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ORIGINAL ARTICLE

Occurrence of plant viruses on pepper cultivated in open fields in R. Macedonia and partial characterization of cucumber mosaic virus isolates

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Abstract
Screening of pepper plants cultivated in open fields for the presence of cucumber mosaic virus (CMV), alfalfa mosaic virus (AMV) and potato virus Y (PVY) in the Republic of Macedonia was performed by DAS-ELISA from 2012 to 2014. During the tested period, the predominant infection was by CMV, ranging from 34 to 61% of the tested samples, followed in 2012 by AMV and in 2013 and 2014 by PVY. Molecular detection and identification of CMV on selected pepper leaf samples was done by Reverse Transcription – Polymerase Chain Reaction (RT-PCR) and sequencing of the coat protein (CP) gene. Genetic relationships of the 18 CMV isolates generated in this study were compared with isolates from other parts of the world. Phylogenetic analysis was conducted, based on the partial sequences of the CP gene. In the constructed phylogenetic tree, all of the 18 Macedonian CMV isolates were clustered in the IA subgroup.

Keywords *Cucumovirus* · Serological detection · RT-PCR · Phylogenetic analysis · Coat protein gene sequences

Introduction

Pepper (*Capsicum annuum* L.) is one of the most important crops in the Republic of Macedonia (Tudzarov 2011), which ranks amongst the top 10 pepper-producing countries in Europe (FAO 2015).

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Pepper cultivated in open fields is more susceptible to virus infections, compared to crops grown in greenhouses (Bogatzevska et al. 2007). Since in Macedonia most of the pepper plants are cultivated in open fields (Tudzarov 2011), viruses represent a major problem and a limiting factor to production (Jovanchev et al. 1996). The most common pepper viruses are: cucumber mosaic virus (CMV), alfalfa mosaic virus (AMV), tobacco mosaic virus (TMV), tomato spotted wilt virus (TSWV), potato virus X (PVX) and Y (PVY) (Jovanchev et al. 1996; Choi et al. 2005; Ormeño et al. 2006; Kim et al. 2010; Milošević 2013). These pathogens can cause crop losses of up to 100% (Jovanchev et al. 1996).

Previous findings on virus occurrence and distribution in pepper cultivated in open fields (Rusevski et al. 2011, 2013) showed that the most widespread was CMV, followed by AMV and PVY.

The widespread distribution of CMV is due to its great adaptability to plant species and environments (Roossinck 2002). Compared to other known plant viruses, in the regions with moderate climate conditions, CMV has the broadest host range, including monocotyledonous and dicotyledonous plants belonging to more than 100 botanical families (Palukaitis and García-Arenal 2003). It causes economic damage on pepper, tomatoes, various cucurbits etc. (Gallitelli 2000).

CMV belongs to the family *Bromoviridae* (Büchen-Osmond 2006), representing a leading species in the genus *Cucumovirus* (Palukaitis and García-Arenal 2003). The virus has a tripartite genome, consisting of a linear single-stranded RNA (Palukaitis and García-Arenal 2003; Büchen-Osmond 2006), where RNA 1 is the largest, RNA 2 is smaller and RNA 3 is the smallest. RNA 1 and RNA 2 encode proteins involved in viral replication (proteins 1a and 2a, respectively). RNA 3 is dicistronic, the 5′ cistron encoding a cell-to-cell movement protein (3a) and the 3′ cistron (3b) encoding the coat protein CP. In addition to the genomic RNA molecules, CMV also contains the sub-genomic RNA 4 which serves as a messenger RNA for CP synthesis (Palukaitis et al. 1992).

The CP enables the virus movement through the host plant (Taliansky and García Arenal 1995; Kaplan et al. 1998), symptom expression (Shintaku et al. 1992) and virus transmission by insect vectors in a non-persistent manner (Chen and Francki 1990; Callaway et al. 2001). It has been observed that mutations of the CP gene which affect its amino acid sequence can alter aphid transmissibility (Ng and Perry 1999, 2004), even if the change occurs in only one amino acid (Perry et al. 1998).

CMV has a large number of strains and isolates (Palukaitis et al. 1992; Rodriguez-Alvarado et al. 1995; Büchen-Osmond 2006) divided into group I and group II (Owen and Palukaitis 1988; Palukaitis et al. 1992). A further division of group I in subgroups IA and IB was suggested by Roossinck et al. (1999) based on phylogenetic analysis of the CP gene and the 5' non translated region (NTR) of RNA-3.

Building up on previous findings on CMV occurrence in pepper cultivated in open fields in Macedonia (Rusevski et al. 2011, 2013), this study was conducted to uncover the phylogenetic relationship of Macedonian CMV isolates to those from other parts of the world.

Material and methods

Plant samples and serological testing The study was conducted during 2012, 2013 and 2014 on pepper plants cultivated in open fields in eight important pepper-producing regions, i.e. areas around Skopje (one locality), Kumanovo (one locality), Sveti Nikole (two localities in 2012 and one locality in 2013 and 2014), Kochani (two localities), Strumica (two localities), Radovish (two localities), Prilep (one locality) and Bitola (two localities). Each year, the same localities were surveyed, conducting field inspection along a transect and sampling seven randomly chosen plants per locality. Leaves from the uppermost 2–3 branches of each plant were collected and subjected to DAS-ELISA using commercial kits (Bioreba AG, Switzerland), as described by Clark and Adams (1977) and modified as proposed by the manufacturer. Plant tissue samples were homogenized in an extraction buffer (1:10 *w*/*v*). Positive and negative controls produced by the same manufacturer were included in each plate. Samples were considered positive if the average optical density (OD) after one hour incubation at room temperature in the dark was higher than twice the average OD of the negative control, measured with an ELISA microplate reader Multiskan Ascent, 354 (Thermo Scientific, USA) at absorbance of 405 nm. T-test was used to assess whether the occurrence of the analyzed viruses, either in single or mixed infections, differed significantly during the three year trial.

RNA extraction and RT-PCR of the coat protein gene of CMV Total RNA was extracted using TRIzol® Reagent (Ambion, Life Technologies, USA) according to the manufacturer's instructions (Chen et al. 2011). Homogenization of the plant material was performed in liquid nitrogen to prevent RNA degradation (Bertolini et al. 2003).

Reverse transcription (RT) was performed according to Van Dongen et al. (1999), in a total volume of 20 μl reaction mixture using 3 μl of total RNA, which was added to 2 μl 10xPCR Buffer Gold, 4 μl $MgCl_2$ (25 mM), 8 μl dNTPs (2.5 mM), 1 μl (50 pM/μl) of reverse primer R2 CMV (5'-CTGGATGGACAACCCGTTC-3′) (Deyong et al. 2005), complementary to positions 2012–2030 of CMV-Fny RNA-3 (D10538), 1 μl RNase Inhibitor and 1 μl of MuLV Reverse Transcriptase (Applied Biosystems, USA). PCR of the CMV coat protein gene was done in a 25 μl volume containing 5 μl cDNA, 2.5 μl 10xPCR Buffer II, 2.5 μl $MgCl_2$ (25 mM), 2 μl dNTPs (2.5 mM), 0.5 μl (100 pM/μl) of forward primer F2 CMV (5'-ATGGACAA ATCTGRATCWMCC-3′) (Deyong et al. 2005), homologous to positions 1257–1277 of CMV-Fny RNA-3 (D10538), 0.5 μl (100 pM/μl) of reverse primer R2 CMV and 0.25 μl of Taq DNA Polymerase (Sigma-Aldrich, USA). CMV primers covered the whole CP gene and part of the 3′ NTR. The PCR protocol for amplification of the CP gene was customized to cycling conditions specific for the CMV primer pair: initial melting at 95 °C for 15 min, 35 cycles of 95 °C for 45 s (melting), 59 °C for 45 s (annealing) and 72 °C for 90 s (extension) and final extension at 72 °C for 10 min. RT-PCR was performed on thermocycler Techne, TC – 512 (Fisher Scientific, USA).

The extracted RNA, as well as the amplified products, were analyzed on 1.5% agarose gel electrophoresis, in 1xTBE buffer, stained with ethidium bromide and visualized under a UV transilluminator (Popovski et al. 2013).

Sequencing and phylogenetic analysis The F2 CMV primer was used for sequencing the CP gene. PCR products were purified using a BigDye XTerminator Purification Kit (Applied Biosystems, USA) and the purified products were sequenced using a DNA-analyzer (Genetic Analyzer 3500, Applied Biosystems).

The sequences generated in this study were compared with sequences from the NCBI database using the ClustalW program (Thompson et al. 1994) and MEGA5 software (Tamura et al. 2011). The best-fitted models for estimating nucleotide (nt) and amino acid (aa) diversities were determined by performing a model selection analysis in MEGA5. The Kimura 2-parameter model Gamma distributed (K2 + G) (Kimura 1980) was established for nt sequence analysis and the Jones-Taylor-Thornton model (JTT) (Jones et al. 1992) was chosen for aa analysis.

Using the CMV CP gene partial sequences generated in this study and 41 CP gene sequences of CMV retrieved from GenBank (Supplementary Table S1), a maximum likelihood phylogenetic tree (number of bootstrap trials: 1000; bootstrap values <80% were omitted) was constructed. A sequence of isolate V of tomato aspermy virus (TAV), with GenBank accession No. NC_003836 was used as an outgroup. Intra- and inter-group diversity values were calculated as the average genetic distance, using Kimura 2-parameter model Gamma distributed (K2 + G).

Results

Serological analysis From a total of 259 tested pepper samples (91 samples in 2012, 84 samples in 2013 and 84 samples in 2014), CMV was found to be the most prevalent virus in Macedonian open fields as it was detected in 46 samples (51% of the sampled plants): 37 with single (41%) and nine with mixed infections (10%) in 2012; 28 samples (34%): 24 with single (29%) and four with mixed infections (5%) in 2013 and in 51 samples (61%): 49 with single (59%) and two with mixed infections (2%) in 2014 (Table 1).

In 2012, AMV was found to be the second most prevalent virus, as it was detected in 14 plants (15%): four with single (4%) and 10 with mixed infections (11%). For the following two years, PVY was the second most prevalent virus [six samples (7%): two with single (2%) and four with mixed infections (5%) in 2013; seven plants (8%): five with single (6%) and two with mixed infections (2%) in 2014]. During the inspected period, viruses were found more often in single, rather than in mixed infections. These were primarily observed in 2012 on 11 plant samples (12%). Their frequency declined during the examined years, so in 2014 mixed infections were observed in only 2% of the tested samples (two plants).

Most infected plants exhibited leaf mosaic and necrosis, leaf and fruit deformation and stunting. In several instances, however, symptoms were not obvious. Most of the virus infections were detected in the regions of Bitola, Skopje, Kumanovo, Strumica and Sveti Nikole. In some regions (Prilep in 2012 and in 2013 and Radovish in 2012), no virus infection was detected in the inspected localities.

A t-test was applied to the obtained data, revealing a statistically significant prevalence of single CMV infections, compared to all other single or mixed infections, for which no statistically significant difference was detected (Table 1).

Molecular detection and identification of CMV After performing RT-PCR on selected ELISA-positive leaf samples, the presence of CMV was confirmed following the amplification of the complete CP gene with expected size of 773 bp. In the negative controls, where cDNA was omitted or cDNA from ELISA-negative leaf samples was used, no amplification products were observed.

Eighteen partial nucleotide sequences of the CP gene of CMV isolates were generated by sequencing and were submitted to the NCBI database (Table 2). The isolates were collected in different years and from various regions of Macedonia. In 2012, the only collected isolate was from Skopje (SKA3–2012). In 2013, isolates were collected from areas around Skopje (SKA4–2013, SKA5–2013), Sveti Nikole (SVNA3–2013, SVNA6–2013, SVNA7–2013), Strumica (STRA4–2013) and Kochani (KOCA2–2013, KOCA5–2013, KOCB1–2013, KOCB2–2013, KOCB3–2013, KOCB6–2013). In 2014, isolates were collected from areas around Prilep (PRA1–2014), Kumanovo (KUA4–2014), Bitola (BITB1–2014), Sveti Nikole (SVNA3–2014) and Kochani (KOCB2–2014).

Table 1 Incidence of cucumber mosaic virus (CMV), alfalfa mosaic virus (AMV) and potato virus Y (PVY) on pepper plants cultivated in open fields in R. Macedonia during 2012–2014

Year	Number of tested samples	Single infection			Mixed infection			
		CMV	AMV	PVY	CMV + AMV	CMV + PVY	AMV + PVY	CMV + AMV + PVY
2012	91	37 (41%)	4 (4%)	1 (1%)	7 (8%)	1 (1%)	2 (2%)	1 (1%)
2013	84	24 (29%)	1 (1%)	2 (2%)	0	3 (4%)	0	1 (1%)
2014	84	49 (59%)	2 (2%)	5 (6%)	0	2 (2%)	0	0
Average		43%[a]	2.33%	3%	2.67%	2.33%	0.67%	0.67%

[a] Significantly different ($p = 0.01$). The results are obtained using a t-test

Table 2 List of cucumber mosaic virus isolates from R. Macedonia analysed in this study

Isolate	Region	Year of isolation	Accession number
SKA4–2013	Skopje	2013	KY985404
SKA5–2013	Skopje	2013	KY985405
SVNA3–2013	Sveti Nikole	2013	KY985406
SVNA6–2013	Sveti Nikole	2013	KY985407
SVNA7–2013	Sveti Nikole	2013	KY985408
STRA4–2013	Strumica	2013	KY985409
KOCB2–2013	Kocani	2013	KY985410
PRA1–2014	Prilep	2014	KY985411
KOCA2–2013	Kocani	2013	KY985412
KOCA5–2013	Kocani	2013	KY985413
KOCB1–2013	Kocani	2013	KY985414
KOCB3–2013	Kocani	2013	KY985415
KOCB6–2013	Kocani	2013	KY985416
SKA3–2012	Skopje	2012	KY985417
SVNA3–2014	Sveti Nikole	2014	KY985418
KUA4–2014	Kumanovo	2014	KY985419
KOCB2–2014	Kocani	2014	KY985420
BITB1–2014	Bitola	2014	KY985421

The partial CP sequences of the 18 Macedonian CMV isolates showed nt identities of 99.4 to 100%. Compared with the sequences of isolates from the NCBI database included in the analysis, the isolates from this study shared 73.6–100% similarities at the nt level. The highest nt identity (99.7–100%) was observed with two Serbian isolates (KC878465 and GQ340670), one isolate from the USA (D10538, a typical representative of the IA subgroup), one French isolate (X16386), one Hungarian (AJ511990) and two Spanish isolates (AJ829770 and AJ829768).

At aa level, the CMV isolates from this study showed 99.1–100% identities. In fact, all of them, except for isolate STRA4–2013, were identical and did not show any differences at the aa level. The isolate STRA4–2013 differed from the other isolates in one amino acid, at position 137, where instead of an alanine (A) a threonine (T) was observed. At the aa level, our isolates compared to those from NCBI showed an identity of 79.8–100%. Except for isolate STRA4–2013, the highest identity (100%), was determined with five isolates from Serbia (GQ340670, HM065509, HM065510, KC878465, JX280942), three from USA (D10538, AF523344, M98500), two from Spain (AJ829770 and AJ829768) and China (AJ006988 and EF159146) and one from France (X16386), Hungary (AJ511990), Poland (DQ018286), Japan (D43800) and South Korea (AJ296154). The isolate STRA4–2013 shared aa identity of 100% with one isolate from South Korea (AJ276481).

Phylogenetic analysis of CMV A maximum likelihood phylogenetic tree was constructed based on the partial sequences of the CP gene of 18 CMV isolates obtained in this study and 41 isolates from NCBI (Fig. 1). All of the CMV isolates were clustered into two groups, I and II. The reliability of the groups was assessed with a high bootstrap value of 100% for group I and above 80% for group II. Group I included two subgroups: subgroup IA and IB, with bootstrap support of above 80%.

The average genetic diversity of the CMV sequences in the phylogenetic tree was 0.070 ± 0.008. The genetic diversity between the two main groups I and II was 0.235 ± 0.029. The genetic diversity between each of the subgroups (IA and IB) and the second group was 0.236 ± 0.030 for IA and 0.233 ± 0.029 for IB. The genetic diversity between the subgroups IA and IB was 0.067 ± 0.013. The diversity within the groups and subgroups was: 0.026 ± 0.004 for group I, 0.011 ± 0.003 for group II, 0.015 ± 0.003 for subgroup IA and 0.020 ± 0.006 for subgroup IB.

Most of the CMV isolates were clustered in group I (52 of 59) coming from 15 different countries. Subgroup IA was noticeably bigger than subgroup IB, with 46 isolates from 13 different countries. The 18 CMV isolates from Macedonia were situated in subgroup IA, together with isolates from: Serbia, Hungary, Poland, Great Britain, Spain, France, Holland, USA, Colombia, Japan, South Korea and China. Our isolates shared nt identities of 92.8% and above with all of the CMV isolates of group I. Three isolates from China and one from Taiwan, USA and Italy clustered in subgroup IB whereas group II was formed with one isolate each from Australia, Japan, USA, China, Poland, India and Holland.

Discussion

In Macedonia and other countries (Choi et al. 2005; Kim et al. 2010; Milošević 2013) plant viruses regularly occur on pepper plants throughout the cropping period (Jovanchev et al. 1996; Rusevski et al. 2011, 2013) causing economic damage and representing a major threat for pepper production. The susceptibility of pepper plants to plant viruses was confirmed during this three-year study. With the help of DAS-ELISA and the t-test on the infection data, it was determined that CMV was significantly prevalent in Macedonia with an incidence ranging from 34% for 2013, 51% for 2012, to 61% for 2014. This is in agreement with studies that have found CMV to be the most frequent virus affecting pepper production (Choi et al. 2005; Ormeño et al. 2006; Kim et al. 2010).

Mixed infections with CMV, AMV and PVY are quite common (Kim et al. 2010; Milošević 2013). In this study, CMV, AMV and PVY were mostly present in single infections, but mixed infections were also detected, most notably in 2012 (12% of the tested samples). Mixed infections can cause more severe virus symptoms (Kim et al. 2010) and exhibit a

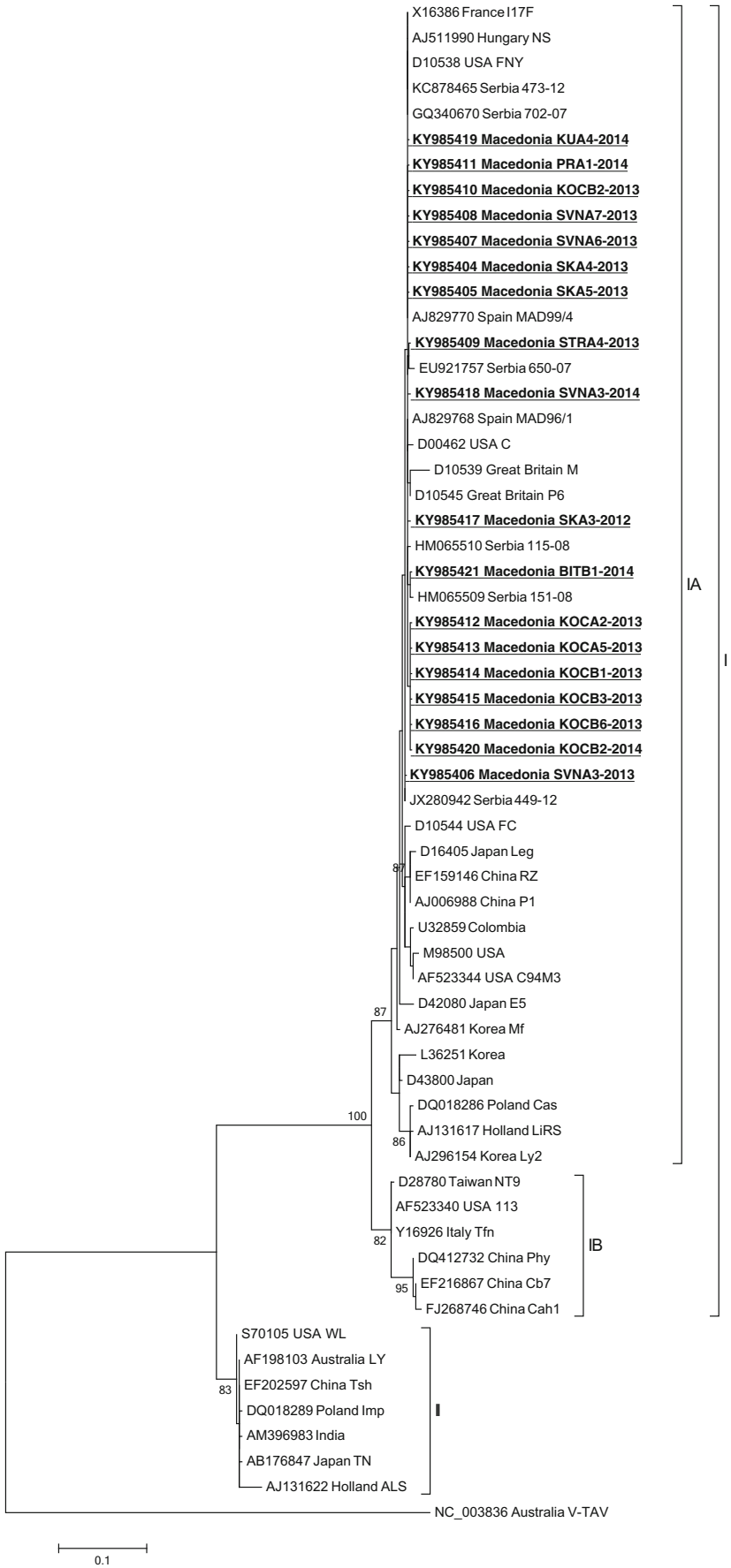

Fig. 1 Maximum likelihood phylogenetic tree (number of bootstrap trials: 1000; bootstrap values >80% are shown next to relevant branches), based on the partial nucleotide sequences of the CP gene of 59 CMV isolates. The isolate V of tomato aspermy virus (V-TAV) is included as outgroup. The Macedonian CMV isolates are underlined and bolded. The scale units (0.1) are substitutions per site

synergistic pathological interaction between viruses (Wang et al. 2002). That is why mixed infections can cause more damage and yield loss to the plants, compared to single infections. However, in this study, mixed infections were only scarcely present in the inspected fields, especially in 2013 and 2014. Further wider research on the spread of mixed viral infections in R. Macedonia should be considered for the future.

Total RNA extraction and amplification of the CP gene, generating amplicons of 773 bp for CMV were performed on chosen isolates. The obtained CP gene partial sequences of the isolates from R. Macedonia were compared to CP gene sequences from other parts of the world, determining nt and aa identities. The identities between different CMV isolates ranged from 73.6 to 100% at the nt level and 79.8–100% at the aa level. According to Palukaitis et al. (1992), the genetic similarity between different CMV groups varied from 69 to 77%, while within the groups, it was above 90%. Those results corresponded with our findings (73.6–77.9% nt identity between groups and above 92.8% in group I).

Phylogenetic clustering of CMV isolates worldwide is considered to be very stable and has been confirmed by many studies (Moury 2004; Deyong et al. 2005). CMV is clustered in two major groups: group I and II (Owen and Palukaitis 1988; Palukaitis et al. 1992; Roossinck et al. 1999). Group I has been divided in two subgroups: subgroup IA and IB (Finetti-Sialer et al. 1999; Dubey et al. 2010). Group I represents a very wide group, in which most of the CMV isolates from different parts of the world have been clustered (Roossinck et al. 1999). Group I accounts for 98% of the CMV world's isolates, with 64% of the isolates clustered in subgroup IA (Bonnet et al. 2005). Subgroup IB mostly contains CMV isolates from Asia (Khan et al. 2008; Gautam et al. 2012), although isolates from this group have also been found in Europe (Sclavounos et al. 2006) and USA (Lin et al. 2003). Group II also contains CMV isolates from different parts of the world (Moury 2004).

The overall shape of the CMV phylogenetic tree, constructed in this study, correlated with the previous findings of the above mentioned authors, clustering isolates in groups with high bootstrap values of 100% for group I and above 80% for the other group and subgroups. All of the 18 isolates gained in this study were clustered in subgroup IA, sharing genetic identities with the rest of the CMV isolates in group I of 92.8% and above. Since isolates belonging to group I shared nt identities greater than 88% (Palukaitis and García-Arenal 2003; Yu et al. 2005), it confirmed the placement of our isolates in group I. The isolates gained in this study showed the highest nt identity (99.7–100%) with the isolate FNY from USA (D10538), which was a typical representative of the IA subgroup. This similarity confirmed that our isolates were typical IA representatives, without exhibiting notable genetic diversity. The only aa mutation was observed from the only isolate collected from the region around Strumica (STRA4–2013). This isolate shared 100% aa identity with only one isolate from South Korea (AJ276481), out of 46 isolates forming the IA subgroup in this phylogenetic tree. Further investigation of CMV isolates from Strumica region may result in less uniform findings about the CMV population in R. Macedonia.

In this study, CMV was observed to be the most frequent virus on pepper plants. For the first time, Macedonian isolates of CMV were sequenced and identified at the molecular level, broadening the field of plant virus research in R. Macedonia.

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