divergent clades of *S. frugiperda* in districts of middle Gujarat that can be considered as divergent haplotypes both belonging to the C-strain.

4. Discussion

The diversity studies on FAW, *S. frugiperda* in Gujarat are scanty, however, research outcomes from India and abroad are discussed to draw conclusive insights. One of the foremost diversity studies carried out by Nayyar *et al*. (2021) [17] based on *COXI* revealed the prevalence of two haplotypes in Gujarat *viz*., haplotype 1 and 13 which are closely in compliance with the present diversity results. Similarly, Shylesha *et al*. (2018) [21] also identified fall armyworm from Bangalore, Karnataka that exhibited 100 per cent similarity with GU095403 (Vancouver, Canada), which is utilised as reference sequence under the current phylogenic analysis. Moreover, Nelly *et al*. (2021) [18] studied the genetic diversity of FAW in West Sumatra, Indonesia based on *COXI* gene that indicated the phylogenic divergence into two groups which is completely in accordance with the divergence of C-strain of *S. frugiperda* from Gujarat, India. Whereas, in states like Karnataka, Tamil nadu, Andhra Pradesh, Telangana, Madhya Pradesh, Maharashtra (Swamy *et al*., 2018) [23] and Goa (Maruthadurai and Ramesh, 2022) [12], presence of both strains *viz*., C and R-strain of *S. frugiperda* have been reported.

5. Conclusion

Thus, two haplotypes of *S. frugiperda* C-strain were found from middle Gujarat and consequently, also reveal the absence of R-strain. Moreover, the similarity of FAW populations of middle Gujarat with geographically distant populations indicates the possible source of introduction *via* migration or displacement. Further, DNA barcoding carried out characterizes the specimen sequences as tools for fast-track identification, authentication of insect-pests and complementing traditional taxonomic studies. Species identity and strain variations are important constraints that make it difficult to properly address the problem of insect-pests in context of integrated pest management and insect-pest monitoring. The survey and analysis performed under this study provide a picture of FAW, *S. frugiperda* identification alongside its distribution into two divergent haplotypes (clades), which is not in alignment with its first report (Sisodiya *et al*., 2018) [22] alongwith its diversity with populations from Indian and abroad. This information generates basis that could serve for better for the monitoring of future patterns of FAW population diversity, migration, abundance and displacement and finally aid in successful management of FAW in India.

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