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Identification and characterization of hypovirus-infected *Cryphonectria parasitica* isolates from biological control plots in İzmir, Kütahya, and Sinop

Seçil AKILLI ŞİMŞEK[1,*], Y. Zekai KATIRCIOĞLU[2], Nora BORST[3], Deniz ÇAKAR[4], Simone PROSPERO[3], Daniel RIGLING[3], Salih MADEN[2]

[1]Department of Biology, Faculty of Science, Çankırı Karatekin University, Çankırı, Turkey
[2]Department of Plant Protection, Faculty of Agriculture, Ankara University, Ankara, Turkey
[3]Swiss Federal Research Institute, Birmensdorf, Switzerland
[4]Forestry Research Institute of Western Black Sea Region, Bolu, Turkey

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Abstract: In a pilot study, a biological control with hypovirus-infected *Cryphonectria parasitica* was applied in 3 study plots in Turkey, in June 2013. The aim of this study was to evaluate the success of the applied biological control by verifying the establishment of the released hypovirus (CHV-1) strains using molecular markers. *C. parasitica* isolates were sampled from cankers at 3 different time points: before the biological control treatments (April 2013), and 5 months (October 2013) and 11 months (May 2014) after the treatment. In total, 255 *C. parasitica* isolates were recovered and characterized. First, the culture morphology and vegetative compatibility type of these isolates were assessed. Next, the presence of hypoviruses in white isolates was checked by RNA extraction and subsequent RT-PCR. Finally, a sequence analysis was performed to compare the hypoviruses to the released biological control hypoviruses by examining single nucleotide markers and reconstructing their phylogenetic relationship. The study sites in İzmir and Kütahya were found to be free of hypoviruses prior to the biological control, whereas in Sinop, the occurrence of hypoviruses of the Italian subtype was observed. Reisolations of the treated cankers and subsequent molecular analysis resulted in the detection of the released biocontrol hypovirus strains in all 3 study sites. The reisolated hypoviruses in İzmir and Kütahya originated from either one of the released biocontrol strains. In Sinop, both natural and artificially introduced hypoviruses were detected. Our study showed that the released biological control hypoviruses persisted in the treated cankers, which is promising for the biological control of chestnut blight in Turkey.

Key words: Chestnut blight, biological control, *Cryphonectria parasitica*, CHV-1, Turkey

1. Introduction

In Turkey, sweet chestnut, *Castanea sativa* Mill., with an acreage of about 262,000 ha, as in pure stands (11.3%), stands mixed with deciduous trees (73.4%), and in stands mixed with conifers (9.3%), is an important tree species with horticultural benefits. The Aegean and Marmara regions, with a chestnut area of 69,243 ha, comprise 26% of all chestnut stands, but produce more than 90% of high-quality nuts in Turkey, which ranks second in world production (Orman Genel Müdürlüğü, 2014).

Chestnut blight caused by the fungus *Cryphonectria parasitica* (Murrill) Barr is a serious disease resulting in bark cankers and dieback on almost all chestnut species worldwide, including Turkey (Anagnostakis, 1988; Robin and Heiniger, 2001; Akıllı et al., 2013; Rigling and Prospero, 2018). The disease was first reported in Turkey in the 1960s (Delen, 1975) and until recently, it has spread all over the country, resulting in considerable damage to the native *C. sativa* population, particularly in the Aegean region (Çeliker and Onoğur, 2001; Erincik et al., 2008).

After a great expansion and damage, the infected trees began to recover from the disease in many stands in Europe, as indicated by the occurrence of superficial nonlethal bark cankers. This recovery has been attributed to hypovirulence, a phenomenon in which fungal viruses infect *C. parasitica* and significantly reduce its virulence and sporulation capacity (Grente, 1965; Choi and Nuss, 1992; Heiniger and Rigling, 1994; Milgroom and Cortesi, 2004).

Although since the 2000s, naturally healing cankers have been observed in Turkey, starting from the west part of Anatolia and spreading eastwards, along the Black Sea region (Çeliker and Onoğur, 2001; Gürer et al., 2001a, 2001b; Akilli et al., 2009), no healing cankers have been observed in the Aegean region thus far.

Hypovirulence in *C. parasitica* is caused by an infection of virulent fungal isolates by mycoviruses named

* Correspondence: secilakilli@gmail.com

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Cryphonectria hypoviruses (CHVs); CHV-1, -2, -3, and -4). The most investigated hypovirus is CHV-1, which is the only hypovirus reported in Europe (Allemann et al., 1999; Nuss, 2005). CHV-1 has also been found in Japan and China (Peever et al., 1998; Hillman and Suzuki, 2004). The infection of *C. parasitica* by CHV-1 causes a reduction of virulence that can vary depending on the virus strain and the fungal isolate (Rigling et al., 1989; Peever et al., 2000; Robin et al., 2010; Bryner and Rigling, 2011). Aside from reduced virulence, fungal isolates infected by CHV-1 also exhibit reduced pigmentation and reduced sporulation, giving them a typical white cultural appearance.

Hypovirulence has been successful in many areas of Europe, either naturally or after artificial introduction of the hypovirus in virulent *C. parasitica* populations. Since CHV-1 is transmitted horizontally from an infected fungal strain to an uninfected strain via hyphal anastomosis (Van Alfen et al., 1975), biological control of chestnut blight can be achieved by treating virulent cankers with vegetative compatible (vc) hypovirulent strains. To establish a basis for the biological control of chestnut blight in Turkey, vc types of *C. parasitica* were widely investigated and the EU-1 and EU-12 vc types were found to be the most widespread, the former being common in the Marmara and Black Sea regions, and the latter in the Aegean region (Çeliker and Onoğur, 2001; Gürer et al., 2001a, 2001b; Erincik et al., 2008; Akilli, 2009). In order to implement the biological control of chestnut blight in Turkey, a project funded by the Food and Agriculture Organization of the United Nations (FAO) was started at 3 chestnut sites; 2 in the Aegean region (1 in İzmir, and 1 in Kütahya) and 1 in the Black Sea region (in Sinop) (FAO, 2014). At these 3 sites, active cankers were selected, *C. parasitica* was isolated, and their vc types were determined. The local virulent isolates of these sites were converted to hypovirulence by compatible hypovirulent isolates of EU-1 and EU-12, and these local hypovirulent isolates were used for the biological control treatments.

The objective of this study was to determine the success of the applied biological control at the three study sites. For this the cankers were sampled before the biological control treatments and again 5 and 11 months after the treatment. Molecular markers based on single nucleotide polymorphisms (SNPs) were then used to verify the establishment of the biological control hypovirus in the treated cankers.

2. Material and methods

2.1. Fungal isolates

Fungal isolates were sampled at the 3 study sites at 3 different time points. Cankers were first sampled prior to the biological control treatments in April 2013. Isolates obtained from this first sampling were indicated as original (ORIG) isolates. Subsequently, in June 2013, the biocontrol strain Z1 (vc type EU-1) was used to treat cankers of vc type EU-1 and biocontrol strain Z2.1 (M7055) (vc type EU-12) was used to treat cankers of vc type EU-12. To evaluate the success of the biological control, a first reisolation (R1) was made in October 2013. Subsequently, a second reisolation (R2) was made in May 2014 to evaluate the persistence of the applied hypovirus in the treated cankers. Agar plugs of successfully isolated *C. parasitica* cultures from the 3 samplings were sent to the Swiss Federal Institute for Forest, Snow, and Landscape Research (WSL) for assessments of their culture morphology, vc types, and identification and characterization of the CHV. In total, 255 *C. parasitica* isolates were successfully recovered and analyzed (Table 1).

2.2. Determination of the vc types

The vc type was determined by pairing all of the isolates on potato dextrose agar (PDA; Difco™) plates against the vc type testers EU-1 and EU-12 (Milgroom and Cortesi, 1999). Six pairings per plate were performed, as described by Bissegger et al. (1997). After incubation, the pairings were evaluated based on the presence or absence of a barrage line between the isolate and the tester strain. If the 2 cultures merged with no barrage line visible, they were considered compatible (i.e. of the same vc type; see Figure 1, to the left). If a barrage line formed between the 2 cultures, they were considered incompatible (i.e. of different vc type; see Figure 1, to the right).

2.3. Assessment of the culture morphology of *C. parasitica* isolates

Culture morphology of the isolates was assessed as part of the vc type testing. The conditions used for the vc testing

Table 1. Overview of the *Cryphonectria parasitica* isolates analyzed in this study.

Study plot	Original isolates (ORIG)	First reisolation (R1)	Second reisolation (R2)
İzmir	25	18	42
Kütahya	45	5	43
Sinop	46	23	8
Total	116	46	93

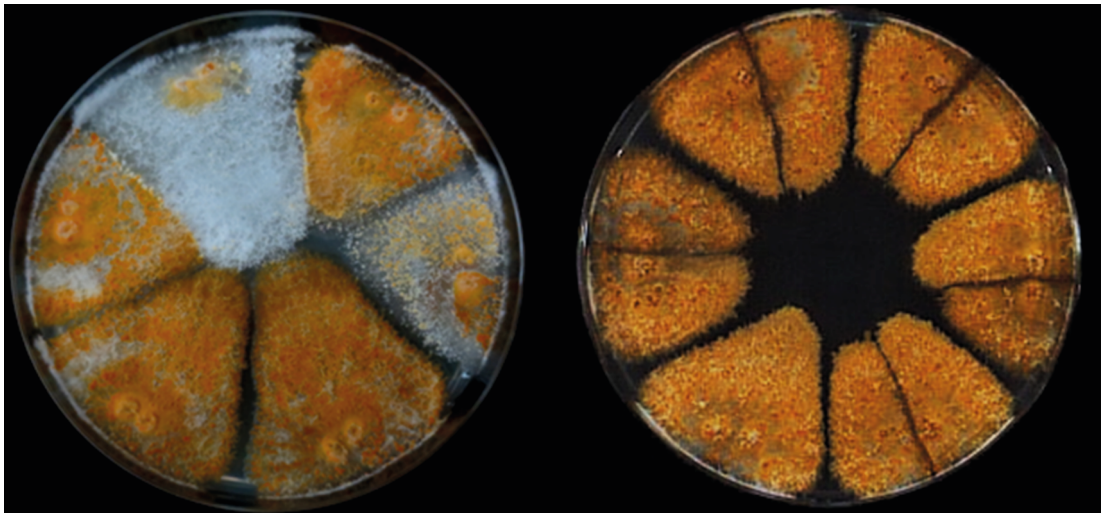

Figure 1. Example of vc type testing plates. No barrage zones were formed between the tester strains and the strains of interest in the plate to the left. Therefore, they were all considered as compatible (i.e. of the same vc type). If a barrage line formed between the 2 cultures as shown in 5 out of the 6 tests in the plate to the right, they were considered as incompatible (i.e. of a different vc type). Transmission of the hypovirus to the tester strain was indicated by the conversion of the tester strain from an orange to a white morphology, as is shown in the picture to the left.

allowed us to distinguish between the orange and white *C. parasitica* isolates. Because the hypovirus suppresses pigmentation and sporulation in *C. parasitica,* hypovirus-infected isolates can be identified on PDA plates by their whitish culture morphology (Figure 1). Under the same conditions, virus-free isolates (including the vc type tester strains) produce a strong orange pigmentation and abundant asexual sporulation. In addition, transmission of the hypovirus to a compatible tester strain can be observed as indicated by the conversion of the tester strain from an orange to white culture morphology.

2.4. Characterization of the hypoviruses by sequence analysis

2.4.1. RNA extraction and cDNA synthesis

The white, presumably hypovirus-infected, *C. parasitica* isolates and the ambiguous ones were used for the molecular analysis. In addition, a few orange, presumably hypovirus-free, *C. parasitica* isolates were included in the analysis as a control. The mycelia used for RNA extraction were obtained after growth of the isolates on a PDA plate covered with a cellophane sheet (Celloclair Inc., Liestal, Switzerland). The mycelia were lyophilized and ground into fine powder in a mixer mill (Retsch® MM 300; Retsch GmbH, Haan, Germany). The total RNA was extracted from approximately 20 mg of mycelial powder using the Norgen Plant/Fungi RNA Purification Kit (Cat. 25800; Norgen Biotek, Thorold, ON, Canada,). The Maxima First Strand cDNA Synthesis Kit with random hexamer primers (Cat. K1641; Thermo Scientific, Waltham, MA, USA) was used to synthesize the complementary DNA (cDNA) from the total RNA.

2.4.2. PCR and sequencing

A region within the ORF A of the CHV-1 genome was analyzed by sequencing. This region of 695 bp in length corresponded to positions 1471–2165 in the nucleotide sequence of CHV-1/Euro7 (Table 2; Chen and Nuss, 1999) and was sequenced essentially as described by Bryner et al. (2012). For the polymerase chain reaction (PCR), 1 µL of the cDNA solution was mixed with 19 µL of the master mix, consisting of 5 µL Jump Start™ REDTaq® DNA polymerase (Sigma-Aldrich®, St. Louis, Missouri, USA), 0.5 µL forward primer (hvep 1; 20 pmol/µL, Table 2), 0.5 µL reverse primer (EP 721-4; 20 pmol/µL, Table 2), and 13 µL of Millipore water (Merck, Burlington, MA, USA). In each PCR series, a CHV-1-positive control and a negative nontemplate water control was included. Agarose gel electrophoresis was used to analyze the PCR products and verify the presence of the hypovirus.

For the Sanger sequencing, the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Cat. 4337455; Applied Biosystems®, Foster City, CA, USA) was used. The sequencing reactions were performed in both directions using the same primers, as for PCR (Table 2). The cycle sequencing products were analyzed on the ABI 3130 Genetic Analyzer (Applied Biosystems®).

2.4.3. Sequence analysis

The assembled sequences were checked for ambiguous nucleotides, aligned, and phylogenetically analyzed using the CLC Main Workbench v.7.0.2 (CLC Bio, Aarhus, Denmark). The International Union of Pure and Applied Chemistry (IUPAC) ambiguity code for nucleotides was

used for the double-peaks (i.e. Y = C or T; R = A or G; M = A or C; and K = G or T). For the phylogenetic analysis, sequences were trimmed to equal length (601 bp) as the reference sequence of CHV-1/Z1, which corresponded to positions 1526–2126 in the nucleotide sequence of CHV-1/Euro7 (Chen and Nuss, 1999). Each phylogenetic tree was created using the Kimura 80 model (Kimura, 1980) and the unweighted pair group method (UPGMA). Branching order stability was estimated by a bootstrap analysis of 1000 replicates. Phylogenetic trees are shown as rooted phylograms. For the phylogenetic analysis of the resident Sinop hypoviruses before the biological control, reference sequences from the NCBI database were included (Gene bank accession numbers: CHV-1/Euro7: AF082191; CHV-1/EP721: DQ861913; CHV-1/EP713: M57938; CHV-1/2103: JF795813; CHV-1/M1372: JF795847; and CHV-1/TR-84: not yet uploaded).

3. Results

3.1. Original populations

In order to determine the occurrence of the vc types and CHV-1 at the 3 study sites (İzmir, Kütahya, and Sinop), the ORIG *C. parasitica* populations were characterized prior to the biological control with hypovirulence. Most of the original isolates were of orange morphology and were not infected by CHV-1 (Table 3).

All of the *C. parasitica* isolates from İzmir were hypovirus-free and of vc type EU-12. In Kütahya, all of the isolates were also hypovirus-free. In this population, vc types EU-1 and EU-12 were dominant. Of the isolates, 4 did not belong to 1 of these 2 vc types and pairing tests with 8 isolates showed unclear results. In Sinop, all but 1 of the *C. parasitica* isolates were vc type EU-1. Isolate SIN-55/ORIG could not be assigned, neither to EU-1 nor to EU-12. Among the original isolates from Sinop, 24 were hypovirus-infected (Table 3). Local strains of CHV-1 differed by at least 3 SNPs from the strain CHV-1/Z1, which subsequently was used for the biological treatment of cankers in this plot.

3.2. First reisolation

At the R1, 5 months after treatment, 46 *C. parasitica* isolates were obtained and characterized**. A**mong them, 42 showed a white culture morphology and 4 had an orange one. From the white isolates, 40 hypoviruses were sequenced (Table 4).

In İzmir, the biocontrol strain Z1 was found in the only EU-1 canker that was treated. In all of the other reisolates, the hypovirus strain Z2.1 (M7055) used for the treatment of cankers with vc type EU-12 was found. Of the 5 reisolates obtained in Kütahya, 4 were white and found to be infected by the released biocontrol strain. In Sinop, many CHV-1 sequences represented hypovirus strains naturally occurring in this area. They were found along with the biological control strain Z1 in the treated cankers. Depending on the canker location, either the natural or the biocontrol CHV-1 strain was recovered. In total, 11

Table 2. Primers used for the PCR and cycle sequencing.

Primers	Map coordinates[1]	Primer sequence (5'-3')	References
hvep 1	1451–1470	TGACACGGAAGCTGAGTGTC	Gobbin et al. (2003)
EP 721-4	2166–2184	GGAAGTCGGACATGCCCTG	Bryner et al. (2012)

[1]Nucleotide map coordinates of the primers correspond to positions in the nucleotide sequence of CHV-1/Euro7 (Chen and Nuss, 1999).

Table 3. Characterization of the *Cryphonectria parasitica* isolates from the 3 study plots prior to the biological control treatments (ORIG).

Population	No. of isolates	Morphology			No. of CHV[1]	vc types[2]		
		Orange	Intermediate	White		EU-1	EU-12	Others
İzmir	25	25	0	0	0	0	25	0
Kütahya	45	45	0	0	0	27	6	4 (8)
Sinop	46	23	1	22	24	45	0	1
Total	116	93	1	22	24	72	31	13

[1]Number of CHV-1 strains analyzed.
[2]vc types; number of isolates with unclear results in brackets.

sequences were identical to Z1 and clearly represented the applied biocontrol strain (i.e. SIN-74/R1, SIN-35/R1, SIN-39/R1, SIN-52/R1, SIN-59/R1, SIN-67/R1, SIN-68/R1, SIN-69/R1, SIN-72/R1, SIN-77/R1, and SIN-78/R1). The naturally occurring CHV-1 strains differed by 3-10 SNPs from biocontrol strain Z1 (Figure 2).

3.3. Second reisolation

At 11 months after treatment, 93 *C. parasitica* isolates were successfully recovered from the treated cankers. CHV-1 sequences could be obtained from 63 isolates. No PCR product was obtained for the white isolate, IZ-24/R2, or the intermediary isolates, KUET-6/R2i and KUET-9/R2.

Table 4. Characterization of the *Cryphonectria parasitica* isolates recovered from the treated cankers at the R1 (5 months after the biological control treatments).

Population	No. of isolates	Morphology			No. of CHV[1]	vc types[2]		
		Orange	Intermediate	White		EU-1	EU-12	Others
İzmir	18	2	0	16	15	1	17	0
Kütahya	5	1	0	4	4	3	2	0
Sinop	23	1	0	22	21	23	0	0
Total	46	4	0	42	40	27	19	0

[1]Number of CHV-1 strains analyzed.
[2]vc types.

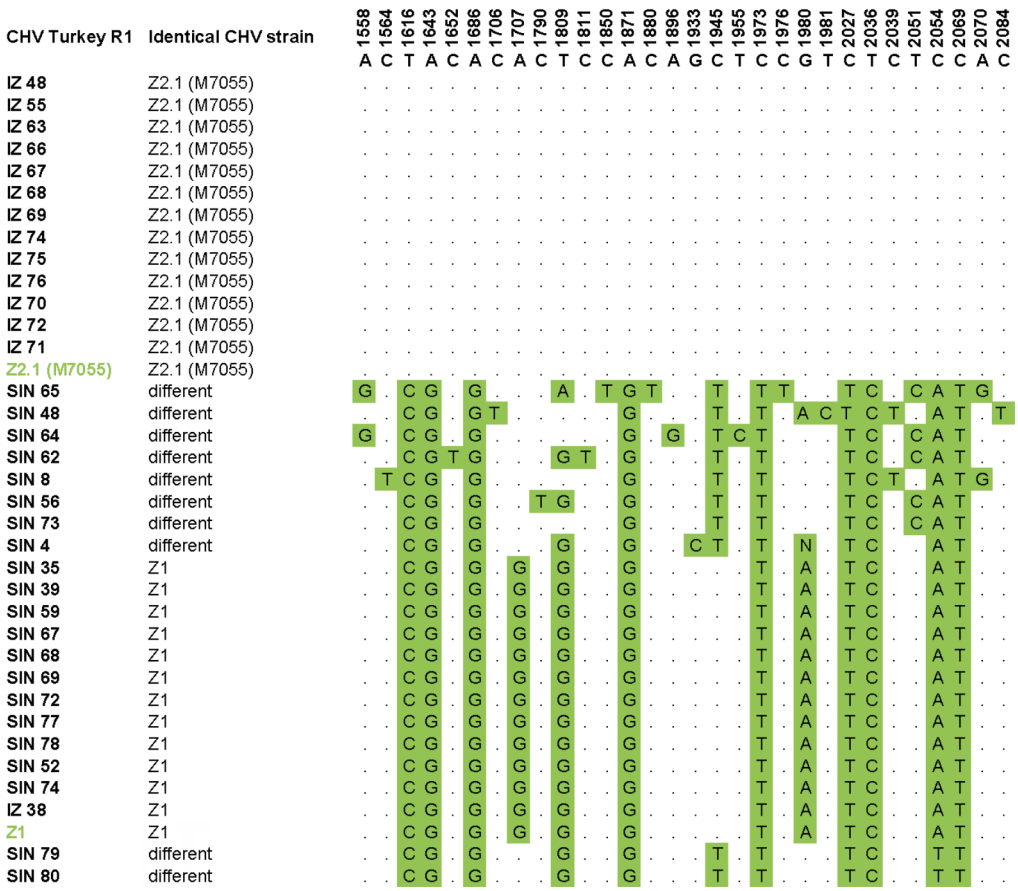

Figure 2. Comparison of SNPs of the CHV-1 sequences found in İzmir (IZ) and Sinop (SIN) in the R1. The applied biocontrol strains were Z2.1 (M7055) and Z1 (indicated in green). The numbers on the top refer to the corresponding nucleotide positions of the reference sequence CHV-1/Euro7 (Chen and Nuss, 1999). Only SNPs within the positions 1526–2126 (601 bp) are shown.

Furthermore, the hypovirus of the white *C. parasitica* isolate, IZ-1/R2, was not sequenced. An overview of the characterization of these isolates is given in Table 5.

In İzmir, only CHV-1 sequences identical to the biocontrol strain, Z2.1 (M7055), were obtained. Both biocontrol strains, Z1 and Z2.1 (M7055), were found in Kütahya. Of the CHV-1 sequences, 6 differed by 1 SNP from the released biocontrol strain, Z1, whereas the remaining sequences were identical to either Z1 or Z2.1. However, in 3 cases (KUET-17/R2, KUET-18/R2, and KUET-52/R2), the sequence did not match the CHV-1 biocontrol strain, which was used to treat the specific cankers. Unexpectedly, the biocontrol strain of the alternate vc type was identified. Of the sequences, 3 (KUET-44b/R2, KUET-3/R2, and KUET-40/R2) showed several double peaks, resulting in ambiguous nucleotides according to the IUPAC ambiguity code (Figure 3). A close inspection of these double peaks at all of the characteristic SNP sites revealed that isolate KUET-44b/R2 was most likely infected with both of the released CHV-1 biocontrol strains (Figure 3). The SNPs of the other 2 sequences apparently represented single mutations in the applied biocontrol strain. Kütahya was the only population where *C. parasitica* cultures with intermediate morphology were found. After the molecular analysis, 2 of these cultures turned out to be virus-free and 3 (KUET-14/R1, KUET-44a/R2, and KUET-44b/R2) were virus-infected. In 3 cankers (KUET-33, KUET-39, and KUET-41), a different vc type was found when compared to the original isolates from the same cankers. This could be due to multiple infections in a single canker by different vc types of *C. parasitica*. With the exception of isolates SIN-57/R2 and SIN-X/R2, the CHV-1 strains obtained from the treated cankers in Sinop were identical to the biocontrol strain previously applied (Z1). Unexpectedly, the strain found in the *C. parasitica* isolate, SIN-57/R2, matched the biocontrol strain, Z2.1 (M7055). This could be due to a mix-up of isolates or contamination. To resolve this ambiguity, canker SIN-57 would need to be reisolated and the identity of the CHV-1 strain double-checked. The hypovirus found in isolate SIN-X/R2 most likely was of natural origin, as it differed by 5 SNPs from Z1 (Figure 3).

3.4. Phylogenetic analysis of the original CHV-1 strains

The phylogenetic relationship of the CHV-1 strains obtained from the original *C. parasitica* isolates in Sinop was investigated. For this, strains Z1 and Z2.1 (M7055), which subsequently were used for our biocontrol study, together with the reference sequences of the CHV-1 subtypes present in Europe (indicated in green) were included in this analysis (Figure 4). All of the sequences from Sinop and the 2 biocontrol strains clearly belonged to the cluster of the Italian CHV-1 subtype (subtype I).

3.5. Phylogenetic analysis of first reisolates

All of the CHV-1 sequences obtained from the hypovirus-infected *C. parasitica* cultures of vc type EU-12 were identical to the biocontrol strain, Z2.1 (M7055). On the other hand, hypovirus-infected *C. parasitica* cultures of vc type EU-1 contained strains identical to the biocontrol strain, Z1, or those that differed at 3 or more nucleotide positions from Z1 (Figure 2). In the latter case, these strains represented the local natural hypovirus population.

3.6. Phylogenetic analysis of second reisolates

Almost all of the CHV-1 strains formed the R2 clustered with either 1 of the 2 released biocontrol strains (Figure 5). These strains were either identical to 1 of the biocontrol strains or differed by only 1 SNP (Figure 3). Only 1 strain from Sinop (SIN-X/R2) was clearly different from the 2 biocontrol strains, and most likely originated from the local CHV-1 population. Because of its mixed sequence, resulting from an infection with both biocontrol strains, strain KUET-44b/R2 was not included in the phylogenetic analysis (Figure 5).

4. Discussion

Cryphonectria parasitica is the pathogenic fungus responsible for the devastating disease of chestnuts

Table 5. Characterization of the *Cryphonectria parasitica* isolates recovered from the treated cankers at the R2 (11 months after the biological control treatments).

Population	No. of isolates	Morphology			No. of CHV[1]	vc types[2]		
		Orange	Intermediate	White		EU-1	EU-12	Others
İzmir	42	10	0	32	28	0	42	0
Kütahya	43	12	5	26	29	30	12	1
Sinop	8	2	0	6	6	8	0	0
Total	93	24	5	64	63	38	54	1

[1]Number of CHV-1 strains analyzed.
[2]vc types.

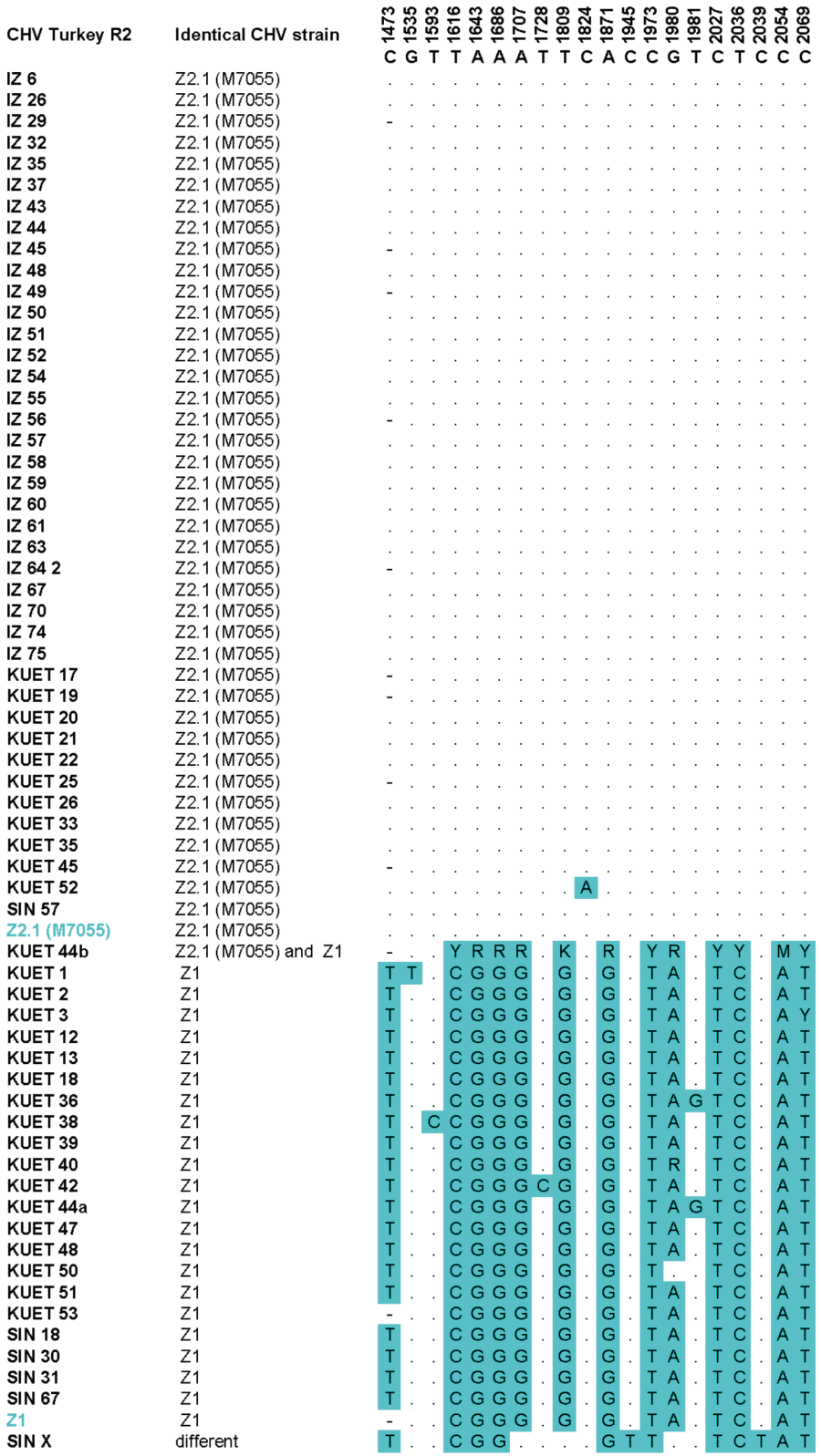

Figure 3. Comparison of SNPs of the CHV-1 sequences found in İzmir (IZ), Kütahya (KUET), and Sinop (SIN) in the R2. The applied biocontrol strains were Z2.1 (M7055) and Z1 (indicated in blue). The numbers on the top refer to the corresponding nucleotide positions of the reference sequence CHV-1/Euro7 (Chen and Nuss, 1999).

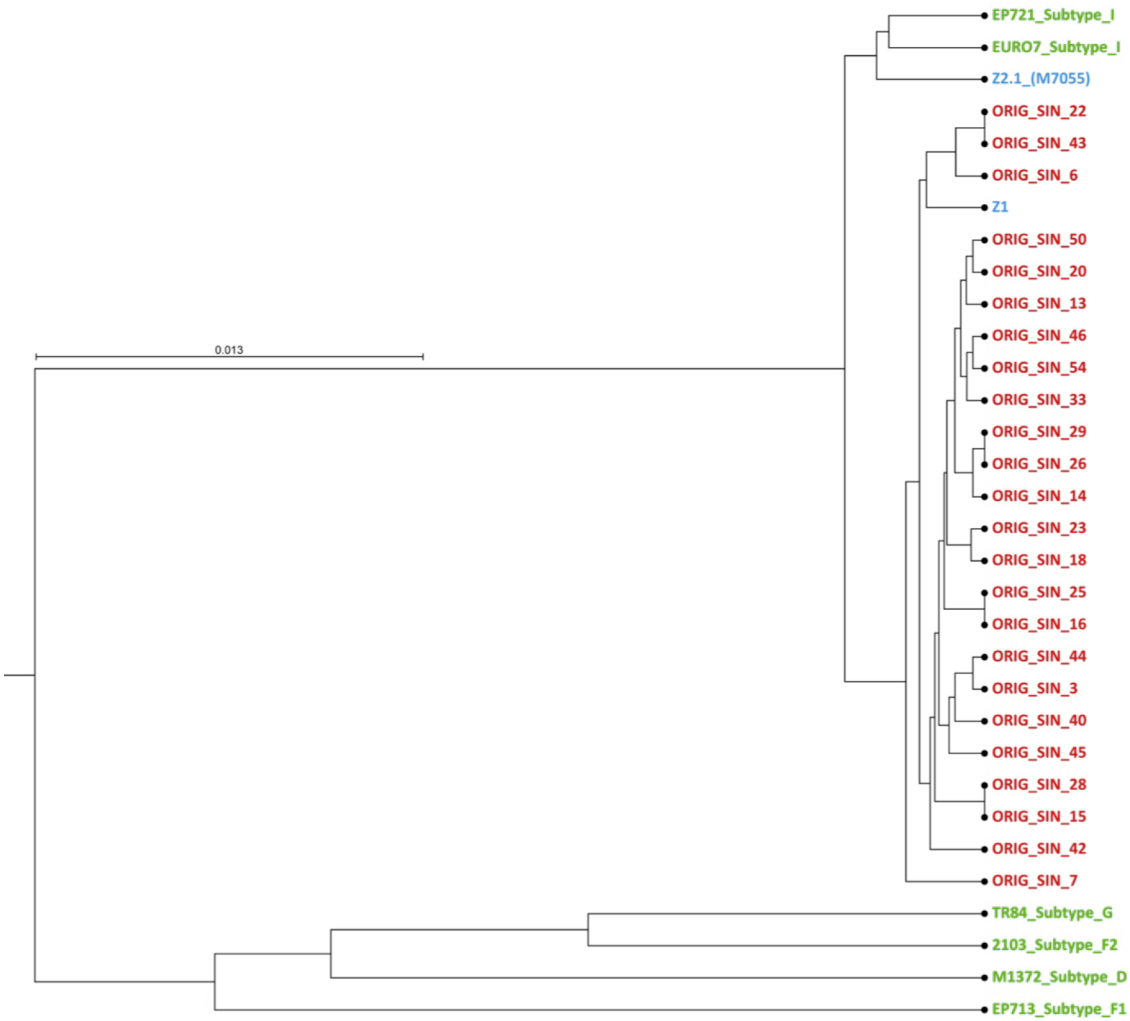

Figure 4. Phylogenetic tree of the CHV-1 strains found before the biocontrol treatments in Sinop (SIN), including the CHV biocontrol strains (in blue), Z1 and Z2.1 (M7055), and 6 CHV-1 subtype reference sequences (in green: EP721/subtype I, Euro7/subtype I, TR-84/subtype G, 2103/subtype F2, M1372/subtype D, and EP713/subtype F1; Gobbin et al., 2003; Akilli et al., 2012). The rooted phylogram was based on the unweighted pair group method with arithmetic means (UPGMA) of a 601 bp region of ORF A. The bootstrap consensus tree was inferred from 1000 replicates. Branch lengths were drawn to scale with a scale bar indicating the number of nucleotide substitutions per site.

(*Castanea* spp.), known as chestnut blight (Rigling and Prospero, 2018). Infection by this necrotrophic pathogen leads to the formation of bark cankers, which cause the wilting and dieback of chestnut trees. In Europe, however, the disease may be successfully controlled by so-called hypovirulence. This phenomenon is caused by a hyperparasitic hypovirus (CHV-1) that infects *C. parasitica*, thereby acting as a biological control agent against chestnut blight. Natural dissemination and active biological control applications have led to a high prevalence of the hypovirus and to the recovery of many chestnut stands in Europe (Rigling and Prospero, 2018).

In Turkey, chestnut blight was first observed in the 1960s and hypovirulence is now present in chestnut stands in the Black Sea and Marmara regions (Akilli et al., 2013). By contrast, in the Aegean region of Turkey, only the virulent form of the pathogen has been found to date. For this reason, biological control treatments were conducted in 2 chestnut sites in the Aegean region (İzmir and Kütahya) and for comparison, in 1 site in the Black Sea region (Sinop). For the canker treatments, 2 specific CHV-1 strains contained in *C. parasitica* isolates of locally dominant vc types EU-1 and EU-12 were used. Resampling of the treated cankers, at 5 and 11 months after treatment, and the subsequent analysis of the *C. parasitica* reisolates showed that the applied biocontrol strains were still present at all 3 sites. This result was in line with previous studies in Europe (Hoegger et al., 2003; Perlerou and Diamandis,

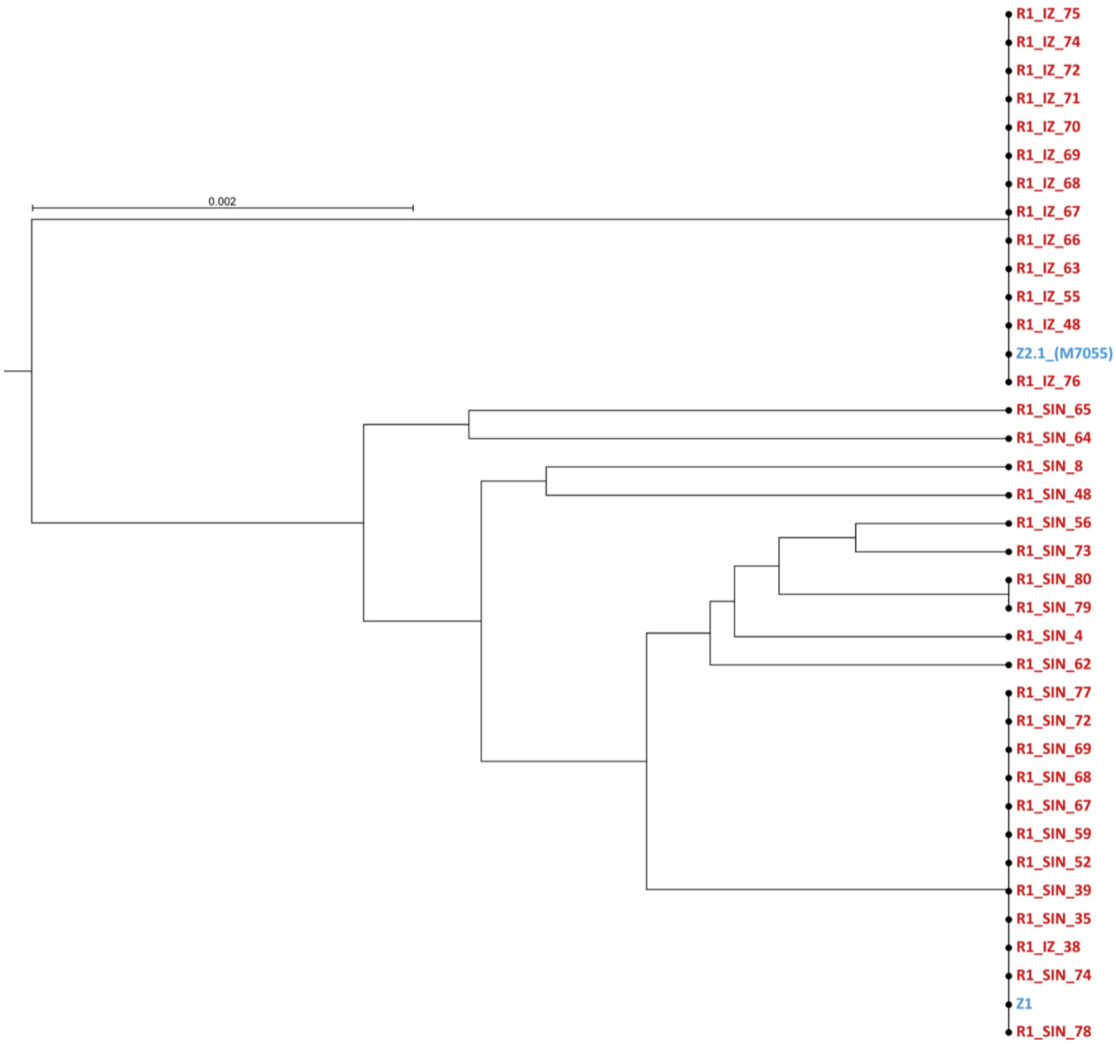

Figure 5. Phylogenetic tree of the CHV-1 strains found in İzmir (IZ), Kütahya (KUET), and Sinop (SIN) in the R1, including the biocontrol strains Z2.1 (M7055) and Z1. The rooted phylogram was based on the unweighted pair group method with arithmetic means (UPGMA) of a 601 bp region of ORF A. The bootstrap consensus tree was inferred from 1000 replicates. Branch lengths were drawn to scale with a scale bar indicating the number of nucleotide substitutions per site.

2010; Prospero and Rigling, 2016), showing that applied biological control strains of CHV-1 were able to become established in the treated cankers. In addition, our study demonstrated that the CHV-1 biocontrol strains could persist in treated cankers under the climatic conditions of the Aegean region. This was particularly relevant for this area, which has thus far experienced a lack of natural hypovirulence. The reason for the absence of natural hypovirulence in the Aegean region is unknown. Possibly, only the virulent form was accidently introduced into this region, without further introductions from areas with natural hypovirulence. Alternatively, some unknown environmental factors (e.g., climatic conditions) might have prevented the establishment of hypovirulence. For example, Bryner & Rigling (2011) showed that temperature could have a significant effect on *C. parasitica* hypovirus interactions. Our study has now shown that climatic conditions in the Aegean region were most likely not crucial for canker treatments, as the hypovirus persisted for at least 1 year in the treated cankers. Such therapeutic canker treatments are often the best choice for controlling existing cankers in high-value chestnut orchards, such as those present in the Aegean region of Turkey (Prospero and Rigling, 2013).

Recently, Çeliker et al. (2017) tested the efficacy and spread of hypovirulence in a chestnut stand near Turgutlu, Manisa, where vc type EU-1 was dominant. Ten years after a hypovirulence application, they observed healing cankers on 47 noninoculated trees, and when combined with dsRNA extractions, concluded that hypovirulence

had naturally spread at the study site. To what extent the applied hypovirulence could spread to other areas in the Aegean region remains to be seen. With respect to vegetative incompatibility barriers, the perspective for spread of hypovirulence is good because vc type diversity is low in the Aegean region. However, vc type diversity could increase in the future through sexual recombination between the dominant vc types in Turkey, EU-1 and EU-12. This probably already happened in Kütahya, where EU-1 and EU-12 were present and new vc types were found in our study.

At the 2 study sites, in İzmir and Kütahya, no hypovirus-infected isolates were found prior the biological control treatments and among the reisolates from the treated cankers, only the applied biocontrol strains were detected. In contrast, in Sinop, hypovirus-infected isolates were found before the treatments and not all of the reisolated CHV-1 strains corresponded to the strain used for biocontrol. Thus, our study confirmed that in İzmir and Kütahya no natural hypovirulence occurred, whereas in Sinop, hypovirulence was already present before the treatments. Canker treatments in areas where natural hypovirulence is already present could promote the recovery of chestnut stands from chestnut blight. In France, for example, canker treatments have been widely applied in areas where natural hypovirulence is present (Robin et al., 2000). However, the impact of such treatments may have been overestimated, as many treated cankers become infected by naturally occurring hypoviruses, as shown in Sinop and a previous study in France (Robin et al., 2010).

Our study highlights the importance of genetic markers to assess the persistence and spread of the applied CHV-1 strains, which is important for evaluating the success of artificial canker treatments (Prospero and Rigling, 2016). This result, together with the observation that most of the treated cankers showed recovery, is very promising for the biological control of chestnut blight in Turkey.

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