Genetic diversity of subpopulations *of Cryphonectria parasitica* in two chestnut-growing regions in Turkey

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Abstract
A total of 134 *Cryphonectria parasitica* isolates were sampled from the two most important chestnut-growing areas of Turkey. Isolates were screened for vegetative compatibility (vc) by pairing them with the 64 EU testers. All isolates belonged to one vegetative compatibility type, which was compatible with the vc type EU-1. Twenty-four randomly selected isolates were screened for mating type, using a PCR-based method. We found isolates of the two mating types, although *MAT-1* isolates prevailed. The skewed ratio of the two mating types is an indicator of the lack or limited sexual reproduction of the fungus in nature. The absence of diversity for vegetative incompatibility types indicates that the introduction of the pathogen has been seldom, and has probably been followed by a strong founder effect. In addition, all isolates were screened for *Cryphonectria* hypovirus 1 (CHV1) infection, visually assessing the colour of the mycelium grown on PDA. Nineteen out of 134 isolates were virus infected. The presence of virus-infected isolates, coupled with the characteristics of the *C. parasitica* population, indicates that there is a great potential in Turkey for successfully controlling chestnut blight biologically.

Keywords: chestnut blight, dsRNA, vc types, genetic diversity, mating type

1 Introduction

Cryphonectria parasitica (Murrill) Barr, the causal agent of chestnut blight, was first reported in Turkey in the 60's (AKDOGAN and ERKAM 1968). After its introduction, the disease caused considerable damage to the native population of the European chestnut (*Castanea sativa* Mill.), particularly in the Marmara Region (DELEN 1975), in spite of the presence of healed and healing cankers. Hypovirulence of *C. parasitica* is due to the presence of double stranded dsRNA (VAN ALFEN *et al.* 1975; HILLMAN *et al.* 1995; ANAGNOSTAKIS 1988). The transmission of dsRNA between isolates of *C. parasitica*, converting the recipient isolate to hypovirulent, forms the basis for the biological control of the disease (VAN ALFEN *et al.* 1975, HEINIGER and RIGLING 1994). However, the efficacy of biological control is hampered by vegetative incompatibility (ANAGNOSTAKIS and DAY 1979, LIU and MILGROOM 1994, CORTESI *et al.* 2001).

In preparation for introducing the biological control of chestnut blight into Turkey, we have analysed the diversity of vegetative compatibility (vc) types, the occurrence of hypovirulence and the distribution of the mating types in subpopulations of *C. parasitica* collected from the two most important chestnut growing areas of Turkey.

2 Materials and methods

Fungal isolates: Bark samples were collected in chestnut forests located in nine geographically separated areas; four in the Marmara region and five in the Black Sea region. In each area, samples were taken in several different chestnut sites. In each site, bark samples were collected from the margin of cankers on at least 10 evenly distributed, blighted stumps. Only one bark sample was collected from each stump to avoid sampling clones.

Individual samples were surface disinfected in 70% ethanol for 10 seconds, followed by 30 seconds in 0.5% sodium hypochlorite and then placed on potato dextrose agar (PDA, 39 g/l potato dextrose agar, Difco, Detroit, Michigan, USA), supplemented with 1 mg l^{-1} biotin and 100 mg l^{-1} methionine. Plates were incubated at 24 °C in the dark for 7 days and then one mycelial plug from each sample was transferred to fresh PDA. A total of 134 isolates of *C. parasitica* were obtained.

Vegetative compatibility: Vegetative compatibility tests were carried out as described by CORTESI *et al.* (1996). The presence of a barrage line of mycelium and dark coloration in the agar were interpreted as evidence of incompatibility. All isolates from each subpopulation were paired with the EU testers (CORTESI and MILGROOM 1998) of the most common vc types found in Europe (CORTESI *et al.* 1998) (ATCC # MYA-1044-1075). To ensure the reliability of the results, each assay was repeated three times.

Hypovirulence: All isolates were grown on PDA at 25 °C in the dark for seven days and then exposed to daylight for seven days. After incubation, isolates were scored for the white phenotype of mycelium and absence, or with a few large pycnidia, as an indicator of infection of *Cryphonectria* hypovirus 1 (CHV-1) (HILLMAN *et al.* 2000).

Extraction and detection of dsRNA: A sample of 10 randomly selected *C. parasitica* isolates that were considered hypovirulent on account of their mycelium phenotype were screened for the presence of dsRNA. The extraction of nucleic acids from fungal mycelium and the purification of the dsRNA fraction by CF11-cellulose (Whatman, Maidstone, Kent, England) were performed essentially according to the method of MORRIS and DODDS (1979). DsRNA was visualized in agarose gel stained with ethidium bromide. Two CHV-1 infected isolates, E13, isolated in 1976 from Valesone (Domodossola, Italy) (BISIACH *et al.* 1988) and HTE9, isolated in 1994 from Teano (Caserta, Italy) (CORTESI *et al.* 1996) and one virus-free ascospore isolate P2-4 (ATCC # MYA-1045) (CORTESI and MILGROOM 1998) were used as positive and negative controls.

Mating types: Twenty-four randomly selected isolates were analysed for their mating types (*MAT-1, MAT-2*) using molecular techniques. DNA extraction from fungal mycelium was performed as described by MILGROOM *et al.* (1992), and purified DNA was amplified by nested PCR according to MARRA and MILGROOM (1999) using sets of primers kindly provided by Dr. Milgroom.

3 Results

All the 134 isolates belonged to the same vegetative compatibility type, which was compatible with EU-1 vc type (ATCC # MYA-1044). The two mating types were found in a subsample of 24 randomly selected isolates. However, isolates with the *MAT-1* allele were more common (20 isolates) than those with the *MAT-2* allele (four isolates). Among all 134 isolates, 19 were considered morphologically hypovirulent. Eighteen hypovirulent isolates were found in three out of four areas in the Marmara region and one was found in the Black Sea region (Table 1). The presence of dsRNA was confirmed for all the 10 isolates screened.

Table 1. Characteristics of two subpopulations of *Cryphonectria parasitica* in Turkey.

	Marmara region	Black Sea region
Areas sampled	4	5
Isolates	89	45
Isolates tested for mating type	15	9
Mating types ratio *(MAT-1:MAT-2)*	14:1	6:3
Hypovirus-infected isolates	18	1

4 Discussion

Our results demonstrated the absence of diversity for vc types in a population of *C. parasitica* composed of 134 isolates from the Marmara and the Black Sea regions in Turkey. In fact, all the isolates belonged to the vc type, EU-1. A previous study carried out with a larger popu-

lation of the fungus (324 isolates) collected in the Marmara, the Black Sea and the Aegean regions, revealed that 96% of isolates were in the vc type, EU-1, and 4% were in the vc type, EU-12 (CELIKER and ONOGUR 2000). Nevertheless, these results are in agreement with our findings, because isolates in the vc type, EU-12, were restricted to the Aegean region. The absence or low diversity of vc types indicates that the pathogen has seldom been introduced into Turkey, but that where it has been introduced it is followed by a strong founder effect of the immigrants.

Cryphonectria parasitica has a bipolar, self-incompatibility mating system with two alleles at a single *MAT* locus (MARRA and MILGROOM 2001). The fungus in nature outcrosses (MILGROOM *et al.* 1993), and sexual reproduction in populations where *vic* loci are polymorphic could result in an increase in the number of vc types (MILGROOM and CORTESI 1999). The population of *C. parasitica* in Turkey has the potential to outcross as shown by the presence of isolates of the two mating types. However, this does not necessarily mean that the fungus reproduces sexually in nature. In fact, we have observed a ratio of isolates of the two mating types skewed from 1:1, which provides evidence of the absence or limited sexual reproduction of the fungus. In Italy, *C. parasitica* reproduces mainly asexually, despite the widespread presence of perithecia in the field, probably because environmental factors hamper the survival of the ascospore progenies (MILGROOM and CORTESI 1999). It is possible that in Turkey the fungus reproduces mainly asexually as well. Finding perithecia in the field could clarify its reproductive biology.

The population of *C. parasitica* in Turkey is subdivided: the Marmara-Black Sea subpopulation, characterized by the absence of diversity for vc type, and the Aegean subpopulation with low diversity (this study; ÇELIKER and ONOĞUR 2000). The occurrence of sexual reproduction will not determine any increase in the genetic diversity of vc types in the Marmara-Black Sea subpopulation, whereas the risk of increase in the diversity of vc types is high for the Aegean subpopulation. In the Aegean subpopulation there are four polymorphic *vic* loci. Therefore, crosses between isolates in vc types EU-1 (ATCC # MYA-1044) and EU-12 (ATCC # MYA-1055) could generate offspring with 14 new vc types. Therefore, we recommend the population should be continuously monitored to detect any increase in vc type diversity.

Isolates infected with CHV-1 hypoviruses were found in Turkey in a range from 4.3% (ÇELIKER and ONOĞUR 2000) to 7.5% (this study). Almost all hypovirulent isolates were found in three out of four areas in the Marmara region, whereas in the Black Sea region hypovirulence is extremely limited. It is known that the lack of diversity of vc types between individuals always results in virus transmission (CORTESI *et al.* 2001). Therefore, it is likely that the limited percentage of hypovirulent isolates in the Black Sea region is due to natural barriers within and between the two regions, which probably hamper the natural spread of hypovirulence. For this reason the biological control of chestnut blight by releasing hypovirulent isolates is highly desirable and will be highly effective.

Absence of diversity of vc types must be preserved by planning biological control carefully. This means: 1) using local hypovirulent isolates with EU-1 vc type and *MAT-1* mating type, 2) in the absence of local hypovirulent isolates, preferring the conversion to hypovirulence of the local virulent isolate rather than using hypovirulent isolates from other regions, 3) prohibiting the introduction of hypovirulent isolates from other countries.

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