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Short communication

Simultaneous detection of *Cherry necrotic rusty mottle virus* and *Cherry green ring mottle virus* using real-time PCR and high resolution melting analysis

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ABSTRACT

In this study, the real-time PCR assays were combined with high resolution melting (HRM) analysis for the simultaneous detection of *Cherry necrotic rusty mottle virus* (CNRMV) and *Cherry green ring mottle virus* (CGRMV) infection in sweet cherry trees. Detection of CNRMV and CGRMV was performed in a real-time PCR using a primer set for both of them or duplex real-time PCR that included one specific primer set for each virus. These two strategies allowed us to confirmed virus infection in all tested samples. In 17 field samples the technique revealed samples positive for CNRMV or CGRMV as well as positive for both viruses. In addition, the HRM analysis made it possible to differentiate clearly between CNRMV and CGRMV. Sequence variations among CNRMV and CGRMV isolates observed from the HRM peaks were confirmed by sequencing. To test the capability to use this method in field, forty one sweet cherry samples were examined by HRM analysis. The HRM data showed that seven samples were positive for CNRMV and three were infected with CGRMV. The results presented in this study indicated that real-time PCR followed by HRM analysis provides sensitive, automated and rapid tool to detect and differentiate between CNRMV and CGRMV isolates.

Cherry green ring mottle virus (CGRMV) and *Cherry necrotic rusty mottle virus* (CNRMV), two unassigned members of the family *Betaflexiviridae*, are flexuous, filamentous plant viruses with a single-stranded, positive-sense RNA genome of approximately 8.4 kb [1]. Both viruses have a very similar morphology and genomic organization and share about 60% identity at the nucleotide level over the entire genome [7,11,19,26,28]. The alignments of the available sequences indicated that genetic variations among the isolates of each virus are high. CNRMV infects sweet cherry (*Prunus avium*) and has been reported in North America, Europe, Japan and Chile [6,7,10,12,25]. CGRMV infects several *Prunus* species and causes diseases on susceptible hosts such as sour cherry (*Prunus cerasus* L.) and flowering cherry (*Prunus serrulata* Lindl.) [7,28]. The virus has been found in North America, Europe, Africa, Asia, New Zealand and Chile [6,7,10,15,26]. Based on phylogenetic analyses of CGRMV coat protein sequences, the virus isolates were divided into three groups [28]. There are no known vectors for either viruses [14]. Therefore, the use of healthy plant material for propagation is a very effective way for management of the diseases caused by these two viruses. The initial methods used to determine infection of CNRMV and CGRMV employed bioassay with woody indicators, the only method accepted by inspection services during quarantine and certification procedures [11]. However, indexing is laborious and time consuming and is not appropriate for screening a large number of samples. Since commercial antisera for serological detection of these viruses are not available, immunological assays can not be used for their detection. Later, reverse transcription–polymerase chain reaction (RT-PCR) method using CNRMV or CGRMV-specific primers [11,29] as well as degenerate primers [19] were introduced for detection of CNRMV and CGRMV. These two viruses often occur as mixed infection, thus it will be useful to develop an effective method for their simultaneous detection and diagnosis.

High resolution melting (HRM) analysis is a post-PCR technique developed for identifying variations in nucleic acid sequences. It is a cost-efficient, gel-free and closed-tube system that allows high-throughput analysis [27]. The HRM analysis is based on the

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Table 1
Isolates of CNRMV and CGRMV used in this study.

Isolate	Geographical origin	Virus	GenBank accession no.	Co-infection with other viruses
XIII/9	Poland	CNRMV	KC136845 [b]	PDV[d]
2-38-70	Poland	CNRMV	KC136837 [b]	–
19-U	Poland	CNRMV	KC136839 [b]	CVA[e]
2-38	Poland	CNRMV	KC136840 [b]	PDV[d]
II-37	Poland	CNRMV	KC136841 [b]	–
I-18	Poland	CNRMV	KC136842 [b]	CVA[e]
VI-19	Poland	CNRMV	KC136843 [b]	PDV[d], CVA[e]
FC5	USA	CNRMV	EU188439 [c]	–
IV/20	Poland	CNRMV/CGRMV/II[a]	KC136838 [b]/JX468871 [b]	PDV[d], CVA[e]
HI16	Poland	CGRMV/II[a]	JX468874 [b]	PDV[d]
IH17	Poland	CGRMV/II[a]	JX468873 [b]	PDV[d], LChV-1[f]
IH14	Poland	CGRMV/II[a]	JX468872 [b]	PDV[d]
XIII/17	Poland	CGRMV/II[a]	JX468870 [b]	PDV[d], CVA[e]
HI28	Poland	CGRMV/II[a]	JX468875 [b]	PDV[d], LChV-1[f]
10039	Chile	CGRMV/II[a]	KC513747 [b]	–
10040	Chile	CGRMV/II[a]	KC513748 [b]	–
N	USA	CGRMV/III[a]	–	–

[a] Roman numeral indicates the group to which CGRMV isolates belong.
[b] GenBank accession nos. of viral coat protein gene sequences.
[c] GenBank accession no. of CNRMV complete genome sequence.
[d] Prune dwarf virus.
[e] Cherry virus A.
[f] Little cherry virus-1.

determination of changes in fluorescence as a result of melting double-stranded PCR products in response to steps of increasing temperature. The melting temperature (T_m) and the characteristic shape in the melting curve profile of amplified products are highly depended on nucleotide sequence of the PCR products. A single-base substitution can change the T_m of an amplified product, and this change is detected using instrumentation capable of real-time fluorescence monitoring combined with high resolution temperature change [5]. The assay is, therefore, a more efficient technique that should enhance resolution for almost identical sequences [24]. The technique has been applied widely for human genotyping and mutation analysis in clinical diagnostics and medical research especially [2,20]. It has also been applied for detection and identification of human, animal and plant viruses [3,9,13,16,21–23].

In this study, a real-time PCR combined with HRM (real-time PCR HRM) analysis was developed for simultaneous detection and identification of CNRMV and CGRMV.

The CNRMV and CGRMV isolates originated from sweet cherry cultivar collection maintained in Horticulture Institute and from commercial orchards in Poland and Chile (Table 1). They were selected on the basis of the symptoms (only for CNRMV), results of biological indexing, non-isotopic molecular hybridization assays [6] and RT-PCR. Some cherry trees selected for projecting real-time PCR HRM method were co-infected with other viruses (Table 1). The healthy plants were included as negative controls. Total nucleic acids were extracted from the leaves or phloem scrapings from twigs using the silica capture (SC) method described originally by Boom et al. [4]. Two RNA extracts, one from a CNRMV-infected plant (FC5) and another from a CGRMV-infected plant (N), were obtained from the National Germplasm Resources Laboratory of USDA-ARS in Beltsville, Maryland of the United States. The correct primer choice is critical to increase the resolution of the amplicons since small variations in their sequences can affect dramatically the HRM results [5]. Three pairs of primers targeting the most conservative regions of coat protein (CP) gene of the two viruses were selected based on criteria recommended by Roche's protocol as well as their specificity, segregation capacity and accurate reproducibility in the HRM analyses (Table 2). Primer pair NEG1U/NEG1L [19] allows amplification of cDNA fragments of both viruses, but does not discriminate between them in conventional RT-PCR. Primers CNRMVF2 and CNRMVR2 anneal only to the CNRMV genome, while primers CGRMVF1 and CGRMV3/R only to the CGRMV genome. These two pairs of virus-specific primers were used in a duplex PCR for simultaneous detection and identification of CNRMV and CGRMV. Two microliters of nucleic acids preparation were used for reverse transcription (RT) in total volume 20 μl. RT was performed using Transcriptor High Fidelity cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) with oligo $(dT)_{18}$ as a primer according to manufacturer's instructions. Real-time PCR and HRM analysis were performed using LightCycler 480 II system (Roche Diagnostics, Mannheim, Germany). The reaction mixture contained 5 μl of cDNA, 2 mM $MgCl_2$, 200 nM of each primer and 10 μl of LightCycler 480 High Resolution Melting Master mix, which includes ResoLight HRM dye (Roche Diagnostics, Mannheim, Germany) in total volume of 20 μl. The thermocycling parameters were as follows for all tested primer pairs: pre-incubation for 10 min at

Table 2
Primers used in this study.

Primer	Primer sequence	Virus detected	Product size (bp)	Reference
NEG1U	5′-AGTTCGCAGCYTTTGAYTTYTTTG	CNRMV/CGRMV	255	Rott and Jelkmann, 2001
NEG1L	5′-GAKTGGRWTTGCAGRGGTTTATCA			
CNRMVF2[a]	5′-GAGTGTGTGTGAGCTTTCAAGTTT	CNRMV	107	This study
CNRMVR2[a]	5′-CTTCTTCTTCGGGATCTTGTTRTT			
CGRMVF1[a]	5′-GGCGCAGACGGACCCTAAGT	CGRMV	80	This study
CGRMV3/R[a,b]	5′-TTCTGGCTCGCAGTATGTC			Li and Mock, 2005

[a] The primers used in multiplex real-time PCR assay.
[b] CGRMV3/R was designed originally as a forward primer.

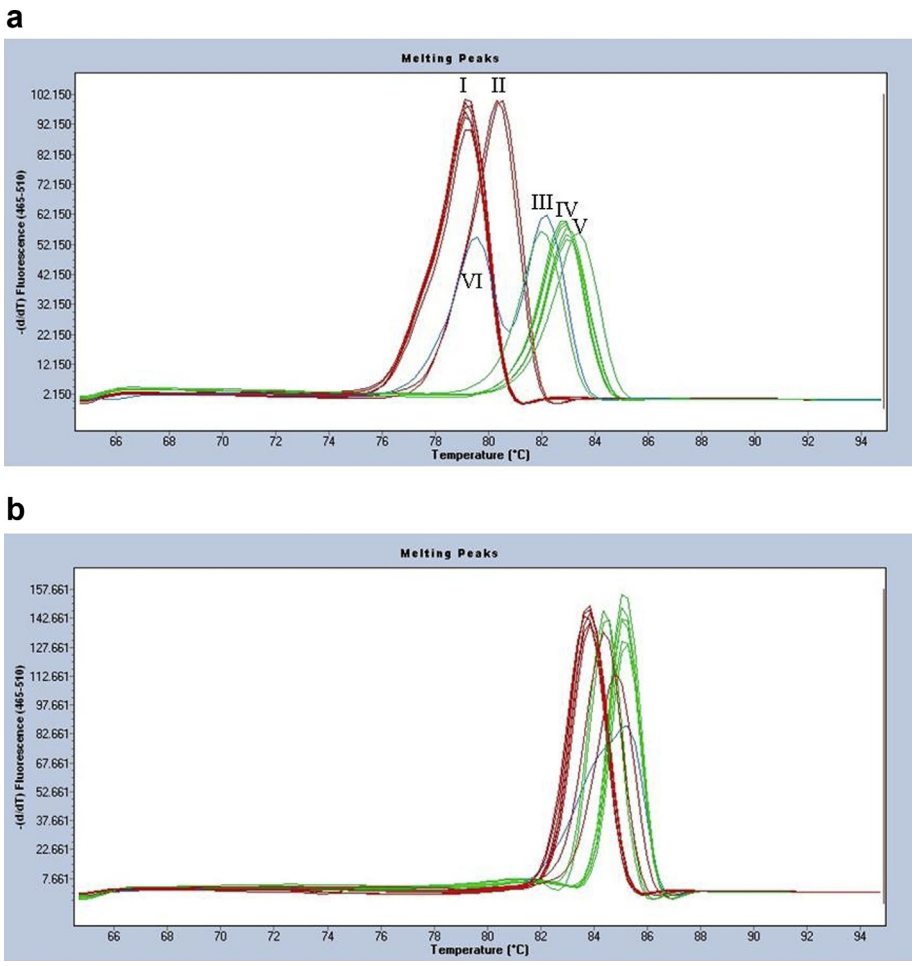

Fig. 1. Traditional melting curve analysis of real-time PCR products amplified by duplex real-time PCR using two pairs of virus-specific primers (a) and a uniplex real time PCR using a pair of degenerate primes (b), respectively. Red lines represent CNRMV isolates, green lines CGRMV isolates, and blue line mixed infection. In Fig. 1a, peak I includes the CNRMV isolates XIII/9, 2-38-70, 19-U, 2-38, II-37 and I-18; peak II the CNRMV isolates IV-19 and FC5; peak VI the CNRMV isolate IV/20; peak III the CGRMV isolates HI28 and IV/20; peak IV HI16, IH17, IH14, XIII/17, 10039 and 10040; peak V the CGRMV isolate N. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

95 °C followed by 45 cycles of quantitation with denaturation at 95 °C for 10s, annealing at 55 °C for 15 s and extension at 72 °C for 18 s. After the amplification process was completed, either traditional meting curve analysis or the HRM analysis was performed by increasing the temperature in steps of 0.1 °C over the range from 65 °C to 95 °C with 25 signal acquisitions per degree. Data generated after HRM analysis was interpreted using the LightCycler Software Version 1.5. The cDNA obtained and the primers designed were analyzed in parallel by standard PCR using the FastStart Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany). The master mix contained 5 μl of cDNA, 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each primer and 1 unit Taq DNA Polymerase in total volume of 20 μl. PCR conditions were as follows for all tested primer pairs: (1) initial denaturation at 95 °C for 4 min; (2) 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30s; and (3) a final extension step at 72 °C for 7 min. Agarose gel electrophoresis of PCR amplicons obtained by standard PCR enables detection of all the studied isolates either in uniplex or duplex PCR (data not shown). However, the approach with the primers NEG1U/NEG1L [19] does not differentiate between CNRMV and CGRMV isolates because the amplicons length for these two viruses is identical.

Fig. 1a and b shows the results of traditional melting curve analysis of the real-time PCR products. A monophasic melting curve was generated from the plants infected either with CNRMV (red lines) or CGRMV (green lines), whereas a biphasic curve (blue line) was obtained from mixed infection followed the duplex real-time PCR (Fig. 1a). Although relatively high variations in the T_m profiles were observed, it was possible to discriminate between CNRMV and CGRMV isolates. The CNRMV isolates generated two melting peaks (I and II) with T_m values of 79.15 °C and 80.46 °C, and the CGRMV isolates produced three melting peaks (III, IV and V) with T_m values of 81.98 °C, 82.82 °C and 83.36 °C, respectively. Sample containing mixed infection generated one peak (VI) at 79.50 °C for CNRMV and the second (III) at 81.98 °C for CGRMV. The T_m profiles suggested that genetic variations exist among different isolates of the two viruses. Traditional melting curve analysis after the real-time PCR with primers NEG1U-NEG1L yielded peaks in a narrow range of T_m values. The curves for two CNRMV isolates were overlapped with those for the CGRMV isolates. Furthermore, the melting curve for the mixed infection was monophasic. Thus, it was impossible to differentiate between CNRMV and CGRMV isolates (Fig. 1b). The results showed that the traditional melting curve analysis is limited in its ability to differentiate the target viruses.

The HRM analysis was then tested for clear differentiation of CNRMV and CGRMV. The studied samples were clustered into three groups of similar melting curve shape in the resulting difference plots. Red lines represent CNRMV isolates, blue lines CGRMV

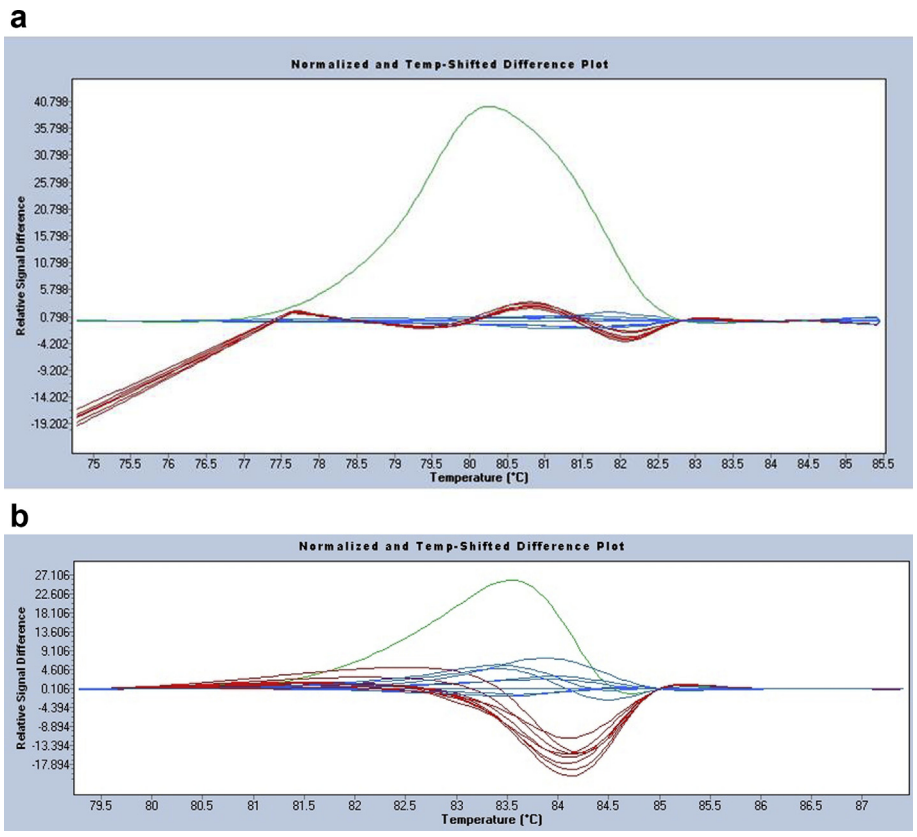

Fig. 2. Difference plots of real-time PCR products amplified by duplex real-time PCR using two pairs of virus-specific primers (a) and uniplex real time PCR using a pair of degenerate primes (b), respectively. Red lines represent CNRMV isolates, blue lines CGRMV isolates, and green line mixed infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

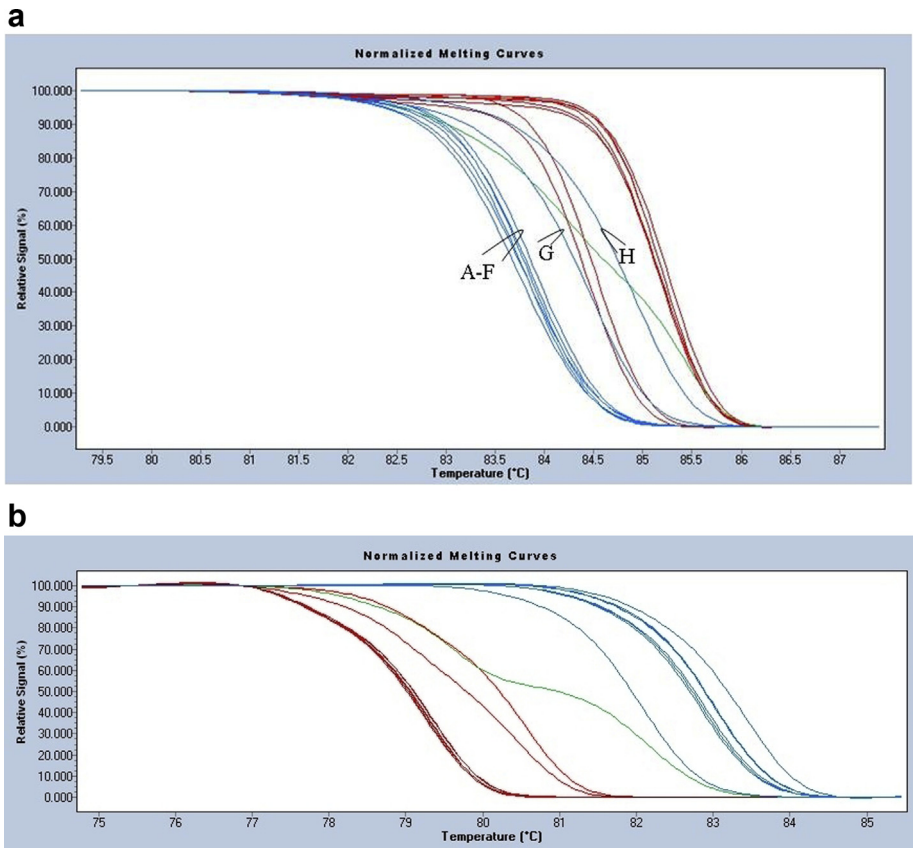

Fig. 3. Normalized HRM curves of real-time PCR products by duplex real-time PCR using two pairs of virus-specific primers (a) and uniplex real time PCR using a pair of degenerate primes (b), respectively. Blue lines represent CNRMV isolates, red lines CGRMV isolates, and green line mix infection. Curves A-F represent the CNRMV isolates (XIII/9, 2-38-70, 19-U, 2-38, II-37, I-18); G (FC5) and H (VI-19). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

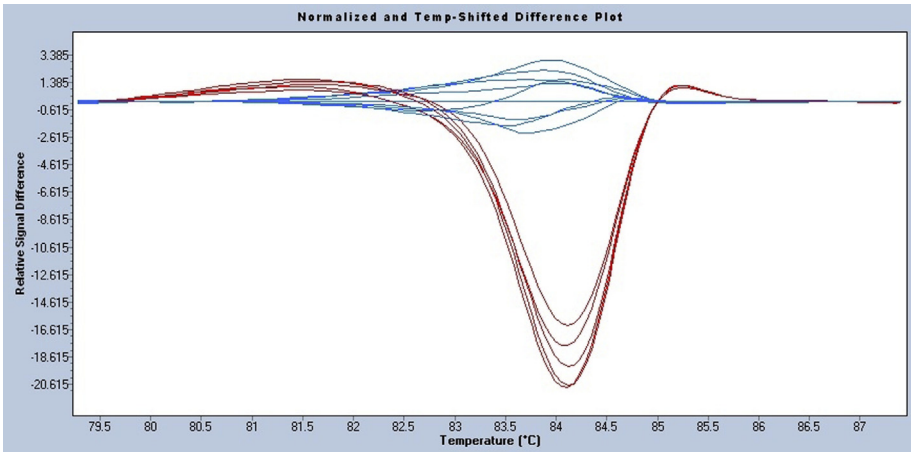

Fig. 4. Difference plot of real-time PCR products amplified by uniplex real time PCR using a pair of degenerate primes. Red lines represent CGRNV isolates and blue lines CNRMV isolates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isolates, and green line mixed infection (Fig. 2a, b). The HRM analysis made it possible to differentiate clearly between CNRMV and CGRMV isolates by HRM curves from the duplex real-time PCR (Fig. 2a) or the uniplex real-time PCR using primers NEG1U-NEG1L (Fig. 2b). When the HRM profiles were displayed in normalized melting curve format, three distinctly different patterns could be seen corresponding to each of the target viruses and the virus mix, respectively (Fig. 3a, b). The HRM profiles confirmed that eight samples were infected with CNRMV, eight samples with CGRMV and one sample was positive for both CNRMV and CGRMV. The results obtained in this study provide evidence that HRM method is a valid approach for detection and differentiation of the studied CNRMV and CGRMV isolates. On the other hand, there is still a risk that a new, atypical isolate will be tested and the limitations may appear. In such a case, the LightCycler 480 software will not cluster the isolate into any recognized groups, indicating that sequencing is required.

To examine further the possibility of sequence variation in the real-time PCR products, the 255-bp amplicons from eight CNRMV isolates (A-H) (Fig. 3b) were used as representatives for sequence analysis. The results revealed that isolates XIII/9, 2-38-70, 19-U, 2-38, II-37 and I-18 (A–F) were 100% identical at the region, and they had 20- and 19-nucleotide differences with isolates VI-19 (H) and FC5 (G), respectively. The nucleotide sequences of isolates VI-19 and FC5 differed from each other by 5 nucleotides. Of the differences, only one corresponds to an inverted position of nucleotides (A::T) and did not influence the GC/AT content. The overall GC contents of the analyzed amplicons were from 44.3% to 45.5%, and their distribution levels varied along the length of the amplicon. The sequence data corresponded well with the HRM profiles (Fig. 3b), indicating that the HRM analysis is able to detect genetic variations in the target sequences. It is important to mention that under HRM analysis the melting curve profiles obtained are not related to the size of amplicons or the sequence identity, but instead other parameters are more relevant such as GC content and nucleotide distribution [17,18].

After optimization of the HRM analysis with the sequenced CNRMV and CGRMV isolates, the set of 41 field samples from sweet cherry commercial orchard were analyzed. The processing of field samples was carried out together with the reference isolates (I-18, VI-19, HI16, HI28), to ensure uniform conditions and avoid bias in the analysis. Figs. 4 and 5 show that seven samples were positive for CNRMV and three for CGRMV. All the positive samples were resolved in an optimal way, in the same way as the references samples.

This is the first report on successful application of the real-time PCR HRM technology for simultaneous detection and differentiation of CNRMV and CGRMV in sweet cherry trees. The capability of this method to detect simultaneously the two viruses in one reaction tube reduced cost and time when compared to standard RT-PCR assay. The complete process from the nucleic acid extraction

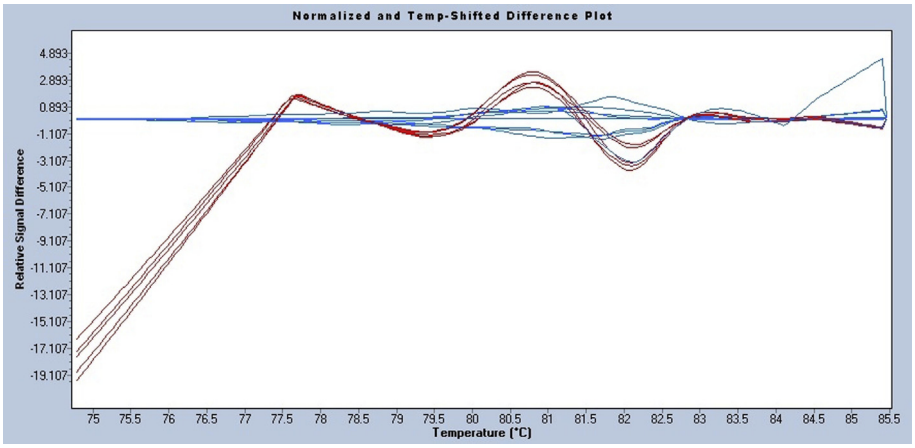

Fig. 5. Difference plot of real-time PCR products amplified by duplex real time PCR using two pairs of virus-specific primers. Red lines represent CGRNV isolates and blue lines CNRMV isolates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to the HRM analysis took approximately only 4 h. The real-time PCR HRM technique is simple and fast due to its closed system format and avoidance of post-PCR manipulation. In addition, the method requires specific primers for each virus but avoids the use of fluorescently labeled probes which reduces the cost greatly [27]. The method is able to detect small sequence variations in the amplicons. Finally, it also allows evaluation of large numbers of samples as a presequencing screening technique. Although it does not replace DNA sequencing as a standard technique in differentiation between variants, this method stands as a useful technique for determining distinct genetic variants to be sequenced.

In conclusion, the real-time PCR HRM provides a fast, sensitive and specific detection and identification of CNRMV and CGRMV, making it a useful tool for diagnosis and epidemiological studies of these viruses.

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