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

Neha Sharma*, Satya Vrat Bhardwaj, Anju Sharma, Manica Tomar, Rajinder Kaur, Pritam Dass Thakur and Anil Handa
Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

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 EXOMETM 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; Phylogenams; 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, cucurbitis 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 EXOMETM 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

*Corresponding author: Neha Sharma, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India, E-mail: angelnbt@gmail.com

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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.

**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
trees EXOME™ was used. Test virus nucleotide sequence and polypeptide sequence analysed countrywide 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.

Symptomatology

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, motting 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

| 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                    | AA707674                      | 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                    | CAB03753                      | Hungary     |
| 41    | GQ251520                    | ACS36116                      | India       |
| 42    | EFO62582                    | ABL01531                      | Israel      |
| 43    | EFO62583                    | ABL01532                      | Israel      |
| 44    | AB063251                    | BAB29274                      | Japan       |
| 45    | AB458595                    | BAH97116                      | Japan       |

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

```
GCTACGAACCTACGGGATAGCAGTCTCAACTT-GACGCTTTGAGTTTCTATGAAATCACAATCTCAACTCT-GAAAGACCGCTGTAGCTGTAGCGCAGATGAAAGCAG-CAGCCTCTTAGCAATGTTTCTTCAAGGCGGTTTGGCATAGG-
GAAAGAGCCCGTGTAGCTGTAGCGCAGATGAAAGCAG-
GACGCTTTCGATTTCTATGAAGTCAATTCTACAACTCCT-
```

**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’-FLRNLRDSLTLDAFDFYEVNSTTPERAVAMETKAALSNVSSRFGID
```

**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
Table 4: DAC ELISA results for detection of potyvirus using potyvirus group specific immunoglobulins (O.D value at A\textsubscript{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 5: DAS ELISA results against detection of Zucchini yellow mosaic virus using ZYMV specific immunoglobulins (O.D value at A\textsubscript{405} nm).

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% |
| A8188116 (Japan) | Zucchini yellow mosaic virus genomic RNA, complete genome, isolate:2002 | 89% |
| A8188115 (Japan) | Zucchini yellow mosaic virus genomic RNA, complete genome, isolate:ZS-1 | 89% |
| AJ316229 (China) | Zucchini yellow mosaic virus gene for polyprotein, genomic RNA, isolate WG | 89% |
| AJ420020 (Australia) | Zucchini yellow mosaic virus genomic RNA for polyprotein gene, NiL 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 EXOMETM.

**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.
The test virus found protein sequence similarity with ABM65098 and AB197984 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. Pospieszny 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,

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

| 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 virus] | 74.3%    |
| ABM65098 (Poland)            | coat protein [Zucchini yellow mosaic virus] | 74.3%    |
| ABL09422 (Australia)         | polyprotein [Zucchini yellow mosaic virus] | 74.3%    |

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 the phylograms and phylogenetic trees constructed for Japanese isolates
- The test virus found protein sequence similarity with BAE75935, BAE75934, and BAD42017 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 and the results using different methods 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 sequence similarity with CAD22062, AAQ17215 and AAQ17216 protein sequences in the phylograms and phylogenetic trees constructed for Taiwanese sequences
- The test virus found maximum sequence similarity with D13914 in all the phylograms and phylogenetic trees constructed

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

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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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