

## BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF THE POLISH *Zucchini yellow mosaic virus* ISOLATES

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**Abstract.** The diversity of *Zucchini yellow mosaic virus* (ZYMV) isolates from cucumber and zucchini plants growing in different regions of Poland was analyzed using biological tests and molecular biology techniques. The isolates differed in their host range and symptoms induced by them on a series of plant species. In addition, the analysis of the genetic diversity of the coat protein (CP) gene revealed high level of nucleotide variability among the isolates. Comparison of the CP gene sequences of 70 isolates from different geographical regions worldwide showed that the Polish isolates belong to different groups and they do not form a monophyletic cluster with European isolates. Interestingly, among the central European ZYMV isolates lower variability has been observed previously. The ratio of nonsynonymous to synonymous polymorphic sites showed a dominant negative selection however codons which might undergo positive selection were also identified. Moreover, the evidences for recombination in analyzed sequences of the CP gene of the analyzed ZYMV isolates were provided.

**Key words:** ZYMV, coat protein, sequence analysis, genetic diversity, host range

### INTRODUCTION

*Zucchini yellow mosaic virus* (ZYMV), a member of the family Potyviridae and genus *Potyvirus*, causes yield losses in cucurbit crops worldwide. The virus is highly infectious and can be transmitted not only by aphids but also mechanically or by seeds [Schrijnwerkers et al. 1991]. The ZYMV, like other potyviruses, contains of single stranded positive sense RNA of about 9600 nucleotides, encoding a single large polyprotein precursor which is proteolytically processed to yield as many as 10 functional proteins [Revers et al. 1999]. Differences among ZYMV isolates in their host range, pathogenicity, serological and molecular characteristics have been defined worldwide [Glasa et al. 2007, Pfosser and Baumann 2002]. The presence of ZYMV in Poland was

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first reported in 2002 [Pospieszny et al. 2003]. Mosaic and interveinal chlorosis on leaves of cucumber (*Cucumis sativus* cv. 'Śremski F1') growing in one of the commercial greenhouses in the Great Poland region were observed. In our study we used this cucumber isolate (Cu) and two others collected from zucchini plants. Mosaic and yellowing of leaves, as well as yellow spots on green fruits of the zucchini (*Cucurbita pepo* L. var *giromontiina* cv. 'Atena Polka F1') growing in commercial fields in the Kujawsko-Pomorski region of Poland were observed. In 2006, we partially characterized isolate designated Zug, and we showed that it was distinct from the European ZYMV isolates [Pospieszny et al. 2007]. A third isolate, marked Zuy was also obtained from zucchini plant in 2007 in the Kujawsko-Pomorski region of Poland. So far, the cucurbit crops have not been widespread in Poland and for that reason amount of collected samples was limited.

Due to the fact, that now the production of cucurbit crops is increasing we aimed to estimate the extent of genetic diversity among the Polish isolates. The knowledge of ZYMV variability is essential for an understanding the complexity and epidemiology of the pathogen and for designing effective control strategies. In this work, we analyzed the Polish ZYMV isolates on the basis of reaction of tested plant species as well as the sequences of their CP. The coat protein is probably the best marker for investigating closely related isolates because it is the only gene product which shares little sequence identity with the corresponding protein of other virus groups [Berger et al. 1997, Domier et al. 1987]. The coat protein is involved in the encapsidation of viral RNA, vector transmission [Shukla et al. 1991, Urcuqui-Inchima et al. 2001], the regulation of viral RNA amplification and cell-to-cell movement and systemic movement [Urcuqui-Inchima et al. 2001].

Although the ZYMV isolates have been studied before [Glasa et al. 2007, Pfosser and Baumann 2002] the information about their diversity is still limited for the European region, especially in Poland. In this work we present results concerning the phylogenetic relationships, selection pressure and recombination acting on ZYMV isolates population using the coat protein gene as a model. Moreover, we present the biological and genetic diversity observed among naturally occurring Polish ZYMV isolates.

## MATERIALS AND METHODS

**Virus isolates, DAS-ELISA and host range.** Virus isolates were collected from naturally infected zucchini (Zuy, Zug) and cucumber plants (Cu) in the Kujawsko-Pomorski and the Great Poland regions, respectively. Only flexuous virus particles (~750 nm long), typical for potyviruses, were observed in leaf-dip preparations from symptomatic zucchini and cucumber plants. The presence of ZYMV in the original host or in experimental test plants was checked by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) [Clark and Adams 1977] using commercial polyclonal antiserum (AS-0234; DSMZ, Braunschweig, Germany). Due to the fact, that many cucurbit plants are double-infected by cucurbit-infecting viruses as *Cucumber mosaic virus* (CMV) in commercial fields the ELISA test using antiserum against CMV was also performed. At least five zucchini plants were inoculated mechanically with

each isolate for propagation. Young zucchini leaves showing severe symptoms were ground in 0.05 M phosphate buffer pH 7.0 and the extract was used for mechanical inoculation of carboroundum dusted leaves of the test plants. Inoculated plants were maintained in a greenhouse, monitored for presence of the symptoms for six weeks and assayed for viral infection by the ELISA test. The virus in the sap from symptomatic plants was mechanically transmitted on test plants including *C. pepo* var. *giromontiina* cv. 'Astra Polka' and 'Atena Polka F1', *C. maxima* cv. 'Bambino', *Cucumis melo* cv. 'Seledyn F1', *C. sativus* cv. 'Śremski F1', *Citrullus lanatus* cv. 'Bingo', *C. pepo* var. *patissonina* cv. 'Disco' and *Nicotiana benthamiana*. The experiments were performed in triplicate (three times) and at least 5 plants of each species were inoculated in every experiment.

**RNA isolation, RT-PCR, sequencing.** Total RNA (at final concentration of 1 µg/µl) was extracted from infected *C. pepo* var. *giromontiina* cv. 'Astra Polka' plants and purified with phenol-chloroform. RNA was dissolved in 40 µl of sterile water and 1 µl of solution was used for cDNA synthesis. First-strand cDNAs were synthesized using 1 µg total RNA, Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions, and M4T (5'-GTT TTC CCA GTC ACG AC (T)<sub>15</sub>3') as the initial primer [Chen et al. 2001]. An universal primer (Sprimer: 5'-GGN AAY AAY AGY GGN CAR CC-3', where N = A, G, C or T; Y = T or C; R = A or G), designed for the consensus sequence of the GNNSGQP amino acid motif in the NIb region conserve for members of the family *Potyviridae*, was used to amplify by RT-PCR the 3'-terminal genome regions from infected plant samples [Chen et al. 2001]. The PCR mixtures contained 2 µl template cDNA, 0.4 µM of each amplification primer (M4: 5'-GTT TTC CCA GTC ACG AC-3' and Sprimer), 10 mM each dNTP, 5 U *Taq* DNA polymerase (Fermentas), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl pH 8.3 in a total volume of 50 µl. The amplification was for 30 cycles each consisting of 0.5 min at 94°C, 1 min at 47°C and 2 min at 72°C and followed by a final extension step for 10 min at 72°C. RT-PCR products were examined by electrophoresis in 1% agarose gels. RT-PCR products were cloned into pCR<sup>®</sup>-*XL-TOPO*<sup>®</sup> vector, and electro-competent One SHOT Top10 cells (Invitrogen) were transformed according to manufacturer's instructions. Plasmid DNA was purified using PureYield Plasmid MiniPrep System (Promega) and selected clones were sequenced at least twice.

**Restriction Fragment Length Polymorphism (RFLP) analysis.** The part of ZYMV genomes containing nuclear inclusion body (NIb) with function of polymerase and the CP genes were amplified using Sprimer and M4 primers as described above. Obtained products about 1700 bp were subjected to restriction fragment length polymorphism analysis. The restriction enzyme *TruI* (Fermentas) was chosen by *in silico* analysis of the Polish ZYMV sequences using BioEdit program. The digestion was performed according to manufacturer's instructions and obtained restriction fragments were visualized in 1.5% agarose gel.

**Phylogenetic, recombination and selection pressure analyses.** Phylogenetic analyses were conducted with the Polish and other isolates of ZYMV, for which the nucleotide sequence of the CP genes were available in databank. The multiple sequence alignments were performed using the ClustalW program [Thompson et al. 1994]. Phylogenetic analysis was carried out by the Maximum Likelihood algorithm implemented

with MEGA 5 software [Tamura et al. 2011]. The best fit model of nucleotide substitution (Tamura-Nei) was determined by MEGA5. A second aim of this study was to verify the nature of the selective pressure operating on CP of ZYMV. The single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL) and internal branches fixed-effects likelihood (IFEL) methods were also used to evaluate  $\omega$  per codon [Kosakovsky Pond and Frost 2005]. Both SLAC and FEL methods using the default significance level of  $p = 0.1$ ; a Bayes factor of 40 was applied as selection threshold for Random Effects Likelihood (REL). These analyses were performed using the HyPhy package implemented in the Datamonkey web server [Kosakovsky Pond et al. 2004, Kosakovsky Pond and Frost 2005]. Analysis of selective pressures was based on evaluating  $\omega$  for each codon in the nucleotide sequence alignment. ZYMV sequences were initially analyzed for evidence of recombination events using the RDP package [Martin et al. 2010]. The sequences were analyzed using the following methods: 3Seq, Chimaera, Lard, BootScan, RDP, Geneconv, and MaxChi. Only recombination breakpoints supported by more than three methods were considered as valid.

## RESULTS

All diseased plant materials used in this study showed positive reactions in ELISA test, only with antiserum against ZYMV. The host range and the severity of the systemic symptoms induced by Cu, Zuy and Zug isolates on the test plants were different as presented in Table 1. The reaction of *C. sativus* cv. ‘Śremski F’ clearly differentiated the three ZYMV isolates (fig. 1). The severest symptoms displayed plants infected with Zug isolate which induced stunting of the tested plant species and severe malformation of leaves. Milder symptoms were observed during infection with Cu isolate which cau-

Table 1. Symptoms produced on test plants by three ZYMV isolates

Test plants	Isolates of ZYMV		
	Zuy	Cu	Zug
<i>Cucurbita pepo</i> var. <i>giromontiina</i> ‘Astra Polka’	Vc, M, SMI	Vc, M, SMI	Vc, <b>SM</b> , SMI
<i>Cucurbita maxima</i> cv. ‘Bambino’	Lch; N, MM	SM	M, St, MI
<i>Cucumis melo</i> cv. ‘Seledyn F1’	M	M, SMI	St, SMI
<i>Cucumis sativus</i> cv. ‘Śremski F1’	MM	SM, GVB	SM, MI
<i>Cucurbita pepo</i> var. <i>giromontiina</i> cv. ‘Atena Polka F1’	Vc, M	M	M, MI
<i>Citrullus lanatus</i> cv. ‘Bingo’	M, MI	M, MI	M, St
<i>Cucurbita pepo</i> var. <i>patissonina</i> cv. ‘Disco’	Vc, M, MI	M, MI	Vc, <b>SM</b> , MI
<i>Nicotiana benthamiana</i>	s	s	s

SM – severe mosaic (+++), M – mosaic (++) , MM – mild mosaic (+), SMI – severe malformation, MI – malformation, St – stunting, N – leaf necrosis, Lch – local chlorotic spot, Vc – vein clearing, GVB – green vein banding, s – symptomless, systemic infection

sed mosaic and green vein banding of leaves. Isolate Zuy induced milder symptoms than Cu and Zug on *C. sativus* cv. 'Šremski F1' and *C. melo* cv. 'Seledyn F1', however on *Cucurbita maxima* cv. 'Bambino' and *C. pepo* var. *giromontiina* cv. 'Atena Polka F1' caused additional symptoms like leaves necrosis and vein clearing, respectively. The reaction of *C. pepo* var. *giromontiina* cv. 'Astra Polka' and *C. pepo* var. *patissonina* cv. 'Disco' also revealed differences between isolates. In both cases isolate Zug induced severer mosaic on the test plants in comparison to Cu and Zuy isolates.

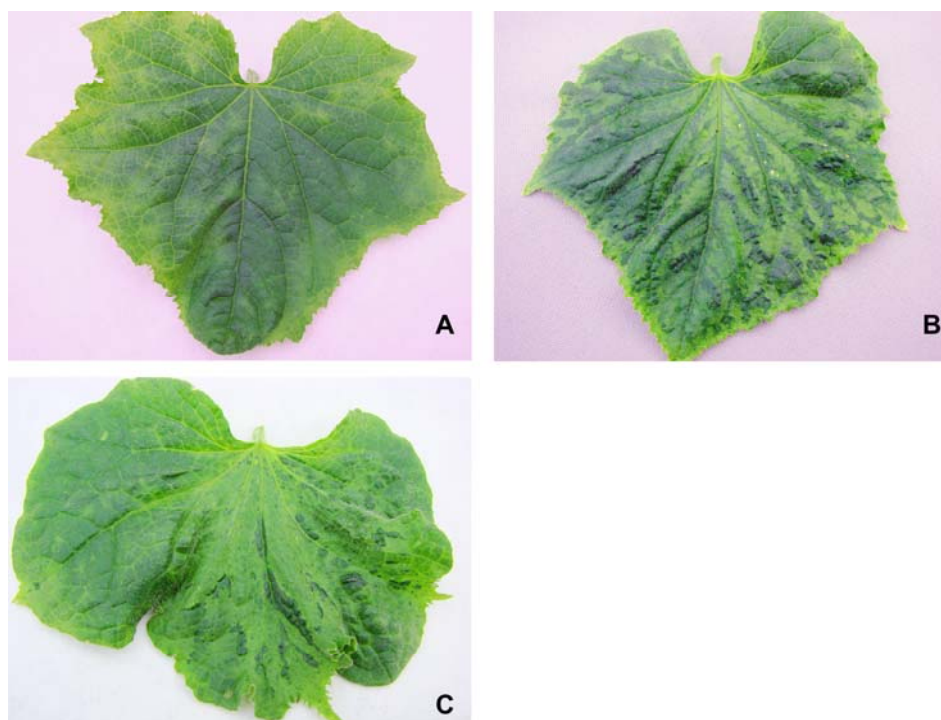


Fig. 1. Symptoms induced by the Polish isolates Cu (A), Zuy (B) and Zug (C) of ZYMV on *C. sativus* cv. 'Šremski F1' plants

The biological differences between the Polish ZYMV isolates were confirmed by the phylogenetic analyses. In total, about 1700 nt sequence representing approximately 18% of the complete viral genome was determined for each isolate. On the basis of the RNA polymerase and coat protein genes sequences the endonuclease *TruII* was used in the restriction analysis in order to differentiate among isolates. The observed restriction fragment length polymorphism is exemplified in Figure 2. Three different RFLPs patterns were detected among the Polish ZYMV isolates which confirms genetic diversity between them.

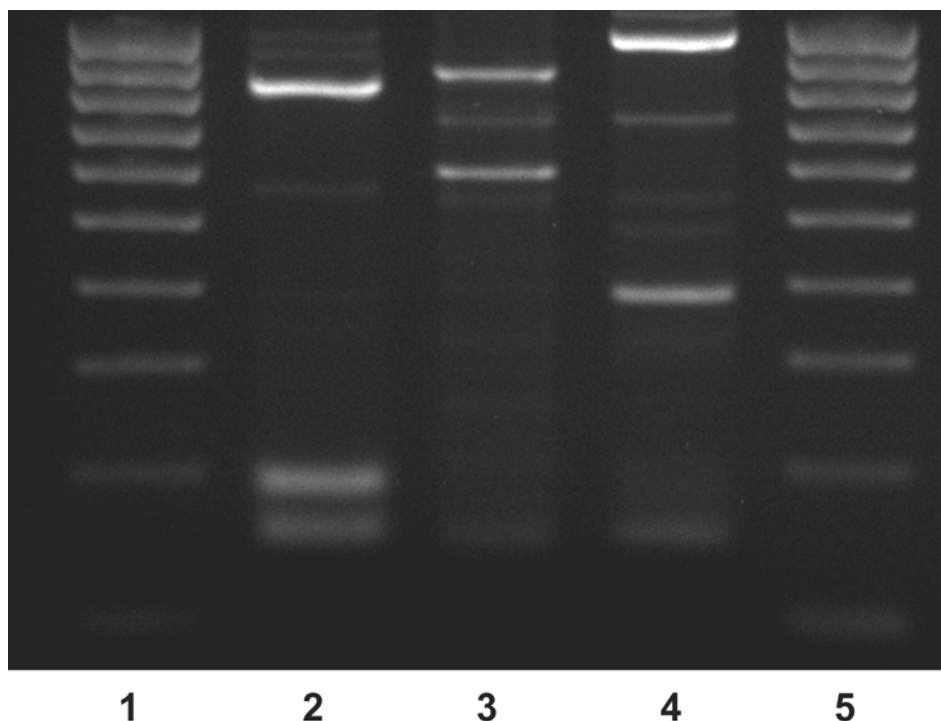


Fig. 2. Restriction patterns of 1700 bp genome fragment of the Polish isolates of the ZYMV after the digestion with the restriction endonuclease *Tru*I: Lane 1 and 5 – molecular weight marker Hyper Ladder IV (Bioline), 2 – isolate Cu, 3 – isolate Zuy, 4 – isolate Zug

The obtained sequences were deposited in the GenBank database under the accession numbers: EU561043 (Cu), EU561044 (Zuy) and EU561045 (Zug). Sequence analysis was carried out in order to investigate the genetic diversity among Polish ZYMV isolates and to establish their molecular relationships with the previously characterized ZYMV isolates from Europe and other parts of the world. Sequences of the CP gene of the three ZYMV isolates, newly acquired here, were combined with 67 isolates collected from GenBank (accession numbers showed on the tree) (fig. 3). We analyzed 837 nt representing complete CP gene. Study of the genetic diversity of Polish ZYMV isolates revealed the presence of nonsynonymous mutations (with effect on amino acid sequence). The Zuy shared 95.3% identity of the CP gene sequence with Cu isolate. At amino acid level, there was an over 98% identity. On the other hand, isolate Zug was much more divergent and shared only 83.6% and about 87% identity with Cu and Zuy, respectively. At the amino acid level, it shared 91% and 88–89% similarity with the CP sequences of the Cu and Zuy isolates. The phylogenetic tree, presented on Figure 3, was calculated and constructed using full length nt sequences of the CP gene. The separation of two groups, A and B could be observed. Interestingly, the Polish

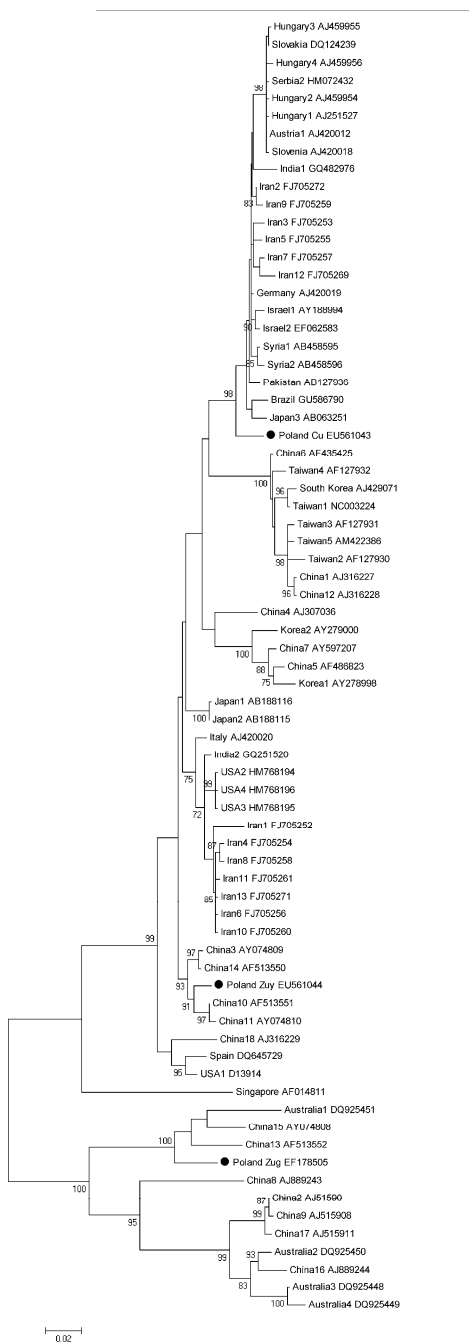


Fig. 3. Maximum-likelihood tree based on nucleotide alignments of the coat protein gene. The Polish isolates are marked with dots. Sequences of other ZYMV obtained from public data bases were included

isolates did not cluster with the Central-European strains, nor were they monophyletic. Within group A, isolate Cu was most closely placed to the Central European strains, however several isolates especially from Asia separated them. Isolate Zuy was also in group A, but formed a common branch with two Chinese isolates. Finally, isolate Zug was clustered with other Chinese isolates within group B. Similar tree topology was obtained when amino acid alignments were used and when the remaining Nib fragment was analyzed (data not shown). The recombination event were identified among analyzed sequences. The analysis revealed that isolates China 2 and China 17 (AJ51590 and AJ515911, respectively) are recombinants probably between Korean and Australian isolates (Korea 1 and Australia 3). The predicted recombination break point was identified as nt 76.

Nucleotides substitutions can be classified into two categories on the basis of their effects on the protein amino acid composition: synonymous and nonsynonymous replacements. In general synonymous substitutions accumulate at rate  $d_S$  per synonymous site. It is generally accepted that synonymous substitutions accumulate neutrally because they have no effect on the amino acids composition and thus shall not affect the folding and function of proteins. In contrast, nonsynonymous substitutions accumulate at rate  $d_N$  per synonymous site. Since these substitutions involve amino acid replacements, they must be subjected to selection. The average ratio of these two substitutions rates  $\omega = d_N/d_S$  across a gene, provides information on whether it has been fixing amino acid replacements in a neutral fashion ( $\omega = 1$ ), amino acids changes have been removed by the action of purifying selection ( $\omega < 1$ ), or changes have been fixed by positive evolution ( $\omega > 1$ ). If a gene evolves under purifying selection most of the time but is occasionally subject to episodes of positive selection, a comparison between two distant related sequences is unlikely to yield a  $\omega$  ratio significantly  $> 1$ . It is well known that recombination affects the estimation of  $\omega$  and interferes with phylogenetic reconstruction. Most CP nucleotide substitutions were silent, with dN/dS ratios  $< 1$  indicating purifying selection pressure on the CP gene. However, both FEL and IFEL methods estimated codons 40 and 73 to be under positive selection FEL and REL indicated also codon 5.

## DISCUSSION

The biological and molecular diversity of ZYMV isolates from different host plants and geographical locations from Poland was established. Overall, sequence analysis revealed a high level of nucleotide diversity between isolates. We also noticed high level of biological variability among isolates. Most of the plants infected with Zug isolate displayed severer symptoms in comparison with those infected by Cu and Zuy isolates. In addition, Zug isolate induced stunting of the test plants. Milder symptoms were induced by Cu isolate which caused mainly mosaic and green vein banding of leaves. *C. sativus* cv. 'Śremski F1' and *C. melo* cv. 'Seledyn F1' infected by Zuy isolate displayed milder symptoms in comparison to those infected by Zug and Cu, however on *C. maxima* cv. 'Bambino' and *C. pepo* var. *giromontiina* cv. 'Atena Polka F1' Zuy caused additional symptoms like leaves necrosis and vein clearing, respectively.



In our and other authors' analyses, most of the isolates from Europe formed a monophyletic group [Glasa et al. 2007, Pfosser and Baumann 2002]. It has previously been shown that Slovak and Czech isolates clustered together with the Central European isolates [Glasa et al. 2007]. The close genetic relationship among ZYMV isolates from the Central European region suggests that they probably had a common ancestor or have spread from a unique infection focus [Glasa et al. 2007]. The results of phylogenetic analyses indicate that the Polish ZYMV isolates are more divergent. The analysis showed that Polish isolates clustered in two separate groups, A and B. It should be mentioned, that within group A, isolate Cu seemed to be more closely related with the Central European cluster, while isolate Zuy was grouped together with several Chinese isolates (fig. 3). The sequence differences between the group A of the Polish isolates of ZYMV may be connected with different host origins (Cu-cucumber, Zuy-zucchini) of the isolates and with specific host-pathogen interactions. The most interesting is isolate Zug, belonging to group B, which is most divergent from the other two Polish and the other European isolates. Isolate Zug showed the highest similarity with Chinese isolates. Our results confirmed that isolates sampled from adjoining locations are not always related, suggesting that biogeographical structure may, to some extent, be determined by the international trading of infected seeds. Comparison of genetic diversity between nucleic acid and deduced amino acids sequences among Polish and other ZYMV isolates revealed presence non-silent mutations with effect on amino acids sequences of both N1b and capsid protein. This was confirmed by RFLP analysis where three different patterns were observed. The variability of virus strains particularly within the capsid protein raises a problem for the development of reliable diagnostic methods, most of which are based on the antigen properties of CP.

In this paper we showed that ratio of synonymous and nonsynonymous mutations suggests purifying selection dominating in case of ZYMV. Different approaches detected strong signal of purifying selection for the majority of codons. Although purifying selection dominates in evolution of ZYMV capsid protein, three sites having a diversifying selection signal were detected. It is not surprising that various codons are under different selection forces. By analogy to animal viruses, is likely that codons 5, 40 or 73 are involved in reaction with host factors. Host resistance may lead to diversifying selection in these positions. It is also possible that the amino acids coded by codons 5, 40 and 73 are on the surface of the capsid protein. Analysis of capsid three-dimensional structure may confirm this hypothesis; unfortunately there are no proper templates which could be applied for homology modeling of this protein to this date. Mutational studies should be conducted to gain more insight about the possible importance of this part of the capsid protein. Moreover, the recombination events were detected in analyzed ZYMV isolates suggesting that both positive selection pressure and recombination are shaping ZYMV population structure.

Our results showed that there is little evidence clustering of the isolates according to their geographical origin. The Polish isolate Zug grouped together with Chinese isolates which could most likely be explained by vegetative propagation and exchanged infected material between different countries. However, it is not known if isolate Zug was transmitted to Poland by seeds or it is native isolate. This again indicates that gathering knowledge about the diversity and variability within a virus group is especially impor-

tant and of interest when virus-resistant cultivars are to be developed. The data obtained during this study help better understand ZYMV epidemiology and develop efficient control strategies.

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## **BIOLOGICZNA I MOLEKULARNA CHARAKTERYSTYKA POLSKICH IZOLATÓW WIRUSA MOZAIKI CUKINII (*Zucchini yellow mosaic virus*)**

**Streszczenie.** Wirus żółtej mozaiki cukinii (*Zucchini yellow mosaic virus*, ZYMV) charakteryzuje się wysokim stopniem zróżnicowania zarówno biologicznego, jak i genetycznego. W pracy analizowano zakres roślin gospodarzy i symptomy wywoływane przez polskie izolaty ZYMV. Ponadto przeprowadzono analizę filogenetyczną i przebiegu rekombinacji z wykorzystaniem sekwencji nukleotydów genu kodującego białko płaszczki polskich i 67 innych izolatów ZYMV, których sekwencje zdeponowano w Banku Genów. Badania wykazały, że polskie izolaty nie tylko różniły się od siebie w teście biologicznym, ale należą do różnych grup filogenetycznych, nie tworząc tym samym jednej grupy z innymi izolatami europejskimi. Analiza presji selekcyjnej działającej na populację izolatów ZYMV wykazała dominację presji negatywnej, aczkolwiek zidentyfikowano również kodony, które mogą podlegać wpływowi pozytywnej presji selekcyjnej.

**Słowa kluczowe:** ZYMV, białko płaszczki, analiza sekwencji, zróżnicowanie genetyczne, zakres roślin gospodarzy

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