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Occurrence and detection of little cherry virus 1, little cherry virus 2, cherry green ring mottle virus, cherry necrotic rusty mottle virus, and cherry virus A in stone fruit trees in Poland

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Summary. – To investigate the occurrence of little cherry virus 1 (LChV-1), little cherry virus 2 (LChV-2), cherry green ring mottle virus (CGRMV), cherry necrotic rusty mottle virus (CNRMV), and cherry virus A (CVA) in stone fruit trees in Poland, leaf samples were collected from sweet and sour cherry, peach, and apricot trees. Two sets of primers were used to increase the effectiveness of virus detection. The RT-PCR results indicated that the most frequently detected virus in all of the tested samples was CVA (60%), followed by CGRMV (13%), CNRMV (12%), LChV-1 (11%), and LChV-2 (4%). CVA and CNRMV were not detected in peaches. Mixed infections of these viruses were frequently detected.

Keywords: little cherry virus 1; little cherry virus 2; cherry green ring mottle virus; cherry necrotic rusty mottle virus; cherry virus A; RT-PCR

Stone fruit trees, including sweet and sour cherries, peaches and apricots, are popular species grown in Poland. Currently, the area of stone fruits cultivation is approximately 56,000 ha, with annual production of about 153,000 tons.

More than 20 different viruses have been reported to infect stone fruit trees worldwide (Diekmann and Putter, 1996). Little cherry virus 1 (LChV-1), little cherry virus 2 (LChV-2), cherry green ring mottle virus (CGRMV), and cherry necrotic rusty mottle virus (CNRMV) can cause severe yield and quality losses. Cherry virus A (CVA) may not be significant when present alone, but it may enhance the severity of the symptoms when combined with other viruses (James and Jelkmann, 1998). LChV-1 and LChV-2 have been associated with little cherry disease (LChD) that has spread worldwide in sweet and sour cherry trees (Rott and Jelkmann, 2001a). Infection of sensitive cultivars results in small, angular, and pointed fruits with reduced sweetness. Plants infected with CNRMV show brown, angular necrotic spots, rusty chlorotic areas, shot holes of the leaves, blisters, gum pockets, and general necrosis of the bark (Wadley and Nyland, 1976). CGRMV causes a yellow and green mottle pattern in mature leaves 4 to 6 weeks after petals fall. Fruits can have necrotic rings and a bitter, off-flavor taste (Nemeth, 1986). The previous reports showed that CVA did not seem to be associated with any symptoms and it was, therefore, described as a latent virus (Jelkmann, 1995).

As stone fruits are important for fruit production in Poland, extensive studies on the occurrence and detection of LChV-1, LChV-2, CNRMV, CGRMV, and CVA by a reverse transcription PCR (RT-PCR) were conducted.

Surveys and sample collections were carried out from the germplasm collections of the Research Institute of Horticulture and from commercial orchards in five different locations during the spring-summer periods of 2014–2015. Leaf samples were taken from 290 stone fruit trees, including 119 sweet cherries, (*Prunus domestica*),

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Abbreviations: cherry green ring mottle virus = CGRMV; cherry necrotic rusty mottle virus = CNRMV; cherry virus A = CVA; little cherry virus 1 = LChV-1; little cherry virus 2 = LChV-2; RT-PCR = reverse transcription-polymerase chain reaction

91 sour cherries (*Prunus cerasus*), 40 peaches (*Prunus persica*), and 40 apricots (*Prunus armeniaca*). The samples were collected from the leaves of trees exhibiting necrotic spots, necrotic rusty mottle, rusty chlorotic areas, reddish and brown discoloration, yellow and green mottle pattern, and apparently healthy trees. Total nucleic acids were isolated using the silica capture method (Boom *et al*., 1990)). Subsequently, RT-PCR was conducted to detect LChV-1, LChV-2, CGRMV, CNRMV, and CVA with two sets of primers in order to improve detection efficacy. The first primer set contained primers referred to in previous reports, and the second set contained newly designed primers (Table). The primers were designed based on the sequences of the studied viruses retrieved from GenBank. In order to determine the optimal annealing temperature, gradient RT-PCR was carried out with the reference isolates of LChV-1, LChV-2, CGRMV, CNRMV (Acc. Nos. HE580104, EU153101, JX468875, KC136845, resp.), and CVA isolate WV/5 (Komorowska and Cieślińska, 2004). Amplification was carried out using a SuperScript One-Step RT-PCR kit (Invitrogen) in a Thermocycler PTC-200 (MJ Research, Watertown, MA, USA). RT-PCR experiments with the published primers were performed according to the original thermal profiles (Jelkmann, 1995; Rott and Jelkmann, 2001a,b; Rybak *et al*., 2004; Glasa *et al*., 2015). The thermal profile of the RT-PCR reaction with the newly designed primers was: reverse transcription at 50°C for 30 min, the initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 68°C for 1 min. and a final elongation for 8 min at 68°C. The RT-PCR products were separated on 1% agarose gel stained with Midori Green (NIPPON Genetics, Düren, Germany). RT-PCR products were purified using the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and the amplicons were directly sequenced in both directions (Genomed, Warsaw, Poland). The sequences were analyzed using DNASTAR (Lasergene, USA). The obtained sequences were compared with others retrieved from GenBank. Multiple sequence alignments and sequence identities were determined using the CLUSTALW, http://www2.ebi.ac.uk/clustalw/ (Thompson *et al*., 1994).

Amplicons of the expected size were obtained for LChV-1, LChV-2, CGRMV, CNRMV, and CVA, but differences in the effectiveness of virus detection using some of the primer pairs were observed. The results of RT-PCR with primers LCV1F-LCV1R as well as LCVU-LCVL showed that 17 sweet cherry trees (14%) and six sour cherry trees (6.6%) were infected with LChV-1. The presence of LChV-2 was confirmed in two sweet cherry trees (1.7%) and in six sour cherry trees (9%) by both primer pairs. CGRMV was detected in 19 sweet cherry trees (16%), 11 sour cherry

Table Primers used for detection of CGRMV, CNRMV, LChV-1, LChV-2 and CVA by RT-PCR

Primer name	Sequence (5'-3')	Viral target (position)	Product size (bp)	Reference
CGRMVF	GGCGCAGACGGACCCTAAGT	AF017780 (7457-7476)	273	This work
CGRMVR	ACAACATCAAAGATGCAGTCAA	AF017780 (7708-7729)		
GRM7950	GCAGCCTTTGACTTTTTTGAG	AF017780 (7950-7970)	390	(9)
GRM8316	CCTATAGCCAGTCTTCATATTATG	AF017780(8316-8339)		
CNRMVF	CAACAAGATCCCGAAGAAGAAG	AF237816 (7494-7515)	439	This work
CNRMVR	ATCACTGGCGCGTATTTAGAACA	AF237816 (7912-7934)		
NRM48U*	TTAATGATCTTCGTGGCTTGTTG		170	(9)
NRM48L*	GAATTGACTCCTCGGTGGGTTTA			
LCV2F	GCTTTAATTTGGTCRGGTACCGAG	AF531505 (13227-13250)	460	This work
LCV2R	CATCGCCATCACCAAAACYTGACC	AF531505 (13688-13665)		
NESup	TCTTTAGTTGATGGGATGG	AF531505 (1170-1188)	376	(10)
NESlo	AATTACAAAAGTACATCCCG	AF531505 (1526-1545)		
LCV1F/ 1LC_12776F**	TCAAGAAAAGTTCTGGTGTGC3	Y10237 (12773-12793)	503	(11)
LCV1R	CTCCTACCGCACGTGGTCC	Y10237 (13252-13271)		This work
LCV1U	TCCGCCTGAAGCACCTAATCCA	Y10237 (16390-16411)	438	(3)
LCV1L	GGTAAGCGGTATAAAAACCCTCCTCT	Y10237 (16809-16783)		
CVAF	GGAGGCATCAGAGGGAGTGG	KY510918 (6568-6587)	279	This work
CVAR	CCTTCTGCACCAACTACACCCC	KY510918 (6825-6846)		
CVAU	GAATACTCAAGCTTACTGAAG	KY510917 (4673-4693)	834	(6)
CVAL	TTGATTCGTCTCCTGCGACTT	KY510917 (5487-5506)		

*There was no similarity between primer sequence and CNRMV sequences from Genbank database found by Blast. **The primer LCV1F had been designed before starting the studies and in 2015 the sequence of the primer 1LC_12776F identical to the primer LCV1F sequence has been published (Glasa *et al*., 2015).

trees (12%), five apricot trees (12.5%) and three peach trees (7.5%) using primers CGRMVF-CGRMVR, whereas the primers GRM7950-GRM8316 did not amplify the product from eight infected trees. The presence of CNRMV was confirmed in 20 sweet cherry trees (17%), 12 sour cherry trees (13%) and three apricot trees (7.5%). The second primer pair (NRM48U-NRM48L) failed to amplify the virus cDNA fragment from any of the infected trees. CVA infection was confirmed in 71 sweet cherry trees (60%), 58 sour cherry trees (64%), and 23 apricot trees (57.5%) by both sets of primers.

To explain the source of the problems observed with the specificity of the primers, analysis of sequence diversity of the corresponding virus genome fragments was carried out using the currently available data. Up to five mismatches in the primer GRM7950 and up to three mismatches in the primer GRM8316, spaced along their entire length were identified. Moreover, the comparison of GRM7950 primer sequence with 135 CGRMV sequence records using BLASTn of NCBI (www.nlm.nih.gov) revealed that only nine of them displayed 100% identity with the primer sequence, whereas for the primers CGRMVF and CGRMVR, there were 73 and 93 sequences, respectively, showing 100% identity. Even few nucleotide mismatches between the primer and the target sequences, especially adjacent to the 3-terminus of the primer, may significantly reduce or even abolish amplification (Caetano-Anollés, 1993; Komorowska *et al*., 2010), which may, in turn, lead to false negative results. There was no similarity between the sequences of NRM48U and NRM48L primers and CNRMV sequences from the GenBank database found by BLAST. The primers CNRMVF and CNRMVR showed 100% identity with most of the virus isolates.

The RT-PCR results showed that CVA was the most prevalent virus with the exception of peach trees. In Japan, the pathogen has been detected in 92% of the tested samples, including asymptomatic trees (Isogai *et al*., 2004). The virus was originally found in a sour cherry tree affected by little cherry disease (Jelkmann, 1995) and later in sweet cherry, peach and apricot of different disease status and in symptomless samples (James and Jelkmann, 1998). CNRMV and CGRMV were the second and third most commonly detected viruses in sweet and sour cherry trees as well as in apricot. As reported by (Isogai *et al*., 2004), infection with CGRMV in Japanese orchards was confirmed in 14% of sweet cherry trees. The occurrence of both viruses was surveyed by RT-PCR in Serbia (Mandic *et al*., 2007) as well as using duplex RT-PCR in South Korea (Li *et al*., 2014). CNRMV and CGRMV infection rates were 30%, 12% in Serbia and 29.6%, 53.6% in South Korea, respectively. The percentage of LChV-1 in sweet cherries was close to those for CNRMV and CGRMV, whereas in the sour cherry it was lower. LChV-1 appears to be far more widely spread in cherry trees than LChV-2 in Poland. In contrast, LChV-2 was found to be prevalent in Japan, where among 49 sweet cherry trees tested, 14% and 65% were infected with LChV-1 and LChV-2, respectively (Isogai *et al*., 2004). Mixed infection of the studied viruses was detected frequently in the tested samples. More than 63% of the samples were infected by at least one virus, whereas 36% and 2.7% by two and three virus species, respectively. Co-infection of a single plant by CVA with CNRMV (12.5%) or CGRMV (10.8%) or LChV-1 (7%) was the most common co-infection detected. Almost 3% of samples were co-infected by CVA, CNRMV and CGRMV. Foliar and fruit symptoms type and severity were observed and recorded during the survey. All trees infected with LChV-1 or LChV-2 exhibited typical symptoms described in the literature (Welsh and Cheney, 1976), including reddish and brown discoloration of the leaves and the small, poorly-colored fruit. The trees infected with CGRMV showed light green mosaic together with green rings on the leaves of both sweet and sour cherry trees. CNRMV infection caused small, necrotic spots on the leaves, but no symptoms on the fruit. The most intensive symptoms were observed on sweet cherry trees infected with both CGRMV and CNRMV. There were no differences in the intensity of symptoms on plants, in which CVA was detected in mixed infections.

The specificity of amplified cDNA viral fragments was confirmed by sequencing and representative sequences excluding primer sequences for selected isolates were deposited in GenBank (Acc. Nos.: MH671297 – MH671307 (CGRMV), MH681783 – MH681793 (CNRMV), MH576595 – MH576603 (LChV-1). MH576605 – MH576612 (LChV-2), MH316600 – MH316612 (CVA). The comparison of nucleotide sequences of CGRMV, CNRMV, LChV-1, LChV-2, and CVA with other described to date revealed the following ranges of identity: 87–100%, 90–100%, 83–99%, 89–99%, and 88–100%, respectively.

The results presented here provide useful data on the infection rates among stone fruit trees in Poland and could be applied to a quarantine system in order to improve the management and selection of virus-free trees. Additionally, the results showed that knowledge of the targeted sequence variability in the virus population and careful validation of new primers is necessary for achieving a reliable detection.

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