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Cherry Necrotic Rusty Mottle and *Cherry Green Ring Mottle Viruses* in Czech Cherry Germplasm

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Abstract

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Using reverse transcription polymerase chain reaction, 160 sweet and sour cherry trees from a germplasm collection, orchards, and wild trees in the Czech Republic were screened for the presence of *Cherry necrotic rusty mottle virus* (CNRMV) and *Cherry green ring mottle virus* (CGRMV). The viruses were detected exclusively in sweet cherry trees in the germplasm collection, with CNRMV determined in two trees and CGRMV in four trees. Using next-generation sequencing, nearly complete genomic sequences (complete ORFs) were obtained for one CNRMV and three CGRMV isolates. Their relatedness to GenBank sequences of isolates from different countries together with negative results from screening outside of the germplasm collection suggests that the viruses had been imported with accessions.

Keywords: CNRMV; CGRMV; next-generation sequencing; *Prunus avium*; *Prunus cerasus*; detection

Cherry necrotic rusty mottle (CNRMV) and *Cherry green ring mottle virus* (CGRMV) are two species belonging to the newly established genus *Robigovirus* within the family *Betaflexiviridae* of ssRNA plant viruses (Villamor *et al.* 2015; ICTV 2016). Both viruses are filamentous with the positive-sense RNA genome ranging from 8.3 kB to 8.9 kB in length and composed of 7 ORFs. They are transmitted by grafting or budding but not mechanically with sap, and there is no known insect vector for either virus (Jelkmann *et al.* 2011; Rott & Jelkmann 2011). For this reason, the propagation and trade of healthy material are generally recommended as an effective system for preventing the spread of these pathogens within certification schemes (EPPO 2016a, b).

CNRMV infects mainly sweet cherries, causing angular chlorotic spots and chlorotic or necrotic rusty mottles on leaves in sensitive cherry cultivars. Reduced growth of infected trees, significant decrease in yield, and early death of trees have also been reported (see the review by Rott & Jelkmann 2011). The virus exhibits mainly sporadic or restricted distribution and has been reported from various European countries, North America, Chile, Japan, Korea, and New Zealand (Fry & Wood 1973; Isogai *et al*. 2004; Sabanadzovic *et al*. 2005; Fiore & Zamorano 2013; Noorani *et al*. 2013; Zhou *et al*. 2013; Cho *et al.* 2014; EPPO 2016a). Other hosts have also been reported recently, including such *Prunus* species as peach, apricot, plum, or flowering cherry

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(Li & Mock 2008; Zhou *et al.* 2013; Zindovic *et al.* 2014). CNRMV has been detected in what probably were naturally infected wild roses and bamboos, and its natural spread between hosts by specific Asian aphids has recently been proposed but not proven in India (Awasthi *et al.* 2015a, b).

CGRMV exhibits a similar host range and distribution as does CNRMV. It infects mainly sweet cherries, but sour cherry, flowering cherry, apricot, plum, and peach have also been reported as hosts (Jelkmann *et al.* 2011). Although the CGRMV infection can lead to leaf necrosis, leaf twisting and curling, and necrosis in fruits of sensitive cherries (Jelkmann *et al.* 2011), in most noted cases it is symptomless. The presence of the virus has been reported either historically or recently from Europe, America, Asia, and New Zealand (Gentit *et al.* 2002; Isogai *et al.* 2004; Wang *et al.* 2009, 2013; Jelkmann *et al.* 2011; Zhou *et al.* 2011; Fiore & Zamorano 2013; Lee *et al.* 2014; EPPO 2016b). In view of the frequency of CGRMV latent infections and its low economic impact, it is believed that CGRMV occurs worldwide (Jelkmann *et al.* 2011).

The Czech Republic is among those European countries with few occurrences of CNRMV and CGRMV, as reported by Czech NPPO in 1994 (EPPO 2016a, b), but in general the situation is unclear because no additional documentation or information exists.

The aim of the present study was to map the occurrence of CNRMV and CGRMV in both wild-growing and cultivated sweet and sour cherry trees in the Czech Republic and to verify the previously published EPPO reports.

MATERIAL AND METHODS

Plant material. Leaf samples were taken preferably from trees exhibiting virus-like symptoms of virus infection, but also from non-symptomatic trees. Representative leaves were taken from several parts of the canopy. The samples were collected in the period from April to August in the years 2014 and 2015 from the germplasm collection at the Research and Breeding Institute of Pomology Ltd., Holovousy, Czech Republic, and from orchards and wild-growing trees at 11 locations in the Czech Republic.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated for RT-PCR screening using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, reverse transcribed using random primers (iScript cDNA Synthesis Kit; Bio-Rad Laboratories, Irvine, USA), then amplified by PCR (TopBio, Prague, Czech Republic) using the primers CGRMV1 (5'-CCTCATTCACATAGCTTAGGTTT-3') and CGRMV2 (5'-ACTTTAGCTTCGCCCCGTG-3') (Li & Mock 2005) targeting a region approximately 950 nt long from both CGRMV and CNRMV viruses. Samples tested positive were examined using RT-PCR also for the presence of other cherry-infecting viruses, namely *Apple chlorotic leaf-spot virus* (ACLSV), *American plum line pattern virus* (APLPV), *Apple mosaic virus* (ApMV), *Arabis mosaic virus* (ArMV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV), *Cherry leaf roll virus* (CLRV), *Cherry mottle leaf virus* (CMLV), *Cherry rasp leaf virus* (CRLV), *Cherry virus A* (CVA), *Little cherry virus 1* (LChV1), *Little cherry virus 2* (LChV2), *Prune dwarf virus* (PDV), *Prunus necrotic ring spot virus* (PNRSV), *Plum pox virus* (PPV), *Strawberry latent ringspot virus* (SLRSV), and *Tomato ring spot virus* (ToRSV) (Supplement Table S1). All PCR products thus obtained were sequenced (GATC, Constance, Germany) using the Sanger sequencing method.

The dsRNA for next-generation sequencing (NGS) was purified from the total nucleic acid by binding to cellulose powder in 16% ethanol (Morris & Dodds 1979; DePaulo & Powel 1995) from about 400 mg of fresh or frozen leaf tissue of collected tree samples.

NGS and sequence analyses. The dsRNA from samples which were positive by RT-PCR for CNRMV and CGRMV was examined by NGS. The sequencing library was prepared using the TruSeq RNA Library Preparation kit (Illumina, San Diego, USA) and sequenced by Illumina HiSeq 2500 (Seqme Ltd., Dobříš, Czech Republic). Sequences thus obtained were processed (trimming low quality sequence, duplicate removal, *de novo* contig creation) using the CLC Genomic WorkBench 7.5 software (QIAGEN, Aarhus C, Denmark). The 5' and 3' ends of the genome with lower coverage and weak consensus were trimmed out of the final sequences.

Sequence identity was analysed using the BLAST algorithm (Zhang *et al.* 2000). Sequences available in GenBank were aligned using the ClustalW algorithm, then examined by phylogenetic analysis and the neighbour-joining method with 1000 bootstrap replications. The final trees were visualised using the Tree Explorer software (all Mega v. 7.014) (Kumar *et al.* 2016).

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RESULTS

To elucidate the occurrence of CNRMV and CGRMV in the sweet and sour cherry plantations, a total of 160 trees were screened: 55 sweet and 22 sour cherry trees from the germplasm collection, 49 sweet and 10 sour cherry trees from orchards, and 20 sweet and 4 sour cherry trees growing in the wild. Both viruses were detected only in six sweet cherry trees from the germplasm collection but they were not found in the other surveyed plantations.

Further RT-PCR screening for another 19 cherry viruses (Supplement Table S1) confirmed co-infection by ACLSV, CLRV, CVA, LChV2, and PDV in both symptomatic and non-symptomatic trees (Table 1). Four selected samples (one CNRMV and three CGRMV) were subjected to the detailed NGS analysis. Sequences of both viruses were obtained by *de novo* assembly of reads, without any unmapped regions. The numbers of reads and average coverage for each sequence are listed in Table 1. The NGS analysis confirmed the presence of all viruses detected by RT-PCR. There were neither false-positive nor false-negative results. However, NGS revealed additional sequences of heretofore undescribed viruses, and these are now being completed or have recently been published (Lenz *et al.* 2017). The NGS analysis also confirmed 100% identity of primer CGRMV1 with all four isolates sequenced and only one mismatch of primer CGRMV2 with sequences of CZ43 and CZ54 (data not shown).

A nearly full-length sequence of the CNRMV isolate CZ31 (GenBank Acc. No. KY178274) was obtained by NGS. It consists of 8 392 nucleotides covering all seven ORFs and parts of both 3' and 5' UTR regions. The genome shared the highest nucleotide (nt) identities with the isolates Ch-yt-13 (China, KR820549) and 120/86 (Switzerland, NC_002468) at 98.7 and 98.4%, respectively. The lowest (86.7%) nt identity was found with isolates FC4 and FC5 (Japan, EU188438 and EU188439). Detailed analysis of each of the seven detected ORFs confirmed the similarity between CZ31 and Chyt-yt-13 and 120/86 CNRMV isolates, showing 98–99.9% nt and 97.4–100.0% deduced amino acid (aa) identities (Supplement Table S2). ORF1 (encoding viral replicase) revealed the highest variability in nt sequence among the analysed CNRMV isolates, showing the identity ranging from 84.0% with FC4 to 98.5% with Ch-yt-13. This relatively wide range of variability was not detected in aa sequences, varying as they did from 92.3% to 99.2%. ORF2 (encoding TGBp1) revealed the most conservative character, showing the highest aa identity (98.6–100.0%). In contrast, the greatest aa variability was detected in ORF5a (encoding hypothetical protein), which showed 72.0–97.5% identity with CZ31.

The sequences of three CGRMV isolates (CZ43, CZ54, and CZ74) obtained by NGS ranged from 8348 to 8431 bp (without poly(A) tail) covering the complete sequence of all seven ORFs. The BLAST analysis revealed that isolates CZ43 (KY178275) and CZ54 (KY178276) were 99.6% identical without regard to their assumed origins from the USA and Moldova. On the other hand, the isolate CZ74 (KY178277) shared with them only 82.9% identity, which was the lowest identity found between the compared CGRMV sequences. The isolates CZ43 and CZ54 were the most similar to the USA 02F23rD (KC218931) isolate (83.9 and 83.8% identity, respectively), while the CZ74 showed the highest identity with the South Korean CGRMV-Korea (LC064752) isolate (94.7%).

A comparison of the individual ORFs of NGS-obtained sequences revealed relatively high variability in nt sequences and lower variability in deduced aa sequences. The most variable was ORF5a (encoding hypothetical protein), with 88.8–97.6% nt and 73.6–93.9% aa identity. ORF5 (encoding capsid protein) was identified as the most conservative ORF with 88.1–97.4% and 95.2–98.9% nt and aa identity, respectively (for detailed data see Supplement Table S2).

Table 1. Viruses detected in sweet cherry trees from germplasm collection

Isolate	Cultivar	Origin	Virus	Sequenced region	Acc. No.	Other viruses detected	Symptoms
CZ31	Kišiněvskaja	Moldova	CNRMV	8392	KY178274	PDV	light mottle spots
CZ43	Lambert	USA	CGRMV	8353	KY178275	ACLSV, CLRV, CVA, PDV	dying, defoliation
CZ54	Dněprovka	Moldova	CGRMV	8365	KY178276	no	no
CZ63	Deacon	USA	CGRMV	820	KY178278	CVA	reddening
CZ71	Mona Cherry	USA	CNRMV	361	KY178279	no	yellowing
CZ74	Rube	Germany	CGRMV	8348	KY178277	LChV2, PDV	no

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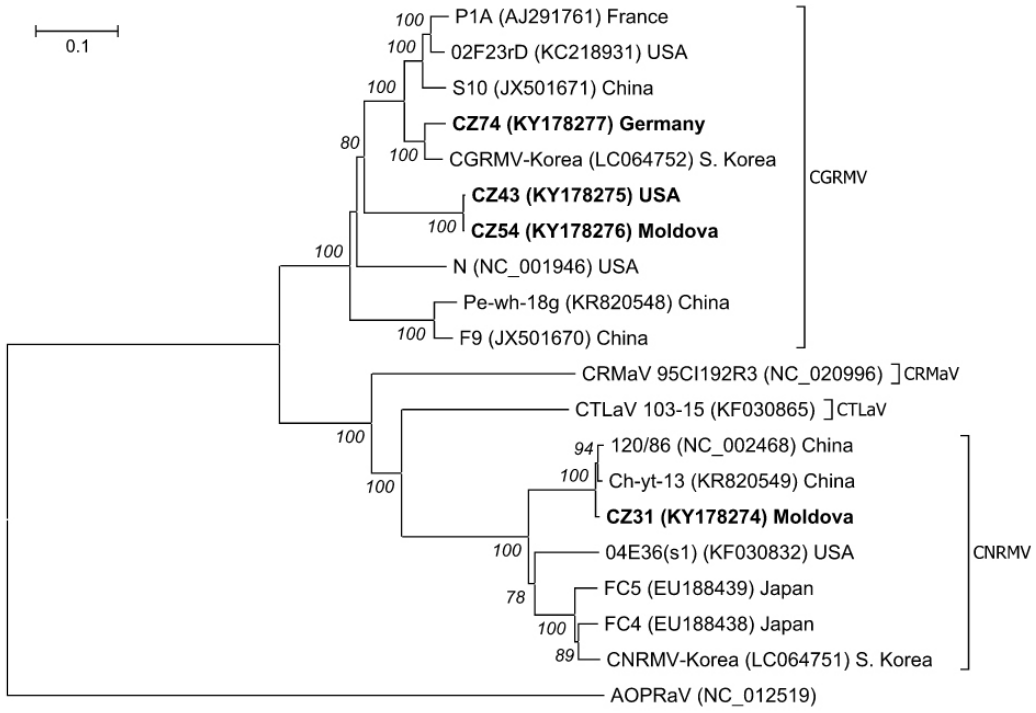

Figure 1. Phylogeny based on full genome sequences of robigoviruses (neighbour-joining method). For each virus, isolate name, GenBank Accession Number, and assumed origin are listed. Isolates from this study are shown in bold. AOPRaV is used as an outgroup. Bootstrap values less than 70% are not shown; the bar represents a phylogenetic distance of 10%

Two isolates, CZ63 and CZ74, were partially sequenced by Sanger sequencing only and were compared with the four isolates analysed by NGS. CZ63 (CGRMV) was the most similar to CZ74 (93.6% nt identity; 98.1% aa identity in the CP-gene), and the nucleotide identity of CZ71 (CNRMV) with the isolate CZ31 was 89.9% (only a partial CP sequence was obtained).

The phylogenetic analysis confirmed the position of Czech CNRMV and CGRMV isolates deduced from the nucleotide sequence analysis (Figure 1). CNRMV isolate CZ31 clustered in a separate branch together with Chinese and Swiss isolates clearly discriminated from the other CNRMV isolates sequenced so far. CGRMV isolates CZ43 and CZ54 formed a distant branch, while CZ74 clustered together with the Korean CGRMV-Korea isolate. All of them, however, were significantly related to the American and Chinese isolates.

DISCUSSION

CNRMV and CGRMV have been reported from various countries worldwide, but their distributions are mostly limited to a few cases or scarce occurrences (EPPO 2016a, b). Nevertheless, due to the fact that non-symptomatic infections occur frequently, it is generally believed that both viruses could occur in any country with the presence of host plants (Jelkmann *et al.* 2011; Rott & Jelkmann 2011).

In this study, we used primers CGRMV1 and CGRMV2 designed for detection of both CGRMV and CNRMV viruses (Li & Mock 2005). The analysis showed the identity of these primers with the majority of sequences of both CNRMV and CGRMV available from GenBank or NGS sequences (Supplementary file, Figure S1 and S2). Furthermore, we found greater variability at the annealing site of other CNRMV primers designed by Isogai *et al.* (2004). Therefore, primers CGRMV1 and CGRMV2 seem to be well suited for detection of CGRMV as well as CNRMV.

The comparison of available GenBank sequences reveals that the most similar isolates of both viruses often originate from distant countries. For example, the Swiss isolate CNRMV 120/86 is the most similar to the Chinese Ch-yt-13, while the CGRMV isolate 02F23rD from the USA is most closely related to the French P1A and the two cluster together with the isolate S10 from China (Figure 1). The sequence

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relatedness of isolates from this study does not reflect the geographic origin: CNRMV isolate CZ31 (origin Moldova) clusters within the group of Swiss and Chinese isolates (120/86 and Ch-yt-13), and CGRMV isolate CZ74 (origin USA) was the most similar to the isolate CGRMV-Korea from South Korea. The other two CGRMV isolates of different origin, infecting USA (CZ43) and Moldovan (CZ54) cherry cultivars, were nearly identical and formed a distinct phylogenetic clade. They differed significantly from the CZ74 isolate of CGRMV coming from the same location. Indeed, their genetic distance is the greatest within the CGRMV group. Despite the relative conservancy of CNRMV genomes described by various authors (Sabanadzovic *et al.* 2005; Zhou *et al.* 2011; Awasthi *et al.* 2015b), genetic similarities observed within both viruses are in accordance with the anticipated fact that both viruses were spread by grafting and/or planting of infected material.

Therefore, the combined evidence of (a) two distant lineages of CGRMV in trees from the same location, one of which was closely related to the isolate from South Korea; (b) absence of CNRMV and CGRMV trees in the vicinity of infected trees; and (c) high genetic similarity of both viruses detected to other known isolates from distant countries supports the conclusion that these viruses detected in the Czech Republic are of imported origin.

The survey for CNRMV and CGRMV, their absence in orchards and wild-growing trees, together with the absence of any documentation as to their previous presence in the Czech Republic did not confirm the few occurrences previously reported here by EPPO (2016a, b). Both viruses occurred only in imported accessions within the germplasm collection.

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