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PARTIAL CHARACTERIZATION OF *Cherry leaf roll virus* (CLRV) ISOLATES INFECTING *Sambucus* spp. PLANTS IN POLAND

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Abstract. *Sambucus nigra*, *S. kamtschatica* and *S. racemosa* plants growing in natural habitats or commercial nurseries in Poland, showing symptoms of vein clearing or chlorotic patterns were found to be naturally infected with *Cherry leaf roll virus* (CLRV). Nine virus isolates were characterized by enzyme-linked immunosorbent assay (ELISA) and sequence analysis of RNA2 fragments. Serological test using ELISA kits raised against elderberry, birch, cherry and ash isolates of CLRV showed that tested virus isolates reacted with all polyclonal antibodies used in the experiment. Genetic analysis of 3' non-coding region fragments (3'NCR) of isolates originating from elderberries revealed high level of their sequence identity (more than 95%). All tested isolates clustered exclusively within phylogenetic group E of CLRV. Analysis of polyprotein open reading frame (P2 ORF) fragments showed higher sequence variability, with nucleotide identity ranging from 88 to 93%. This indicates that analysis of P2 ORF fragments may be more suitable for studying CLRV population diversity than analysis of 3'NCR region. Phylogenetic analysis of CP gene sequences confirmed clustering of tested isolates in monotypic group. Overall, the serological and phylogenetic data suggest a host-specific nature of CLRV variants infecting *Sambucus* spp. plants in Poland.

Key words: elderberry, virus infection, sequence analysis

INTRODUCTION

Sambucus nigra is a common shrub of woodland edge, hedgerows and grassland scrub, but can also be found on waste ground and even on rubbish tips. Several *S. nigra* and *S. racemosa* cultivars are popular ornamental shrubs, valued for their unusual coloration, winter hardiness, ease of propagation and adaptability to many environmental conditions, so they are a common sight in urban areas and cultivated ground. *S. nigra*

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plants are grown commercially on small scale in Poland. Their fruits contain several chemical components that are used in the food, pharmaceutical and cosmetic industries.

Since limited information is available on viral diseases that threaten the production of *Sambucus* plants in Poland, we conducted a survey of elderberry plants for viruses. Our observations indicated that wild growing shrubs as well as commercially cultivated ornamental forms are generally virus-infected [Berniak and Kamińska 2010]. According to our study, the most widespread virus was *Cherry leaf roll virus* (CLRV).

CLRV is a member of the genus *Nepovirus* within the family *Secoviridae*. The virus has a wide natural host range including a variety of herbaceous and woody plants. It was reported that different *Sambucus* species infected with CLRV may display mosaic, chlorotic flecks or bright patterns on the leaves [Jones and Murant 1971, Juergen Hansen and Stace-Smith 1971]. In nature, the virus is transmitted through seed or pollen [Massalski and Cooper 1984]. Unlike the majority of other members of the genus *Nepovirus*, CLRV is not considered to be transmitted by soil-borne nematodes and biological vector of this virus remains undetermined [Wang et al. 2002]. Previous research have indicated that CLRV isolates could be distinguish by virulence on experimental hosts, differences in reactivity with polyclonal and monoclonal antibodies or analysis of 3' non-coding region (NCR) of the genomic RNAs [Rebenstorf et al. 2006].

Although some properties of CLRV isolates found in elderberry in Poland have been described previously [Berniak and Kamińska 2010], only partial information is available on the molecular properties and diversity of virus isolates infecting *Sambucus* spp. plants. In this study some serological and molecular properties of nine CLRV isolates recovered from three elderberry species – *S. nigra*, *S. kamtschatica* and *S. racemosa* have been analysed.

MATERIAL AND METHODS

Virus source material and CLRV isolates. The CLRV isolates were collected during the surveys of elderberry plants for virus diseases done in 2009–2013. Isolates Sn4, Sn5, Sn6, Sn7 and Sn46 were found in *S. nigra* plants growing in natural habitats located in Łódź province in Poland. Isolate Sk9, found in *S. kamtschatica* plant, originated from elderberry collection of the Polish Academy of Sciences Botanical Garden in Powsin. Three other virus isolates were extracted from ornamental elderberry cultivars *S. nigra* 'Aurea' (SnA16) and *S. racemosa* 'Sutherland Gold' (SrSG28 and SrSG29) growing in commercial nurseries, in Piaseczno and Ciechanów, respectively. Symptoms observed on CLRV-infected elderberry plants varied, depending on *Sambucus* species. The most severe symptoms were observed on the leaves of wild growing *S. nigra* plants. They included clear visible chlorotic spots and irregular patterns. Infected shrubs showed reduced fruit production, ranging from almost complete fruit set on every cyme to complete sterility. Ornamental forms of *S. nigra* and *S. racemosa* exhibited interveinal leaf chlorosis, whereas on the leaves of *S. kamtschatica* plants small bright spots were observed.

Leaves of symptomatic elderberries were collected and stored at -70ºC until used. Leaves of healthy elderberry and *Chenopodium quinoa* plants were included as negative controls. The CLRV isolate PV-0278 from ash (obtained from DSMZ, Braunschweig, Germany) was used as a positive control in all experiments.

DAS-ELISA. Serological properties of tested isolates were determined by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) using four commercial kits showing reactivity restricted to a *Cherry leaf roll virus*: CLRV-e, CLRV-ch (Bioreba, Hornik, Poznań, Poland), CLRV-AS-0149 (DSMZ, Braunschweig, Germany) and CLRV-b (Loewe Biochemica GmbH, Sauerlach, Germany). Assays were performed according to manufacturers' protocols. Absorbance values at 405 nm were determined in Multiscan FC plate reader (Thermo Scientific, Alab, Warsaw, Poland).

Nucleic acids isolation and RT-PCR. Total nucleic acids (TNA) were isolated from about 300 mg of virus infected and healthy leaves by using the silica capture method described originally by Boom et al. [1990] and adapted to the diagnosis of plant viruses by Malinowski [1996]. One microliter of TNA was used for reverse transcription-polymerase chain reaction (RT-PCR) in total volume of 10 µl. Amplification was performed using Titan One Tube RT-PCR System (Roche Diagnostics, Poland) according to manufacturer's recommendation. Specific oligonucleotides developed by Werner et al. [1997] were used in RT-PCR targeting fragment of 3' non-coding region (3'NCR) of CLRV. In order to amplify fragments of the RNA2 polyprotein open reading frame (P2 ORF), containing coat protein (CP) coding region, a new RT-PCR primers – forward F8 (5'-GCTAGCAGTCGSTTCGTMGT-3') and reverse R9 (5'-CCTGAAACATTCCCGCTGAAGCA-3'), were designed. The optimized cycling parameters were: 30 min of reverse transcription at 50ºC, 7 min of initial denaturation at 94ºC followed by 35 cycles consisted of denaturation at 94ºC for 10 s, annealing at 55ºC for 30 s, extension for 1'15 min at 68ºC, and final extension for 7 min at 68ºC. The PCR products (5 µl) were separated on 1% TBE-agarose gels and visualized under UV light after staining with ethidium bromide. Additional F6 (5'-GCHCCHTTYGGGCAYACYTC-3') and R5 (5'-AATTGAACATCCTCAAATGCCG-3') oligonucleotides were included as sequencing primers.

Sequence analyses. Sequencing of amplicons was performed in AbiPrism 3100 Genetic Analyzer apparatus (Applied Biosystems, USA), in Maria Skłodowska Memorial Cancer Center and Institute of Oncology, Warsaw, Poland. Assembled nucleotide sequences were analysed and their identities determined using Lasergene v. 7.1 software package (DNASTAR, USA). Comparison of obtained cDNA fragments with sequences available in GenBank was accomplished using BLAST algorithm (http: http://www.ncbi.nlm.nih.gov:80/BLAST/). For phylogenetic analysis Lasergene v. 7.1 software was used to perform sequence editing and compilation. The neighbor-joining method implemented in MEGA version 5.0.5 [Tamura et al. 2011] was used to infer the tree topologies based on nucleotide sequences. Branch support was calculated with 1000 bootstrap replicates. Corresponding nucleotide sequences of 32 other CLRV isolates were included in the study and are indicated by GenBank accession numbers in Table 1.

RESULTS AND DISCUSSION

An effective CLRV control in forest stands, trees in urban areas and commercial nurseries is based on prevention. The virus elimination strategy must include monitoring and early detection of the virus in elderberries, followed by eradication of infected plants. Since this strategy relies on prophylactic control measures, it requires an effective patho-

gen identification methods, based on analysis of viral nucleic acids. For development of reliable and sensitive virus detection protocol a strong basic knowledge of serological characteristics of the virus and genetic diversity of CLRV population is required.

In this study the presence of CLRV has been confirmed in samples of symptomatic elderberry plants collected from natural stands in forests, from rural areas (i.e. alleys, parks, along roadsides) and commercial nurseries using both DAS-ELISA and RT-PCR assays. The analyzed virus variants were characterized on the basis of their host range in the previous work [Berniak and Kamińska 2010]. The result of the bioassay did not show differences in the biological properties of tested isolates, despite the fact that they affected different elderberry species and induced different symptoms on the infected plants. Therefore, more comprehensive studies were needed to fully characterize virus isolates.

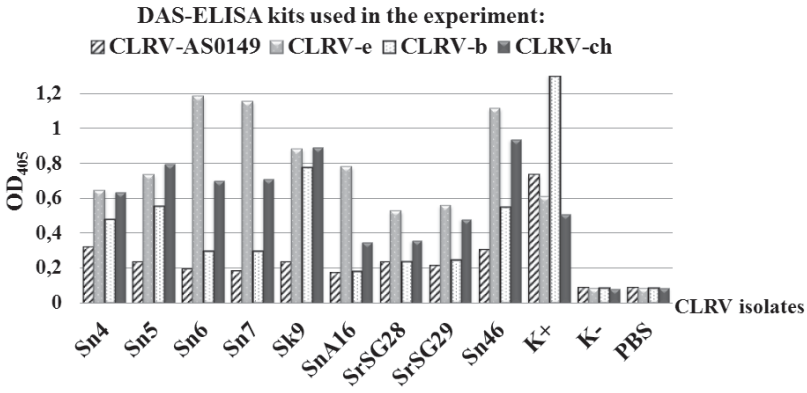

Fig. 1. A comparison of the detection of CLRV isolates found in *Sambucus nigra* (Sn4, Sn5, Sn6, Sn7, SnA16, Sn46), *S. racemosa* (SrSG28, SrSG29) and *S. kamtchatica* (Sk9) plants using DAS-ELISA with four sets of polyclonal antibodies against cherry leaf roll virus isolates from ash (CLRV-AS0149), elderberry (CLRV-e), birch (CLRV-b) and cherry (CLRV-ch). Control samples used in the experiment were: CLRV isolate PV-0278 (K+), healthy *S. nigra* leaves (K-) and PBS-TPO buffer (PBS) as a background control. Each optical density value is a mean of two replications

Results of DAS-ELISA with CLRV-specific polyclonal antibodies are shown in Fig. 1. They indicate that analyzed CLRV variants exhibit similar serological properties. The tested isolates reacted with all polyclonal antibodies used in the experiment, however, reaction of most of isolates with antibodies prepared against CLRV isolates from *Fraxinus* and birch (CLRV-AS1049 and CLRV-b, respectively) was weaker than with two other antibody sets used in experiment. This result is consistent with previous reports on serological variation of CLRV isolates [Walkey et al. 1973, De Zoeten et al. 1982, Rebenstorf et al. 2006]. It was reported that polyclonal antibodies do not recognize all CLRV isolates, and react better with homologous virus isolates than with isolates originating from different plant species. On the other hand, the ELISA set produced against cherry strain gave satisfactory results with the elderberry isolates used in our research, which indicates that tested antibodies against CLRV may vary considerably in their ability to differentiate virus isolates.

Table 1. CLRV isolates used for sequence analyses. Sequences obtained in this study are bold

Isolate	Species	GenBank accession number		Country
		3'NCR	CP coding region	
E120	*Betula pendula*	AJ877118	KF779174	Germany
E327	*Prunus avium*	AJ877127	KF779180	Germany
E603	*Sambucus nigra*	AJ877132	KF779182	Germany
E441	*Sambucus nigra*	AJ877139	KF779181	Germany
E804	*Sambucus canadensis*	AJ877145	KF779183	USA
E326	*Juglans regia*	AJ877146	KF779175	Germany
E648	*Juglans* sp.	AJ877147	KF779176	France
E693	*Sorbus aucuparia*	AJ877155	KF779184	Germany
E395	*Rheum* sp.	FR851462	FR851462	Germany
Sn4	***Sambucus nigra***	**HM165198**	**JX996091**	**Poland**
Sn5	***Sambucus nigra***	**HM165199**	**JX996092**	**Poland**
Sn6	***Sambucus nigra***	**HM165200**	**JX996093**	**Poland**
Sn7	***Sambucus nigra***	**HM165201**	**JX996094**	**Poland**
Sk9	***Sambucus kamtschatica***	**HM165203**	**JX996095**	**Poland**
SnA16	***Sambucus nigra***	**HM165204**	**JX996096**	**Poland**
SrSG28	***Sambucus racemosa***	**HM165205**	**JX996097**	**Poland**
SrSG29	***Sambucus racemosa***	**HM165206**	**JX996098**	**Poland**
Sn46	***Sambucus nigra***	**HM165207**	**JX996099**	**Poland**
Olm1	*Prunus avium*	JN104385	JN104385	USA
Ribes	*Ribes rubrum*	JN371146	KF779168	New Zealand
Rumex1	*Rumex* sp.	JN371147	KF779170	New Zealand
Rumex2	*Rumex* sp.	JN371149	KF779171	New Zealand
Hydrangea	*Hydrangea macrophylla*	JN418885	KF779165	New Zealand
739	*Actinidia chinensis*	KC937026	KC937026	New Zealand
54	*Malus domestica*	KC937027	KC937027	New Zealand
FNW	*Ribes rubrum*	KC937028	KC937028	New Zealand
1978	*Rubus idaeus*	KC937029	KC937029	New Zealand
737	*Rumex obtusifolius*	KC937030	KC937030	New Zealand
441	*Vaccinium darrowii*	KC937031	KC937031	New Zealand
Actinidia2	*Actinidia chinensis*	KF779201	KF779162	New Zealand
Actinidia2	*Actinidia chinensis*	KF779202	KF779163	New Zealand
Actinidia3	*Actinidia chinensis*	KF779203	KF779164	New Zealand
Malus	*Malus* sp.	KF779204	KF779166	New Zealand
Plantago	*Plantago major*	KF779205	KF779167	New Zealand
Rubus	*Rubus idaeus*	KF779206	KF779169	New Zealand
PV0278	*Fraxinus excelsior*	KF779207	KF779172	Germany
PV0276	*Sambucus nigra*	KF779208	KF779173	Germany
Bpen5C	*Betula pendula*	LN714366	LN714425	Germany
Bpub320b	*Betula pubescens*	LN714375	LN714387	Finland
Bpub6	*Betula pubescens*	LN714379	LN714426	Finland
W8	*Juglans* sp.	U24694	KF779179	USA, CA

The 375 bp-long fragments of 3'NCR were amplified for all tested virus isolates and positive controls. No PCR products were obtained from healthy control samples or in water blanks. Similarly, coat protein region fragments were successfully amplified using RT-PCR with newly designed oligonucleotides F8-R9. Those primers generated products of expected size of 1134 bp for all samples containing the virus, but not for negative controls. The specificity of CP-coding amplicons was verified by sequencing. Results of this experiment indicate that the new primers described here may provide a specific test for the presence of CLRV.

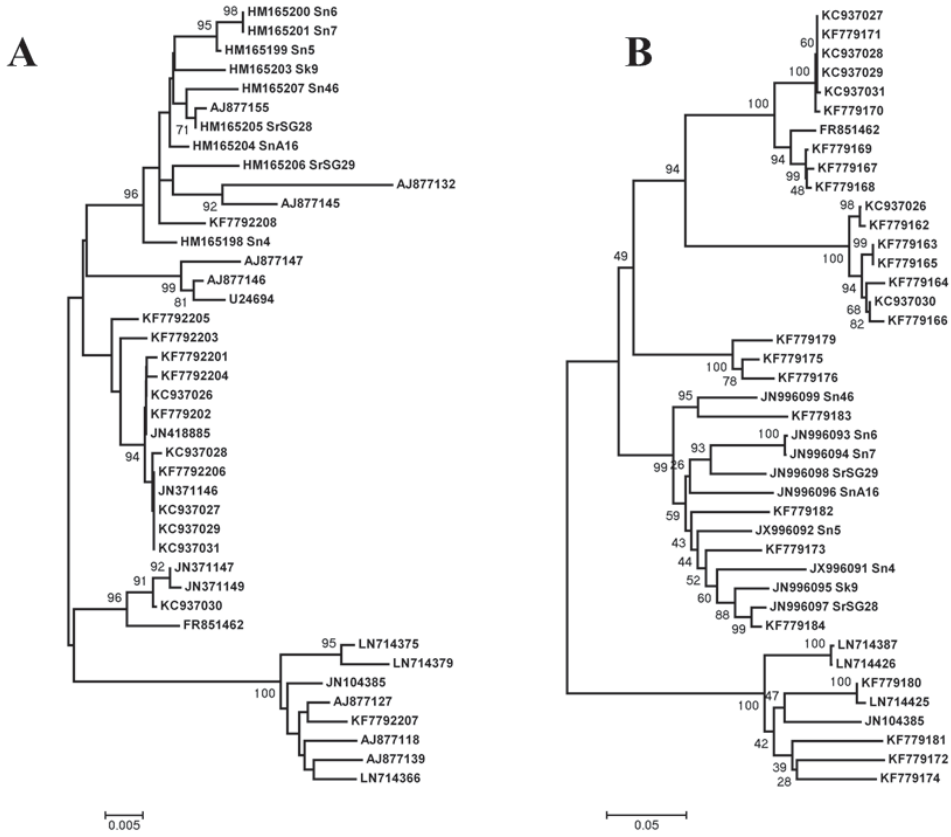

Fig. 2. Phylogenic analysis of the nucleotide sequences of the 3'NCR (A) and CP coding region (B) of CLRV isolates. The trees were created by the neighbor-joining method and bootstrapped with 1,000 replicates using the MEGA 5.0.5 package

The genetic structure of CLRV population found in *Sambucus* sp. plants has been analyzed, based on comparison of CLRV RNA2 sequence fragments of virus isolates described in this paper and previously reported pathogen isolates for which both – 3'NCR and P2 polyprotein coding regions were available (tab. 1). Sequences of amplicons obtained in RT-PCRs have been submitted to the GenBank database of the NCBI (www.ncbi.nlm.nih.gov). Fragments of 3'NCR are available by the accession numbers HM165198-201 and HM165203-207. The percentage of nucleic acid identities among the 3'NCR sequences of CLRV isolates found in Poland ranged from 95.4 to 100%. These sequences shared 90–99.7% identity with sequence of three other virus isolates (E603, E804, PV0276) found in *Sambucus* sp. plants in Germany and USA as well as with isolate E693 from *Sorbus* sp. Interestingly, one CLRV isolate (E441) found in *S. nigra* plant in Germany showed lower 3'NCR sequence identity (87.8–88.9%) to other virus variants found in elderberries worldwide. The comparison of non-coding region sequences of tested isolates with corresponding CLRV sequences available in the

NCBI database showed higher variability among CLRV variants originating from other plant species (identity ranging from 87 to 95.1%).

Although a number of 3'NCR sequences of virus isolates from elderberry have been described [Rebenstorf et al. 2006, Buchhop et al. 2009], limited information is available on the genetic diversity of CLRV strains coding regions. Prior to this work, only four CP coding region sequences of the virus isolates found in *Sambucus* plants have been published in the GenBank (KF779173, KF779181-83). Sequences of P2 ORF fragments of nine isolates reported in this study have been deposited under accession numbers JX996091-JX996099. Analysis of 628 nucleotides long fragments of CP coding region showed that tested CLRV sequences shared 88.1–93.6% identity to each other. When compared to other elderberry isolates, the analyzed CLRV variants showed greatest nucleotide identity (87.3 to 93.9%) to isolates E603, E804 and PV0276. Their identity to elderberry isolate E441 was as low as 77.1–78.8% to. The overall sequence identity of P2 ORF fragments of CLRV isolates from other plant species found worldwide ranged from 77.2 to 86%. This result indicates that CP coding sequences are more diverse than 3'NCR and they probably better reflect genetic variability within CLRV population.

Phylogenetic analysis of 3'NCR sequences led to the classification of the studied isolates as members of elderberry group (E) of CLRV (fig. 2a). This analysis confirmed the report of Rebenstorf et al. [2006] on high level of 3'NCR sequence conservation of the cherry leaf roll virus isolates from *Sambucus* sp.

Similarly, phylogenetic analysis of the CP coding regions showed that a majority of elderberry isolates form a monotypic, host-specific group (fig. 2b). A strong tendency of CLRV isolates from the same host to cluster together in the phylogenetic or serological analyses have been reported previously [Walkey et al. 1973, Rebenstorf et al. 2006, Buchhop et al. 2009]. Such a situation could be explained by two mechanisms – the inability of CLRV isolates from elderberry to infect other hosts, or their inability to be transmitted to a different host through the existence of transmission barriers [Rebenstorf et al. 2006].

However, a problematic elderberry isolate E441 reported previously occurred in phylogenetic group A [Rebenstorf et al. 2006, Buchhop et al. 2009]. Another isolate from *S. nigra* E676 (accession number AJ188130; not included in the analysis since only 3'NCR sequence for that isolate is available), belonged to group B [Rebenstorf et al. 2006]. This indicates that genetic isolation of host-specific CLRV variants from elderberries is partial and not complete. Since the phylogenetic groups A and B consists of virus variants that originate from a range of different host plants, the question arises whether isolates of this type are able to infect a broader range of host plants. It was demonstrated that some group B isolates extracted from herbaceous plants are able to infect woody hosts like elderberry and cherry [Jones 1973]. Similarly, an isolate originating from *S. nigra* 'Aurea' was transmitted by sap inoculation to peach and cherry [Juergen Hansen and Stace-Smith 1971], and from this perspective *Sambucus* plants should be taken into account as a likely source of CLRV spread to other economically or ecologically important woody plants. As a consequence, an integrated strategy relying on prophylactic control measures has to be established in the future to prevent the spread of CLRV in woody plants.

CONCLUSIONS

1. Tested CLRV isolates showed similar serological properties and high level of identity of 3'NCR sequence fragments.

2. Based on the RNA2 sequence fragments analysis, the tested CLRV isolates found in elderberries in Poland were classified as members of phylogroup E of Cherry leaf roll virus.

3. Differences in symptoms in original host plants as well as sequence diversity of the coat protein coding region suggest that the tested isolates exist as biologically distinct strains, even within the same CLRV group.

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CHARAKTERYSTYKA IZOLATÓW WIRUSA LIŚCIOZWOJU CZEREŚNI (*Cherry leaf roll virus*, CLRV) PORAŻAJĄCYCH ROŚLINY Z RODZAJU *Sambucus*

Streszczenie. Przedmiotem badań były rośliny trzech gatunków bzu (*Sambucus nigra*, *S. kamtschatica* i *S. racemosa*) rosnące na stanowiskach naturalnych lub uprawiane w komercyjnych szkółkach w Polsce. W roślinach z objawami przejaśnienia nerwów lub chlorotycznymi wzorami na liściach wykryto wirusa liściozwoju czereśni (*Cherry leaf roll virus*, CLRV). Dziewięć izolatów wirusa scharakteryzowano na podstawie ich właściwości serologicznych oraz analizy sekwencji fragmentów RNA2. W teście DAS-ELISA z użyciem czterech zestawów IgG służących do diagnozowania CLRV w roślinach bzu, brzozy, czereśni i jesionu badane izolaty reagowały ze wszystkimi przeciwciałami poliklonalnymi, co wskazuje na ich zbliżone właściwości serologiczne. Porównanie niekodującego regionu końca 3' (3'NCR) wykazało wysokie podobieństwo sekwencji tego fragmentu (powyżej 95%) dla izolatów wirusa wykrytych w roślinach bzu. Analiza fragmentu sekwencji nukleotydowej poliproteiny (P2) zawierającej region kodujący gen białka płaszcza wirusa (CP) wykazała większe zróżnicowanie tego regionu niż regionu 3'NCR. Podobieństwo sekwencji regionu P2 zawierało się w przedziale od 88 do 93%. Analiza filogenetyczna przeprowadzona dla sekwencji obu regionów wykazała, że badane izolaty z bzów grupowały się na wspólnej gałęzi drzewa filogenetycznego i umożliwiła ich zaklasyfikowanie do grupy E, w której znajduje się większość dotychczas opisanych izolatów CLRV wykrytych w roślinach z rodzaju *Sambucus*. Uzyskane wyniki analiz serologicznych i molekularnych wskazują na wysoką specyficzność izolatów CLRV z bzu względem rośliny gospodarza.

Słowa kluczowe: bez czarny, choroby wirusowe, analiza sekwencji

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