*Full Length Research Paper*

# Detection and partial characterization of two distinct walnut isolates of cherry leaf roll virus (CLRV)

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Two new cherry leaf roll virus (CLRV) isolates (Ah and Ad) were isolated and detected from traditionally-grown walnuts that showed severe apical necrosis and chlorotic spots in systemically infected *Cheneopodium amaranticolor*, in the eastern part of Turkey. The 404 and 405 bp long DNA fragments of the 3'-non-coding region of both isolates from PCR reactions were cloned and sequenced. A significant genetic variability (up to 14% divergence between sequences) was found within the 3' terminal region of viral genome of CLRV Turkish isolates compared with the isolates in databases. The sequence of Ad isolate was found to share 84 to 98% and the Ah isolate was found to share 85 to 97% nucleotide identity with corresponding sequences of the selected world isolates. An RNA riboprobe generated for CLRV-Ad isolate reacted also with the CLRV-Ah isolate in dot blot molecular hybridization test. Positive reactions were still visible in hybridization test when the extracts of infected fresh and dried leaf tissues of *C. amaranticolor* diluted 1:20. Western blot analysis revealed that the molecular mass of the coat protein of about 52 kDa for both isolates.

Key words: Walnut, CLRV, 3' non coding region.

## INTRODUCTION

Cherry leaf roll virus (CLRV) is a serious disease of sweet cherry and walnut found in Europe, Russia, North America, Chile (Herrera and Madariaga, 2001), New Zealand, Australia, China and Japan (Jones, 1986). The virus was first described in 1955 by Posnette and Cropley, as causing a disease of sweet cherry (*Prunus avium* L.) in England (Cropley, 1961). Since then, it has been shown to exhibit a wide natural host range including a variety of herbaceous and woody plants (Cropley, 1961; Rebenstorf et al., 2006). In Turkey, the presence of CLRV was proved in walnut (Ozturk et al., 2008) and in olive trees (Caglayan et al., 2004; Beler and Acikgoz, 2005).

CLRV belongs to the group of nepoviruses consisting of two genomic RNAs and has a bipartite single-stranded positive-sense RNA genome encapsidated in isometric particles (Jones, 1986; Pallás et al., 1992). They are characterized by a large, separately encapsidated RNA-2 with a long 3'noncoding region (NCR) which is identical to that of RNA-1 (Borja et al., 1995). CLRV is mainly trans-

mitted through seeds and pollen (Bandte and Büttner, 2001; Jones, 1986). This virus can be transmitted to many plant species by mechanical inoculation (Jones, 1986). It infects many wild and cultivated woody plants species (Jones, 1986), among which birch (*Betula pendula* Roth), black elderberry (*Sambucus nigra* L.) and sweet cherry (*Prunus avium* L.) (Rebenstorf et al., 2006). The walnut strain of CLRV (CLRV-W) is the causal agent of blackline, an economically important disease affecting English walnuts grafted on black walnuts (*Juglans nigra* L.) (Mircetich and Rowhani, 1984). More than 30 different walnut cultivars are hosts of this virus but they seldom exhibit recognizable leaf symptoms when plants are ungrafted (Savino et al., 1977; Mircetich et al., 1980; Rowhani and Mircetich, 1992).

A strong relationship between the original host, serology and sequence based phylogeny of an approximately 375 bp fragment from the 3' non-coding region (3'-NCR) was shown by Rebenstorf et al. (2006). It has been speculated that this very high conservation of the 3' NCR between the two genomic RNAs could be the result of an RNA recombination mechanism acting as part of the RNA-2 replication process of these viruses (Rott et al., 1991; Scott et al., 1992; Le Gall et al., 1995; Robenstrof

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et al., 2006).

Here, this study present partial characterization of two CLRV-walnut isolates from traditionally grown walnuts. The isolates were analyzed biologically, serologically and genetically and their characteristics were compared with those of birch, cherry, elderberry, walnut, dogwood, blackberry, grapevine, rhubarb and raspberry isolates. The host range following mechanical inoculation of test plants was investigated. Serological and molecular characteristics were determined in western blot analysis, cloning and sequencing of RT-PCR amplified fragments of the 3'-NCR. The sequences were compared with sequences available in the Genbank database. Additionally, the isolates were detected specifically by molecular methods.

## MATERIALS AND METHODS

### Virus sources and mechanical transmission

Two CLRV isolates used in this study were identified from walnut plants growing on field in Ahlat (Ah) and Adilcevaz (Ad), Turkey. The isolates (CLRV-Ah and CLRV-Ad) were maintained on *Chenopodium amaranticolor* by mechanical transmission with phosphate buffer. Leaf tissues of systemically infected *C. amaranticolor* were served as virus source during the trials. *Chenopodium quinoa*, *Cucumis sativus*, *Datura stramonium, Nicotiana benthamiana* and *Nicotiana occidentalis* were attempted to mechanically inoculate with sap from infected *C. amarnticolor*, using 0.1 M phosphate buffer containing 2.5% nicotine (pH 7.2). Plants were grown in a growth chamber at 22 to 24°C with a 14 h photoperiod and were inspected regularly for symptom development; 15 to 20 days after inoculation they were tested by RT-PCR for detection of symptomless infections.

### Sample preparation and RT-PCR

Total RNA extraction of virus isolates was carried out according to the silica-capture method described by Foissac et al. (2000). Oligonucleotide primers designed according to Werner et al. (1997) on conserved region of the 3'-terminal region of viral RNA-2 (F: 5'-tggcgaccgtgtaacggca-3' and R: 5'-gtcggaaagattacgtaaaagg-3') were used for the amplification by RT-PCR. First-strand cDNA synthesis was done in a total reaction volume of 10 μl using 100 units' moloney murine leukemia virus reverse transcriptase, 1 μl of dNTPs (10 mM each) 5 μM reverse primer in M-MuLV-Rtase reaction buffer supplied by Fermentas (Ukraine). PCR amplification was carried out in a total volume of 25 μl containing 1 μl of cDNA, 2.5 μl of 10 X reaction buffer (200 mM Tris-HCL pH: 8.4, 500 mM KCl), 1.5 μl of MgCl₂ (25 mM), 0.5 μl of dNTPs (10 mM each), 0.5 μl of each primer (100 pmol/μl), 0.2 μl of *Taq* DNA polymerase (Promega, Madison, USA) and 18.3 μl of RNase free sterile water. Samples were amplified in a ThermoHybaid PX2 thermo cycler. These primers amplify an approximately 416 bp fragment with the birch strain (Werner et al., 1997) corresponding to the CLRV 3'-NCR. The amplified fragments were analyzed by PAGE electrophoresis (Laemmli, 1970).

### Cloning and sequencing of RT-PCR fragments

The RT-PCR amplified DNA was recovered from agarose gel with Zymoclean™ gel DNA recovery kit (Zymo research) and purified with the DNA clean and concentrator™ kit (Zymo research) according to instructions of the manufacturer. Eluted DNA was then ligated to the AT cloning vector pGEM®-T easy vector (Promega, Medison, WI) and cloned in *Escherichia coli* JM 109 competent cells following the manufacturer's instructions. The nucleotide sequences of clones were determined by sequencing the recombinant plasmids with an automated DNA sequencer (Applied Biosystems).

### Sequence comparison and phylogenetic analysis

Phylogenetic analysis was performed to determine the relationships of CLRV-Ah and Ad isolates with the other CLRV isolates in databases. Partial sequence of 3'-NCR of CLRV isolates were compared using geneious software.

### Riboprobe synthesis and molecular hybridization

A recombinant clone bearing the partial 3'-NCR sequence of the CLRV-Ad RNA was linearized with *Sac*II and used to obtain a digoxigenin-labeled riboprobe, as previously described (Mas and Pallas, 1995). Fresh and dried leaves of *C. amaranticolor* was processed and applied to nylon membrane by using dot blot apparatus (Scie-Plas Ltd. UK). Before use of dried sample, the leaves of infected hosts were dried at 65°C for 48 h and ground into dry powder and stored at 4°C in plastic bags until use. The nucleic acids were further bound by ultraviolet light exposure for 5 min. Prehybridization was carried out for 2 h at 68°C in 50% deionized formamide, 5X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0), 0.1%(w/v) N-laourylsarcosine, 0.02% (w/v) SDS and 5% (w/v) blocking reagent (Roche). The prehybridization solution was removed and replaced with 20 ml of hybridization mixture containing digoxigenin labeled RNA probe denatured by heating at 70°C for 2 min and incubated overnight at 68°C. After hybridization the membrane was washed twice in 2X SSC and 0.1% SDS at room temperature for 5 min, twice in 0.1X SSC and 0.1% SDS at 68°C for 15 min. Binding to anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche) and subsequent chemiluminescent detection using CSPD (Roche) as substrate were used to detect the hybridized probe.

### Western blot analysis

Coat protein fractionation and western blot analysis were carried out essentially by the standard method (Sambrook et al., 1989). Leaf samples were collected from infected and healthy *C. amaranticolor* plants and macerated with precooled mortars and pestles, in homogenizing buffer (0.05 M Tris pH 7.8, 0.01 M MgSO₄, % 0.02 3,3'-iminobispropylamine, 0.5 M urea and 0.01 M 2-mercaptoethanol) at a ratio of 1 g to 10 ml of buffer. Samples were analyzed on discontinuous gel system (5 and 13%) along with polypeptide size standard for immunoblotting analysis (Laemmli, 1970). Coat proteins were electrotransferred to PVDF-plus (GE Water and Process Technologies, USA) membrane. Polyclonal IgG kindly supplied by Dr. Rowhani (Foundation Plant Services, University of California, One Shields Avenue, Davis, CA 95616, USA) was used as primary antibody at 1:5000 dilution and goat anti-rabbit antibody alkaline phosphatase conjugate (Sigma-Aldrich Inc. St Louis, MO) was used as secondary antibody at a 1:10,000 dilution. Visualization of virus-specific coat protein bands was achieved in 15 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; and 5 mM MgCl₂) containing 100 μl of 50 mg of nitro blue tetrazolium (NBT) per ml of 70% dimethylformamide and 50 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per ml of dimethylformamide. Molecular weight of the CP protein of CLRV was estimated from a calibration curve of Log₁₀ molecular weight of

A                                            B
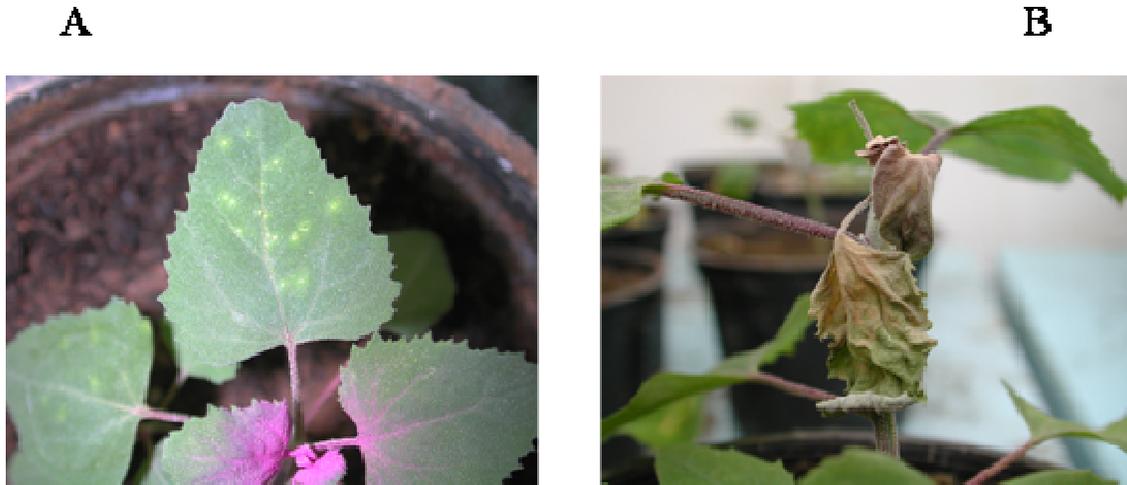


**Figure 1.** Symptoms in *C. amaranticolor* plants approximately 10 days after mechanical inoculation using CLRV-Ad isolate, (a) chlorotic spots, (b) tip necrosis. CLRV-Ah isolate was also caused same symptoms.

standard proteins.

## RESULTS

### Symptoms on herbaceous hosts produced by CLRV isolates

Approximately 10 days after inoculation of both isolates, *C. amaranticolor* plants began to show symptoms of chlorotic leaf spots and severe apical necrosis (Figure 1). Attempts to infect *C. quinoa*, *D. stramonium*, *C. sativus*, *N. benthamiana* and *N. occidentalis* with CLRV isolates failed to result in systemic infection. Mechanical inoculation of herbaceous hosts was not successful when systemically infected *C. amaranticolor* tissue was used as inoculums. This was shown with the lack of symptom appearance and additionally, with the lack of positive reaction after RT-PCR reaction from inoculated herbaceous hosts.

### Sequence of 3'-non-coding region of the CLRV genome and similarity analysis

The 3'- terminal non coding nucleotide sequence of Turkish CLRV-walnut isolates were deposited in the Gen-Bank under the accession numbers FJ785323 and FJ785324. Multiple sequence alignment of these sequences revealed significant variations. Pairwise comparisons revealed an average divergence between sequences in databases of 14 to 1.1% calculated using a nucleotide identity distance. The geographical origin, host and accession numbers of compared world isolates are given in Table 1. About 86 to 98% nucleotide sequence identity was observed when partial CLRV-Ad nucleotide sequences were compared in databases and about 87 to

98% similarity was observed for CLRV-Ah isolate in the analysis with geneious software. Phylogenetic analysis using the maximum likelihood analysis yielded three major phylogenetic clusters (A1, A2 and A3) of CLRV isolates shown in Figure 2, some of them being composed of a large majority of isolates (Group A3) was sharing the same original host (walnut). All CLRV isolates compared, representing phylogenetic groups A1, A2 and A3 originating from Germany, United Kingdom, France, Japan, Hungary, USA, Slovakia and New Zealand. The analysis of the phylogenetic relationships of 3'-NCR Turkish CLRV isolates with other CLRV isolates showed a closer relationship with France, UK and German isolates. Three English (AJ877148, AJ877149 and AJ877126), two French (AJ877151, AJ877147) and one German (AJ877146) isolates cluster within the walnut group.

In order to determine whether several passages of virus isolates through *C. amaranticolor* resulted in genetic change on both isolates (isolates 35 and 36 in Table 1), comparisons were made between cloned sequences. After several passages and subsequent sequencing of the 3'-NCR of both isolates also identified approximately 1% nucleotide divergence. It is not known whether this divergence occurs within the viral genome or whether these small variations represent cloning artifacts.

### RT-PCR analysis

Two-step RT-PCR reactions yielded 404 and 405 bp PCR products for CLRV-Ah and Ad isolates respectively (Figure 3). Both isolates were detected by conventional RT-PCR from systemically infected leaves of *C. amaranticolor* plants. RT-PCR analysis was followed by verification with dot blot hybridization.

**Table 1.** CLRV isolates (Rebenstorf et al., 2006) used to compare Turkish CLRV walnut isolates in this study.

| Nr | Host | Accession number | Geographical origin |
|----|------|------------------|---------------------|
| 1 | Common birch | AJ877121 | Germany |
| 2 | Common birch | AJ877122 | Germany |
| 3 | Common birch | AJ877123 | UK |
| 4 | Common birch | AJ877119 | Germany |
| 5 | Common birch | S84124 | UK |
| 6 | Common birch | S84125 | UK |
| 7 | Common birch | AJ877124 | UK |
| 8 | Sweet cherry | AJ877127 | Germany |
| 9 | Sheep's sorrel | AB168099 | Japan |
| 10 | Chinese chive | AB168098 | Japan |
| 11 | Sheep's sorrel | AB168100 | Japan |
| 12 | Sweet cherry | AJ877128 | UK |
| 13 | Sweet cherry | AJ877129 | UK |
| 14 | Black elderberry | AJ877130 | Germany |
| 15 | Black elderberry | AJ877143 | Hungary |
| 16 | Black elderberry | AJ877140 | Germany |
| 17 | Black elderberry | AJ877131 | Germany |
| 18 | Black elderberry | AJ877141 | Germany |
| 19 | Black elderberry | AJ877132 | Germany |
| 20 | Black elderberry | AJ877136 | Germany |
| 21 | Black elderberry | AJ877133 | Germany |
| 22 | Black elderberry | AJ877137 | Germany |
| 23 | Black elderberry | AJ877138 | Germany |
| 24 | Black elderberry | AJ877134 | Germany |
| 25 | Black elderberry | AJ877135 | Germany |
| 26 | Black elderberry | AJ877144 | Germany |
| 27 | Black elderberry | AJ877142 | Germany |
| 28 | Walnut | AJ877146 | Germany |
| 29 | Walnut | AJ877147 | France |
| 30 | Walnut | AJ877148 | UK |
| 31 | Walnut | AJ877149 | UK |
| 32 | Walnut | AJ877126 | UK |
| 33 | Walnut | AJ877151 | France |
| 34 | Walnut | AJ877150 | Hungary |
| *35* | *Walnut* | *FJ785323* | *Turkey* |
| *36* | *Walnut* | *FJ785324* | *Turkey* |
| 37 | Walnut | AJ877152 | Slovakia |
| 38 | Mountain ash | AJ877154 | Germany |
| 39 | Mountain ash | AJ877155 | Germany |
| 40 | Mountain ash | AJ877153 | Germany |
| 41 | Hombeam | AJ877156 | Germany |
| 42 | Ground elder | AJ877157 | Germany |
| 43 | Golden elderberry | AJ877145 | USA |
| 44 | European ash | AJ888534 | Germany |
| 45 | European ash | AJ877158 | Germany |
| 46 | European ash | AJ888533 | Germany |
| 47 | Flowering dogwood | AJ877161 | USA |
| 48 | Blackberry | AJ877163 | UK |
| 49 | Grapevine | AJ877164 | Germany |
| 50 | Rhubarb | AJ877165 | Germany |
| 51 | Rhubarb | S84126 | UK |
| 52 | Red raspberry | AJ877162 | New Zealand |
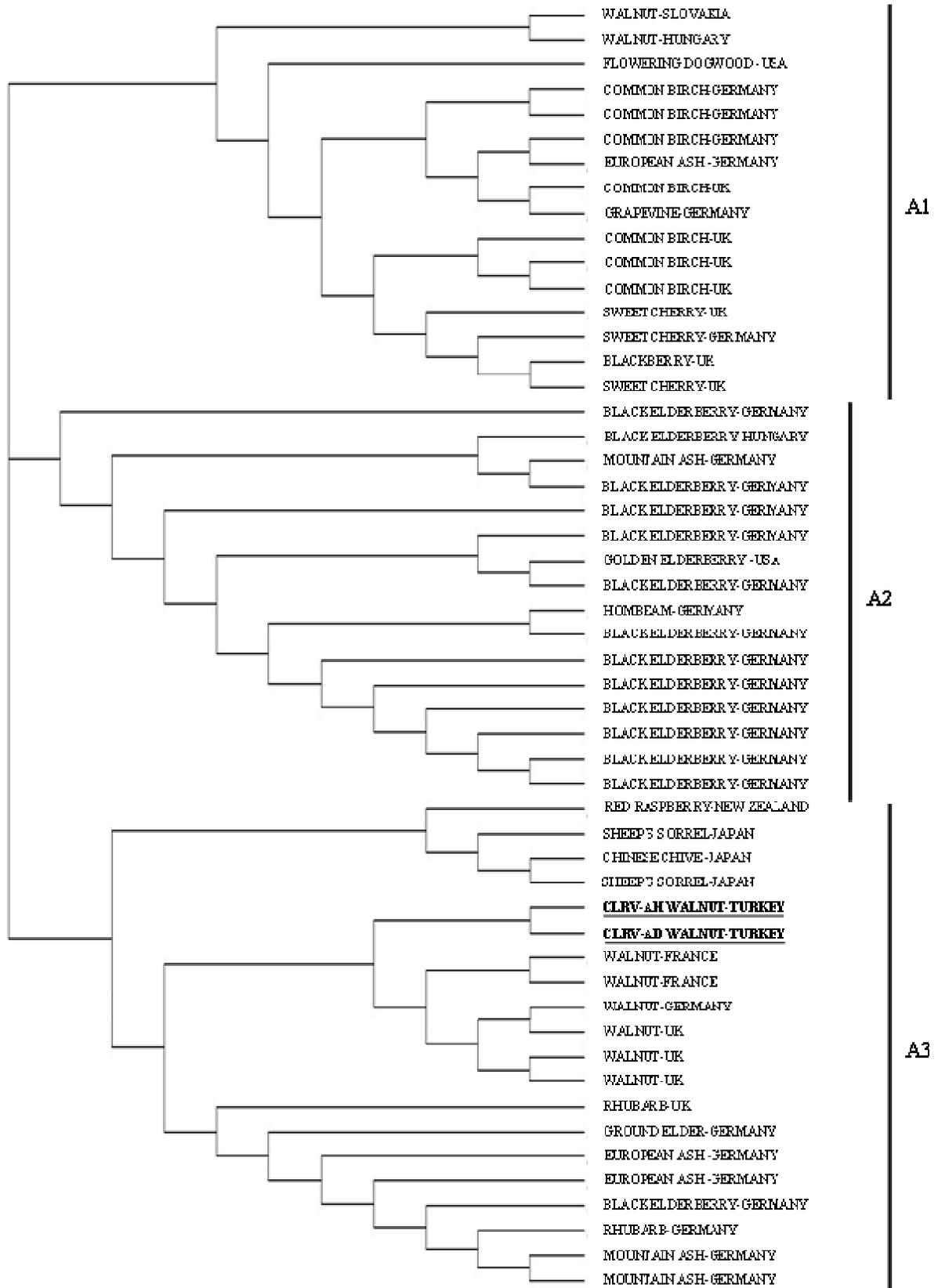
**Figure 2.** Phylogenetic tree based on the nucleotide sequence of a 3'-NCR fragment of the *Cherry leaf roll virus*. All data obtained from the GenBank nucleotide database are indicated by accession numbers. Data analysis and tree construction were done by using the Geneious software. The new CLRV isolates FJ785323 and FJ785324 are marked.
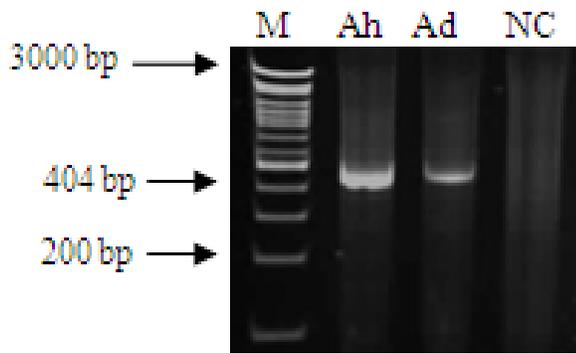
**Figure 3.** PAGE analysis of CLRV walnut isolates detected by RT-PCR, M: (100 bp molecular size markers (Fermentas), Ah: CLRV-Ahlat isolate, Ad: CLRV-Adilcevaz isolate, NC, healthy control.
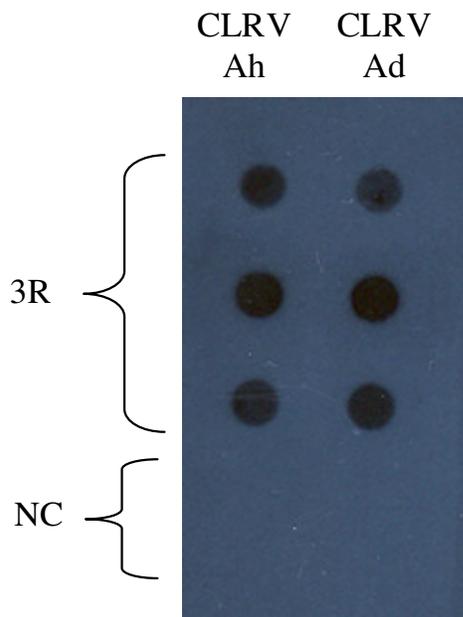


**Figure 4.** Dot-blot hybridization for specific detection of CLRV isolates. 3R: Three replicates of total RNA from CLRV infected *C. amaranticolor* plants, NC, healthy purified tissue extract.



**Figure 5.** Detection of CLRV from infected dry and fresh tissues by using non-isotopic molecular hybridization with genome specific riboprobe. Numbers at the side of the figure represent the dilutions performed on the original undiluted samples. Chemiluminescent detection was carried out after 5 min UV exposure.

## Molecular hybridization

The dot-blot assay detected viral RNA from total RNA purified from infected plants. There were no hybridization signals with the healthy extractions purified from healthy plants, confirming the results obtained by hybridization assay (Figure 4). The sensitivity of dried infected plant material (Sipahioglu et al., 2006) was compared with the infected fresh material by using the same amounts of viral RNA preparation from both tissues. The RNAs of CLRV derived from dried and fresh samples were recovered in all instances. As shown in Figure 5, the sensitivity of infected dried material with respect to the end-point detection limit was similar to fresh material indicating that, the riboprobe can detect CLRV tested without losing sensitivity. The experiment showed that, the dried infected plant material did not affect its sensitivity limit as can be concluded from the comparison of the results obtained with fresh material.

## Western blot analysis

The results of western blot analysis comparing the immunoreactivity of purified CLRV coat protein from virus infected *C. amaranticolor* leaves are presented in Figure 6. CLRV polyclonal antibodies reacted with the coat protein of both CLRV isolates with estimated molecular
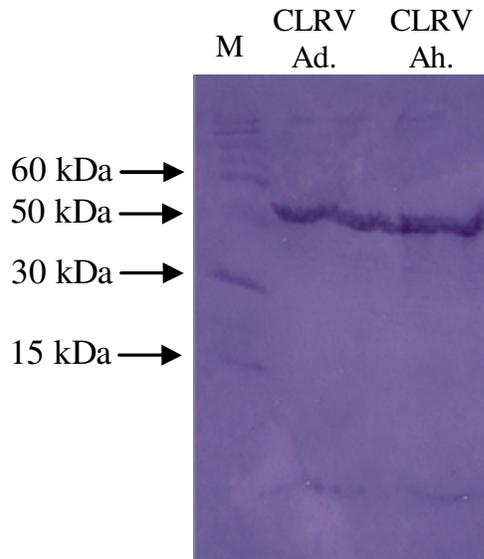
**Figure 6.** Western blot analysis of the coat protein of CLRV Ah and Ad isolates, Lanes M, molecular mass (kDa) markers.

masses of 52 kDa.

## DISCUSSION

CLRV was found for the first time infecting walnut, one of the known natural hosts in 2007 in Turkey (Ozturk et al., 2008). In this report, two distinct isolates of CLRV were described. RT-PCR and dot blot hybridization techniques which successfully confirmed the presence of both isolates in the tested leaves of systemically infected *C. amaranticolor*.

The partial 3'-NCR of the CLRV genome is variable among virus isolates that are serologically diverse and phylogenetically different (Rebenstorf et al., 2006; Buchhop et al., 2009). A 3'-NCR sequence comparison of CLRV walnut (Ah and Ad) isolates showed that, a relatively high level of sequence differences (up to 14%) were observed with its counterpart in other CLRV isolates in databases. The sequence divergences observed reflect the emergence of stable host-specific viral variants or strains from a common viral ancestor sequence possibly due to the evolutionary selection of viral genomic nucleotide compositions adapted to persist in particular host species (Werner et al., 1997). The sequence of the PCR fragment of the CLRV-Ah (404 bp) isolate had 99% homology to the CLRV-Ad isolate (405 bp), but exhibited 1 nucleotide deletion within this fragment. Werner et al. (1997) demonstrated 9 nucleotide deletion between petunia and birch strain of CLRV on the same fragment.

The tree constructed based on the 3'-NCR region showed a tendency to cluster the isolates into three groups on the basis of their geographical and host origin.

The first and second clusters (A1 and A2) contain isolates from Central and Western Europe and the United States and the third cluster (A3) contains the isolates from the Western Europe and Far East. The two Turkish isolates, CLRV-Ah and Ad, cluster with the Western European/Far East isolates. The results obtained are consistent with those results previously reported by Robenstrof et al. (2006). In particular, isolate CLRV-Ah and Ad are very closely related to English isolates. However, these isolates are distinct from other Western European and American isolates and have a higher degree of dissimilarity with partially characterized CLRV isolates from France, Germany and USA (Robenstrof et al., 2006). The analysis placed the isolates Ah and Ad in phylogenetic group A3, which harbors mainly walnut isolates.

The results obtained in the dot blot hybridization assays allowed discrimination between infected and uninfected samples. The sensitivity of the dig-labeled riboprobes used in these reactions was shown to be very high, revealing that they are very specific and reliable with potential to be routinely used in plant sanitary selection programs. The results obtained for the tested isolates are consistent with those in a previously reported paper, where the virus has been detected in tobacco plant (Mas and Pallas, 1996). The authors used dot blot hybridization technique successfully to study CLRV accumulation and translocation in whole plants. Grieco et al. (2002) demonstrated that, dot blot hybridization of denatured dsRNAs with digoxigenin-labeled virus-specific riboprobes is the most reliable detection method currently available for the detection of CLRV. The results of dot blot hybridization assay also showed that, the RNA from dried infected tissue were equal to or more abundant than fresh tissue preparations, indicating that viral RNA degradation did not occur during extraction procedure (Sipahioglu et al., 2006). It was demonstrated that, isolated viral RNA from dried tissue is suitable for diagnostic purpose employing hybridization method. Preliminary experiments showed that the drying of the infected leaves indeed produced a large number of detectable extracts from CLRV-infected host tissue. A rapid drying method is recommended for the extraction of RNA to obtain high quality RNA from the infected hosts. In theory, drying the tissue should limit or reduce the rate of degradation of the cellular components by inactivating proteolytic enzymes and nucleases (Jaiprakash et al., 2003).

CLRV isolates were propagated 5 times since isolation in *C. amaranticolor* and both isolates were almost identical in the 3'-NCR. These results are consistent with previous analyses (Rebenstorf et al., 2006) using a larger number of isolates that demonstrated the sequence composition of the 3'-NCR, seems to be highly stable and not to change rapidly when the virus is propagated in *C. quinoa*. After mechanical inoculation, they propagated several times in *C. quinoa* and all were identical in the

375 bp stretch of the 3-NCR.

On the basis of the host range and phylogenetic analyses, this study showed that the Turkish isolates are very distinct. Most of the mechanically inoculated herbaceous plant species reacted negative to the Turkish CLRV iso-lates. No differences in symptoms were observed in *C. amaranticolor*. The major differences appeared in *C. quinoa* that was not infected by both isolates. Recently, two other walnut isolates exhibiting similar biological behaviors were recovered from another geographical site at the region (Ozturk et al., 2008). Systemically infected *C. amaranticolor* plants grew well for more than two years after mechanical inoculation as previously reported by Polak et al, (2004). They demonstrated that, *C. amaranticolor* plants were more suitable for maintenance of CLRV. Tobias (1995) has recommended *N. rustica* or *N. tabacum* cvs White Burley and Xanthi-*nc* for maintaining CLRV virus cultures.

The biological differences between Turkish CLRV isolates and the other isolates were also confirmed by the western blot analysis of coat protein. Nepoviruses are generally composed of a single CP of about 54 to 55 kDa (Chandrasekar and Johnson, 1998; Hohkuri et al., 2004). When analyzed by western blot, protein extracts of purified virion preparations usually migrated as one clearly defined band of 52 kDa. This unique protein band was consistently observed for both isolates in western blots of purified CLRV preparations when analyzed with a CLRV polyclonal antiserum. Pallas et al. (1992) observed a 56 kDa band for the coat protein of walnut strain of CLRV assayed by SDS-PAGE.

The presented data together with the other available sequences provide useful information concerning CLRV isolates found in Turkey. These findings may be particularly relevant in light of recent reports of the presence of CLRV in Central and Western Europe. The above data indicate a growing distribution of the CLRV infections in Europe and worldwide.

## ACKNOWLEDGEMENTS

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