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Comparative Genomic and Proteomic Phylogenetic Analysis of Indian Isolate of Partial Coat Protein Gene Sequence of Zucchini Yellow Mosaic Virus (ZYMV) Using Data Mining

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Abstract

A viral disease was identified on summer squash (*Cucurbita pepo* L.) plants in the hill state of Himachal Pradesh located in the North Western Himalayan regions, showing symptoms like mosaic, yellowing, shoe stringing of leaves and stunting of plants and infection at early stage of crop could cause as much as 94 per cent reduction of marketable fruits of summer squash. In the present study the causal virus was identified and characterized on the basis of ELISA, RT-PCR and Phylogeny. Partial CP gene was amplified and sequenced. For phylogenetic studies 67 nucleotide and 67 polyprotein sequences of ZYMV belonging to different countries were retrieved from NCBI. Phylogenetic analysis based on nucleotide and protein sequences of each country using Maximum Likelihood (ML), Neighbor Joining (NJ), Maximum Parsimony (MP) and Unweighted Pair Group Method of Arithmetic Averages (UPGMA) methods were achieved via phylip 3.68 and EXOME™ HORIZON, which revealed 91% similarity of the test virus nucleotide sequence with USA ZYMV CP sequence (D13914) and 75.9% similarity with partial polyprotein sequence of Japan (BAE75935).

Keywords: ZYMV; Phylogenetic trees; Phylograms; Nucleotide sequence; Polyprotein sequence; RT-PCR; CP gene sequence; Data mining

Introduction

Crops belonging to family *cucurbitaceae* are generally known as *cucurbits*. As a group, *cucurbits* occupy largest area in India and in other tropical countries amongst vegetable crops. Out of all *cucurbitaceous* crops, summer squash is one of the important crops because it is one of the earliest vegetables reaching markets of India. Amongst different plant pathogens, viral infections are responsible for causing great losses to this crop. In *cucurbit* crops, viruses belonging to Potyvirus genus have severely caused economical damage all over the world [1]. In particular, Zucchini Yellow Mosaic Virus (ZYMV), a member of genus Potyvirus in the family Potyviridae, was subsequently one of the most damaging virus causing epidemics in commercial cucurbits worldwide [2]. In Korea, the disease caused by ZYMV has been considered one of the major limiting factors for production of *cucurbits* [3,4]. In this study the partial coat protein gene sequence of ZYMV of Indian isolate of North Western Himalayan region was determined and phylogenetic analysis of the test sequence at both genomic and proteomic level was carried out to gain insight of the evolutionary pattern of Zucchini yellow mosaic virus and hence phylograms and phylogenetic trees were constructed for all 14 countries viz, Australia, Austria, California, China, France, Hungary, India, Israel, Japan, Korea, Poland, Singapore, Taiwan and USA using phylip 3.68 and EXOME™ HORIZON respectively. The present studies on phylogenetic analysis with other countries isolates have been carried out to suggest world wide distribution of ZYMV and by tracing its phylogeny management of the disease may be understood. This work represents the first detailed phylogenetic study ever conducted with well explained flowcharts for methods used for constructing 64 phylograms and 64 phylogenetic trees.

Materials and Methods

Survey and collection of samples

An extensive survey of different summer squash (*Cucurbita pepo* L.) growing in localities of Himachal Pradesh was conducted. Tender leaves of summer squash plants showing symptoms of ZYMV were collected from the hill state of Himachal Pradesh located in North Western Himalayan regions.

Maintenance of the virus isolate

The virus cultures were maintained on healthy seedlings of summer squash variety Australian Dark Green by mechanical sap inoculation under insect proof glass house conditions.

Enzyme Linked Immunosorbent Assay (ELISA)

ZYMV specific antibodies along with alkaline phosphatase linked antibodies produced from (BIOREBA-AG Switzerland) were used for ELISA and protocols of suppliers of ELISA kits were used (Figure 1). The positive and negative controls were also provided by the antibody suppliers (BIOREBA-AG Switzerland).

RNA isolation

Total RNA from virus infected summer squash leaves was isolated using RNAeasy plant Mini Kit (Qiagen). RNA isolation was also tried at healthy control plant.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and PCR

The above isolated RNA was used as a template for cDNA synthesis

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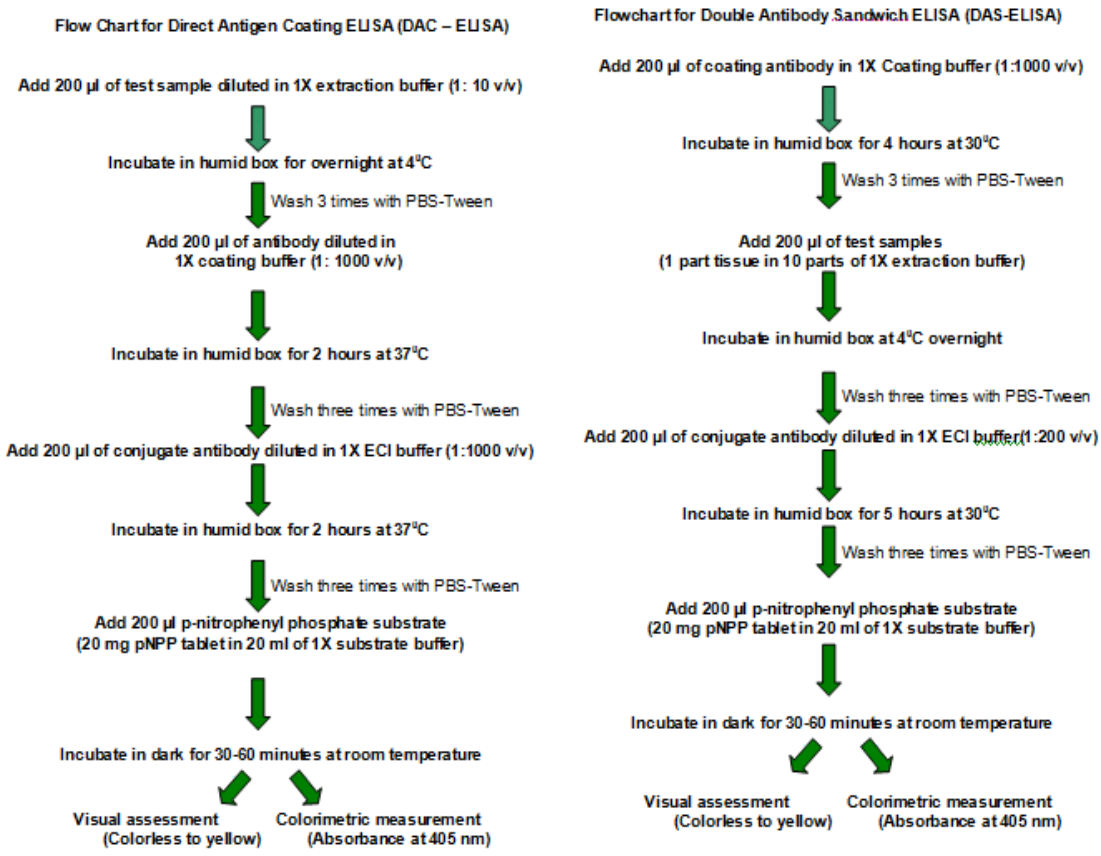

Figure 1

by using specific oligonucleotide primer p9502 shown in Table 1. For the first strand cDNA synthesis RT-PCR was carried out and for further amplification of cDNA, PCR was carried out in a thermal cycler (Applied Biosystem, USA) using specific primers (Table 1). Components of RT-PCR and PCR were standardized (Table 2) and so do the thermal profile and no. of cycles.

Sequencing and translation of the sequenced PCR product

Sequencing using both reverse and forward primers was carried out [5] and the partial coat protein sequence obtained has been submitted to NCBI Database and also the sequence was kept as such for genomic studies at nucleotide level and was also translated to protein using Expert Protein Analysis System (EXPASY) tool for proteomic studies.

Name of the primer	Sequence	Total no. of bases	Reference
P9502	5'- GCGGATCCTTTTTTTTTTTTTTTT – 3' Reverse Primer	25	(Vander Vlugt et al., 1999)
CPUP	5'- TGAGGATCCTGGTGYATHGARAAGG -3' Forward Primer	25	

Table 1: Primers used for PCR amplification and Sequencing.

Component	Concentration
PCR buffer	10X
$MgCl_2$	1.0mM – 4.0mM
dNTPs	1.0 mM – 5.0mM
Primer	30-50 ng
Taq DNA polymerase	0.5U – 1U
DNA	Added separately

Table 2: Standardization of RT-PCR and PCR.

Importing of Sequences

Sequence selection

Both nucleotide and protein sequences of coat protein gene of ZYMV were retrieved from National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (Table 3).

These nucleotide sequences and protein sequences given in Table 3 were later used with test sequence for multiple sequence alignment, phylogenetic analysis using various online/offline bioinformatic tools.

Conversion of selected sequences into FASTA format

All 67 coat protein nucleotide and protein sequences obtained from all over the world in GenBank format were converted into FASTA format [6]. These 'FASTA' formatted sequences were then stored country-wise in separate notepads.

Sequence alignment

During present investigations, multiple sequence alignment of nucleotide and protein sequences of ZYMV and other 67 ZYMV isolates retrieved from NCBI database, was carried out. Multiple sequence alignment was performed using CLUSTAL W program [7].

Phylogenetic analysis

For Phylograms phylip 3.68 Software was used and for phylogenetic

S.No.	Nucleotide Accession number	Polyprotein Accession number	Country
1.	DQ925447	ABL09422	Australia
2.	DQ925448	ABL09423	Australia
3.	DQ925449	ABL09424	Australia
4.	DQ925450	ABL09425	Australia
5.	DQ925451	ABL09426	Australia
6.	AJ420012	CAD12308	Austria
7.	AJ420013	CAD12309	Austria
8.	AJ420014	CAD12310	Austria
9.	AJ420015	CAD12311	Austria
10.	AJ420016	CAD12312	Austria
11.	AJ420017	CAD12313	Austria
12.	AJ420018	CAD12314	Austria
13.	AJ420019	CAD12315	Austria
14.	AJ420020	CAD12316	Austria
15.	L31350	AAA65559	California
16.	EF122498	ABN13960	China
17.	AJ889243	CAI65411	China
18.	AJ889244	CAI65412	China
19.	AJ316228	CAC87635	China
20.	AJ316229	CAC87636	China
21.	AJ307036	CAC85170	China
22.	AJ515911	CAD56800	China
23.	AY597207	AAT07674	China
24.	AJ515907	CAD56796	China
25.	AJ515908	CAD56797	China
26.	AJ316227	CAC87634	China
27.	AF513550	AAM53600	China
28.	AF513551	AAM53601	China
29.	AF513552	AAM53602	China
30.	AF486822	AAL93199	China
31.	AF486823	AAL93200	China
32.	AY074808	AAL71865	China
33.	AY074809	AAL71866	China
34.	AY074810	AAL71867	China
35.	AF435425	AAL30766	China
36.	AY188994	AA061299	France
37.	AJ459954	CAD31056	Hungary
38.	AJ459955	CAD31057	Hungary
39.	AJ459956	CAD31036	Hungary
40.	AJ251527	CAB63753	Hungary
41.	GQ251520	ACS36116	India
42.	EF062582	ABL01531	Israel
43.	EF062583	ABL01532	Israel
44.	AB063251	BAB82974	Japan
45.	AB458595	BAH97116	Japan
46.	AB458596	BAH97118	Japan
47.	AB188115	BAE75934	Japan
48.	AB188116	BAE75935	Japan
49.	AB127936	BAD74201	Japan
50.	AY278998	AAQ17214	Korea
51.	AY278999	AAQ17215	Korea
52.	AY279000	AAQ17216	Korea
53.	AJ429071	CAD22062	Korea
54.	AF062518	AAC77445	Korea
55.	EF178505	ABM65098	Poland
56.	AF014811	AAB72004	Singapore
57.	DQ978272	ABI97984	South Africa
58.	NC003224	NP477522	Taiwan
59.	AM422386	CAM12729	Taiwan
60.	AF127929	AAD44684	Taiwan
61.	AF127930	AAD44685	Taiwan
62.	AF127931	AAD44686	Taiwan
63.	AF127932	AAD44687	Taiwan
64.	AF127933	AAD44688	Taiwan
65.	AF127934	AAD44689	Taiwan
66.	D13914	BAA03010	USA
67.	D00692	BAA00596	USA

Table 3: List of Nucleotide and Protein sequences retrieved from NCBI.

trees EXOME™ was used. Test virus nucleotide sequence and polyprotein sequence analysed countrywise with different sequences retrieved from NCBI using various popular methods like Maximum Likelihood (ML), Neighbor Joining (NJ), Maximum Parsimony (MP) and Unweighted Pair Group Method of Arithmetic Averages (UPGMA) and finally trees were generated and analysed (Figure 2).

Results

Culture identification and collection

Under field conditions, summer squash plants infected with ZYMV develop a variety of symptoms. These symptoms vary from mild to severe mosaic, green blisters on leaves, vein clearing, and shoe stringing of leaves (Figure 3).

For culture collection, survey of various summer squash growing localities of H.P. was conducted.

Mechanical transmission

Indicator plant *Chenopodium amaranticolor* Coste and Reyn was also used to indicate presence of the test virus by observing the lesions.

Symptomatlogy

The first manifestation of the disease on the inoculated plants was observed after 16-18 days of inoculation in the form of vein clearing on the younger leaves. Later, mottling and mild mosaic symptoms were exhibited by the infected plants. As the infestation progressed, leaf lamina was drastically reduced in both shape and size. Leaves were deformed with dark green blisters and distorted mid ribs. Virus


Phylograms and Phylogenetic trees of nucleotide sequences
(Flowcharts to construct Phylograms using phylip 3.68)
Test partial CP and other CP gene sequences from each country taken in Fasta format
'ClustalW' alignment generated
Output file used in 'Seqboot' program
Out file of 'Seqboot' program generated used in 'Dnaml' program
Outree file of 'Dnaml' given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of Maximum Likelihood (ML) phylograms of CP nucleotide sequences of ZYMV using phylip 3.68
Output file of 'Clustal W' alignment used in 'Seqboot' program
Bootstrap data generated applied in 'Dnadist' program
Outfile used in 'Neighbor' program and then 'UPGMA' option selected
Outree file of 'UPGMA' obtained given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of UPGMA phylograms of CP nucleotide sequences of ZYMV using phylip 3.68
Phylograms and Phylogenetic Studies of Protein sequences
(Flowcharts to construct Phylograms using phylip 3.68)
Test protein and other protein sequences from each country taken in Fasta format
'ClustalW' alignment generated
Output file used in 'Seqboot' program
Out file of 'Seqboot' program generated used in 'proml' program
Outree file of 'proml' given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of Maximum Likelihood (ML) phylograms of protein sequences of ZYMV using phylip 3.68
Output file of 'Clustal W' alignment used in 'Seqboot' program
Bootstrap data generated applied in 'prodist' program
Outfile used in 'Neighbor' program and then 'UPGMA' option selected
Outree file of 'UPGMA' obtained given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of UPGMA phylograms of protein sequences of ZYMV using phylip 3.68
Out file of 'Dnadist' generated earlier by UPGMA method used in 'Neighbor' program and then 'Neighbor joining' option selected
Outree file of 'Neighbor joining' given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of Neighbor-joining (NJ) phylograms of CP nucleotide sequences of ZYMV using phylip 3.68
Outfile of 'Seqboot' program generated used in 'Dnapars' program
Outree file of 'Dnapars' given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of Maximum Parsimony (MP) phylograms of CP nucleotide sequences of ZYMV using phylip 3.68
(Flowchart to construct Phylogenetic trees using EXOME™)
Sequence analysis
Phylogeny
Tree Drawing methods
Outree file of 'Consense' of phylip 3.68 of ML/ MP/ NJ/ UPGMA browsed
Plot phylogenetic trees for each country isolates of ZYMV
Flowchart for construction of Phylogenetic trees of CP nucleotide sequences of ZYMV using EXOME™
Out file of 'prodist' generated earlier by UPGMA method used in 'Neighbor' program and then 'Neighbor joining' option selected
Outree file of 'Neighbor joining' given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of Neighbor-joining (NJ) phylograms of protein sequences of ZYMV using phylip 3.68
Outfile of 'Seqboot' program generated used in 'propars' program
Outree file of 'propars' given as an input to 'Consense' program
Outree file of 'Consense' finally plots phylograms
Flowchart for construction of Maximum Parsimony (MP) phylograms protein sequences of ZYMV using phylip 3.68
(Flowchart to construct Phylogenetic trees using EXOME™)
Sequence analysis
Phylogeny
Tree Drawing methods
Outree file of 'Consense' of phylip 3.68 of ML/ MP/ NJ/ UPGMA browsed
Plot phylogenetic trees for each country isolates of ZYMV
Flowchart for construction of phylogenetic trees of protein sequences of ZYMV using EXOME™

Figure 2

Figure 3: Deformed leaf showing blisters and vein clearing symptoms on Summer Squash (*Cucurbita pepo* L.).

infection caused shoe stringing and overall growth reduction in comparison to their healthy counterparts.

Serological detection

Infected leaves of summer squash showing prominent symptoms were subjected to serological indexing and the samples collected from hill state of H.P. produced prominent yellow colour and which was also confirmed by the OD value obtained and as the OD value was so near to the positive control OD it confirmed severe infection of ZYMV in the samples drawn from District Una (HP) (Tables 4 and 5).

RNA isolation and molecular detection of the virus using RT-PCR and amplification of cDNA

Results of serology indicated presence of test virus and concentration of the virus was also high. So, infected and healthy plants were then used for RNA isolation. The isolated RNA was reverse transcribed into cDNA. This RT-PCR was then followed by amplification of cDNA with PCR. The amplified product obtained was of 700 bp and on using this PCR product along with forward and reverse primer for sequencing the sequence so obtained were 154 nucleotides (Sequence in FASTA Format)

Sequence

GCTACGAAACCTACGGGATAGCAGTCTCACACTT-GACGCTTTCGATTTCTATGAAGTCAATTCTACAACTCCT-GAAAGAGCCCGTGTAGCTGTAGCGCAGATGAAAGCAG-CAGCTCTTAGCAATGTTTCTTCAAGGCGGTTTGGCATAGG-TGAT

Translation of the test sequence

The sequence was translated into its amino acid residues using protein translator tool at Target Assisted Iterative Screening (TAIS) network. Analysis of amino acid sequence showed a longest open reading frame (5'-3') of 51 amino acids with Methionine in between. (Protein Product).

Protein sequence

5'-3' L R N L R D S S L T L D A F D F Y E V N S T T P E R A R V A V A Q **Met** K A A A L S N V S S R R F G I G D

Multiple sequence alignment

Multiple sequence alignment of selected nucleotide and protein sequences of zucchini yellow mosaic virus with that of Una (Indian) isolate was performed using CLUSTAL W program [7] available online at European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/) and similarly, country wise CLUSTAL W along with query nucleotide and protein sequence was also performed and these CLUSTAL W outputs were then used in (phylip 3.68 and EXOME™ software) bioinformatics tools for constructing phylograms and phylogenetic trees.

Pairwise percentage similarity score matrices were also drawn for each of the 67 nucleotide and protein sequences when compared with test isolate from Una (India). This data is arranged country wise in tabular form: (Table 6).

The pairwise similarity score of 67 nucleotide sequences with test sequence elucidates that sequences from Australia, Austria, China, Hungary, Japan, Korea, Taiwan and Varied countries were 75%, 77%, 75-77%, 77%, 77%, 67-77%, 75-77%, 75-77% respectively in case of proteins (Table 6).

Phylogenetic Analysis

To trace out the evolutionary patterns of the test virus and to find out relationship of the same with other selected sequences at NCBI (Tables 7 and 8) (Figure 4 (included as supplementary data)) phylograms and phylogenetic trees were constructed using Maximum Likelihood (ML), Maximum Parsimony (MP), Neighbor Joining (NJ) and Unweighted pair group method of mathematical averages (UPGMA) methods using phylip 3.68 and EXOME™ respectively.

Phylograms and phylogenetic trees analysis of nucleotides and proteins

Australia: A total of 5 nucleotide and 5 protein sequences selected from Australian sequences were put to analysis with the test virus, the trees were drawn and the results using different methods are being briefly described

- The test virus found sequence similarity with DQ925447 in all the phylograms and phylogenetic trees constructed for test ZYMV sequences from Australia
- The test virus found protein sequence similarity with ABL09422 in all the phylograms and phylogenetic trees constructed

Austria: A total of 9 nucleotide and 9 protein sequences selected from Austrian sequences were put to analysis with the test virus, the trees were drawn and the results using different methods are being briefly described

- The test virus found sequence similarity with AJ420020 in all the phylograms and phylogenetic trees constructed for Austrian isolates
- The test virus found protein sequence similarity with CAD12315 and CAD12316 in all the phylograms and phylogenetic trees constructed for Austrian isolates

China: A total of 20 nucleotide and 20 protein sequences selected from Chinese sequences were put to analysis with the test virus, the trees were drawn and the results using different methods are being briefly described

- The test virus found least sequence similarity only with AJ316229 out of all the phylograms and phylogenetic trees constructed for Chinese isolates
- The test virus found least protein sequence similarity with some protein sequences from all the phylograms and phylogenetic trees constructed for Chinese isolates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0.03 (N1)		0.04 (N1)		0.045 (H1)		0.034 (E1)				
C												
D		0.08 (P1)		0.04 (P1)		0.07 (P1)		0.06 (H1)				
E												
F		1.36 (U1)		1.37 (U1)		1.40 (+ve)		0.05 (-ve)				
G												
H												

B2: Infected Summer Squash from Dept. of Vegetable Crops UHF Nauni Solan (H.P.) (N1);B4: replica of B2 sample; B6: Healthy Summer squash (H1) and B8: extraction buffer (E1); D2: Infected Summer Squash from university farm Dept. of STPC Pandah (P1); D4 and D6: replica of D2 sample; D8: Healthy Summer squash; F2: Summer Squash samples from District Una (U1); F4: Replica of F2 sample; F6: Positive control (+ve); F8: Negative control (-ve)

Table 4: DAC ELISA results for detection of potyvirus using potyvirus group specific immunoglobulins (O.D value at A_{405} nm).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1.41 (U1)	1.41 (U1)	1.39 (U1)	1.40 (U1)		1.41 (U1)	1.40 (U1)	1.42 (U1)	1.40 (U1)		
C												
D		1.45 (+ve)	1.46 (+ve)									
E												
F		0.06 (H1)	0.04 (H1)									
G		0.034 (E1)	0.05 (-ve)									
H												

B2 to B5: Summer Squash samples from District Una (U1);B7 to B10: Replica of B2 to B5
D2 and D3: Positive control (+ve); F2 and F3: Healthy Summer squash (H1); G2: Extraction buffer (E1); G3: Negative control (-ve)

Table 5: DAS ELISA results against detection of Zucchini yellow mosaic virus using ZYMV specific immunoglobulins (O.D value at A_{405} nm).

Country	Total number of sequences collected	Similarity score (nucleotides)	Similarity Score (proteins)
Australia	05	73-81%	75%
Austria	09	82-86%	77%
China	20	72-87%	75-77%
Hungary	04	82%	77%
Japan	06	82-87%	77%
Korea	05	74-84%	67-77%
Taiwan	08	84-87%	75-77%
Varied countries	10	74-88%	75-77%
Total	67		

Table 6: Nucleotide and protein sequences alignment data generated for different countries by Clustal W tool.

Accession no. with Country	Description	Percent Homology
D13914 (USA)	Zucchini yellow mosaic virus gene for nuclear inclusion protein and coat protein	91%
AF127933 (Taiwan)	Zucchini yellow mosaic virus isolate TW-NT1 polyprotein gene, partial cds	90%
AB188116 (Japan)	Zucchini yellow mosaic virus genomic RNA, complete genome, isolate:2002	89%
AB188115 (Japan)	Zucchini yellow mosaic virus genomic RNA, complete genome, isolate:Z5-1	89%
AJ316229 (China)	Zucchini yellow mosaic virus gene for polyprotein, genomic RNA, isolate WG	89%
AJ420020 (Austria)	Zucchini yellow mosaic virus genomic RNA for polyprotein gene, NIb protein and coat protein region, isolate Italy 1	89%
DQ925447 (Australia)	Zucchini yellow mosaic virus isolate ZYMV-VN/Cm3 polyprotein gene, partial cds	83%
AJ459956 (Hungary)	Zucchini yellow mosaic virus partial CP gene for coat protein, isolate H272-8, genomic RNA.	73%
AJ429071 (Korea)	Zucchini yellow mosaic virus polyprotein gene, strain A, genomic RNA	65%

Table 7: Phylograms and Phylogenetic trees analysis data of nucleotide sequences of ZYMV (with the test sequence) for different countries using Phylip 3.68 and EXOME™.

Hungary: A total of 4 nucleotide and 4 protein sequences selected from Hungarian sequences were put to analysis with the test virus, the trees were drawn and the results using different methods are being briefly described

- The Hungarian sequences found around 60% sequence similarity with the test sequence in all the phylograms and phylogenetic trees constructed for Hungarian isolates
- The test virus found protein sequence similarity with CAD31036, CAD31056 protein sequences in all the phylograms and phylogenetic trees constructed for Hungarian isolates

Accession number with Country	Description	Homology
BAE75935 (Japan)	polyprotein [Zucchini yellow mosaic virus]	75.9%
CAD12315 (Austria)	polyprotein [Zucchini yellow mosaic virus]	75.9%
CAD31036 (Hungary)	coat protein [Zucchini yellow mosaic virus]	75.9%
AAD44688 (Taiwan)	polyprotein [Zucchini yellow mosaic virus]	75.5%
CAD22062 (Korea)	polyprotein [Zucchini yellow mosaic virus]	75.5%
AAM53602 (China)	coat protein [Zucchini yellow mosaic...	74.3%
ABM65098 (Poland)	coat protein [Zucchini yellow mosaic virus]	74.3%
ABL09422 (Australia)	polyprotein [Zucchini yellow mosaic virus]	74.3%

Table 8: Phylograms and Phylogenetic trees analysis data of protein sequences of ZYMV (with test protein) for different countries using Phylip 3.68 and EXOME™.

Japan: A total of 6 nucleotide and 6 protein sequences selected from Japanese sequences were put to analysis with the test virus, the trees were drawn and the results using different methods are being briefly described

- The test virus found sequence similarity with AB188115 and AB188116 in all phylograms and phylogenetic trees constructed for Japanese isolates
- The test virus found protein sequence similarity with BAE75935, BAE75934, and BAD74201 protein sequences in all the phylograms and phylogenetic trees constructed for Japanese isolates

Korea: A total of 5 nucleotide and 5 protein sequences selected from Korean sequences were put to analysis with the test virus, the trees were drawn and the results using different methods are being briefly described

- The test virus found sequence similarity with AJ429071 out of all the phylograms and phylogenetic trees constructed for Korean isolates
- The test virus found protein sequence similarity with CAD22062, AAQ17215 and AAQ17216 protein sequences in the phylograms and phylogenetic trees constructed for Korean isolates

Taiwan: 8 nucleotide and 8 protein sequences of CP ZYMV selected from Taiwan and were put to analysis with the test virus, the trees were drawn are being briefly described

- The test virus found sequence similarity with AF127933 in all the phylograms and phylogenetic trees constructed for Taiwanese sequences
- The test virus found less protein sequence similarity with AAD44688 protein sequence as revealed from all the phylograms and phylogenetic trees constructed for Taiwanese isolates 10 nucleotide and 10 protein CP gene sequences of ZYMV isolates of different countries were studied to analyze with the test virus, the trees were drawn and the results using different methods are being briefly described

Among the various sequences of varied countries, sequences from California, France, India, Israel, Poland, South Africa, Singapore and USA were studied.

- The test virus found maximum sequence similarity with D13914 in all the phylograms and phylogenetic trees constructed
- The test virus found protein sequence similarity with ABM65098 and ABI97984 protein sequences

Discussion

In the present studies, partial CP gene sequence of Una (Indian) isolate of ZYMV compared with other 67 isolates of ZYMV at both genomic and proteomic level to see its evolutionary behavior.

Viral cultures under present investigations were selected on visual symptoms. The zucchini yellow mosaic virus has been known to produce symptoms like vein clearing, yellow mosaic, blistering and shoestringing of leaves, fruit and seed deformations and stunting of plants [8].

DAS-ELISA confirmed the presence of ZYMV in the samples collected. In literature, there are numerous reports (Chalam et al., Auger et al., Malik et al. and Pospieszny et al.) in which DAS-ELISA has been used to confirm presence of ZYMV and other viruses in the infected plant samples [9-12].

There have been many reports of simple and rapid techniques to detect plant viruses using RT-PCR. Lately, in 2007, detection of ZYMV using RT-PCR was carried out in *C. sativus* L. and *Cucumis melo* L. in Poland. Pospiezny et al. and Auger et al. identified a strain of ZYMV on squash by means of DAS ELISA and PCR using ZYMV specific primers ZY-2 and ZY-3 and a segment of 1186 bp was amplified and sequenced [10,12].

There are other numerous reports, where both PCR and RT-PCR have been used for rapid detection of ZYMV [13,14-17]. The amplified product of ~ 700 bp under present investigations is in consonance with the findings of Sharma, who reported similar size (~700 bp) for ZYMV isolates of various infected summer squash plants of H.P [18].

Prieto et al. had also sequenced a fragment of 395 bp in length from the 3' portion of CP gene of Chilean isolate of ZYMV. In the present case however only 154 nucleotide long DNA was amplified confirming only partial amplification and sequencing of the CP gene [19].

Multiple sequence alignment of the test nucleotide and protein sequence of test isolate with other 67 isolates of ZYMV imported from NCBI revealed that alignment score was highest for USA among varied countries and lowest for China in case of nucleotides whereas it was lowest for Korea in case of proteins. Alignment score for Indian sequence of ZYMV was 86% and 77% in case of nucleotides and proteins, respectively on using Clustal W.

Shukla and Ward predicted amino acid sequence of ZYMV coat protein of USA and compared with the published amino acid sequences of other potyviral coat proteins [20]. Overall homology ranged from 47.5 to 67.1%. This was in agreement with 38 to 71% range of homologies observed among distinct potyviruses; while different strains of the same virus showed greater than 90% homologous behavior.

In present studies phylogenetic relationship of the test isolate with 67 isolates of ZYMV retrieved from NCBI database were determined at both nucleotide and protein levels by applying four methods viz., the UPGMA [21], the neighbour joining [22], the maximum likelihood [23,24] and the maximum parsimony using Phylip 3.68 and EXOME™ software [25].

Present phylogenetic analysis at nucleotide level indicated that DQ925447 (Australia), AJ420020 (Austria) with significant bootstrap,

AJ316229 (China), AJ459956 (Hungary) with low bootstrap, (AB188116 and AB188115) Japan, AF127933 (Taiwan), D13914 and DQ978272 (USA among varied countries) showed close proximity with the test partial CP of ZYMV. While at protein level ABL09422 (Australia), CAD12315 and CAD12316 (Austria), CAD31036 (Hungary), BAE75935 and BAE75934 (Japan), AAD44688 (Taiwan) and ABM65098 (Poland among varied countries) showed maximum proximity at protein level. Studies conducted by Lin et al., Pfosser and Baumann, and Zhao et al. supported present investigations [13,16,26].

Auger et al. identified a strain of ZYMV on squash and phylogenetic analysis of this strain with other isolates revealed its 98% identity with Connecticut and California strains [10].

Conclusion

In Conclusion, it was found that the test virus showed maximum similarity with the USA isolate (D13914) of ZYMV. It is however indicating that the virus may have been imported into India from USA.

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