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Genetic diversification of the chestnut blight fungus *Cryphonectria parasitica* and its associated hypovirus in Germany

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ABSTRACT

Chestnut blight in south-western Germany was first reported in 1992 and is since expanding in distribution. Here we investigated the invasion history of *Cryphonectria parasitica* and its associated hypovirus. For this, we characterized 284 isolates collected between 1992 and 2012 for hypovirulence, vegetative compatibility (vc), mating type, and microsatellite haplotype. A total of 27 haplotypes and 15 vc types were observed, although the *C. parasitica* population analyzed is currently dominated to 50 % by one haplotype and to 64 % by the vc type EU-2. Structure analysis indicated two divergent genetic pools. Over 66 % of the haplotypes belonged to a pool probably originating from northern Italy. Further diversification is expected due to ongoing sexual recombination, but also to new migration and additional introductions. *Cryphonectria hypovirus* 1 (CHV-1) was found in four of five *C. parasitica* populations from Baden-Württemberg. Genetic analysis of the 35 CHV-1 isolates obtained revealed that they all belong to the German subtype, although they have clearly diverged from the first German hypovirus isolated in 1992. Our study suggests that *C. parasitica* has been introduced into Germany several times from two different gene pools, whereas the hypovirus most probably has a single origin.

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Introduction

Chestnut blight is a classic example of the destructiveness of invasive organisms. The eradication of nearly the whole native chestnut population (*Castanea dentata* (Marsh.) Borkh.) in the north-eastern United States in the early 20th century after the introduction of the fungal pathogen *Cryphonectria parasitica* (Murrill) Barr from Asia can be considered as one of the most drastic silvicultural catastrophes (Anagnostakis 1982; Griffin & Elkins 1986). *Cryphonectria parasitica* causes bark cankers which can lead to dieback of the distal parts after girdling

branches or the tree trunk (Heiniger & Rigling 1994). In Europe, this pathogen was first recorded in 1938 in Italy and successively in almost all European chestnut growing regions (Robin & Heiniger 2001). Molecular analyses revealed the occurrence of at least two introductions into Europe from different source populations (Dutech et al. 2012). In Europe, however, the epidemic proceeded less drastically than in the United States, in part because the European chestnut (*Castanea sativa* Mill.) is slightly less susceptible to chestnut blight than *C. dentata* (Anagnostakis 1987). In addition, *C. parasitica* populations in Europe have become infected with

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Cryphonectria hypovirus 1 (CHV-1), which contributed considerably to the recovery of European chestnut stands (Bryner et al. 2012).

Infection with the RNA virus CHV-1, termed hypovirulence, reduces the aggressiveness (virulence) of *C. parasitica* towards its host (Hillman & Suzuki 2004), so that infected chestnuts may recover after the appearance of hypovirulence. CHV-1 belongs to the genus Hypoviridae (Nuss et al. 2005) and induces several phenotypic changes in the fungus, such as a reduction in asexual sporulation, inhibition of sexual reproduction, altered colony morphology and reduced pigmentation (Nuss 2005). Hypoviruses lack an extracellular phase and can only be transmitted from one fungal strain to another by cytoplasmic mixing after hyphal anastomosis. Spread of CHV-1 within the fungal population therefore relies on the dispersal of virus-containing asexual spores which may also subsequently transmit the hypovirus to other fungal individuals after hyphal fusion (Hoegger et al. 2003; Milgroom & Cortesi 2004). It is assumed that CHV-1 was introduced to Europe from Asia together with its host (Liu et al. 2007) and has subsequently spread throughout Europe following its host fungus with a time lag of about 20–30 y (Bryner et al. 2012; Heiniger & Rigling 1994; Robin & Heiniger 2001). Hypovirulence has now been detected in most European countries (Akili et al. 2013; Bissegger et al. 1997; Bragança et al. 2007; Bryner & Rigling 2012; Krstin et al. 2008, 2011; Montenegro et al. 2008; Perlerou & Diamandis 2006; Robin et al. 2000; Sotirovski et al. 2006; Vidóczy et al. 2007; Wronski et al. 1997; Zamora et al. 2012) and at low incidence also in south-western Germany (Peters et al. 2012). Four genetically distinct subtypes of CHV-1 (subtype I, F1, F2, D/E) were described from Europe (Allemann et al. 1999; Gobbin et al. 2003), which had assumedly diverged before the introduction of the host fungus and must therefore each have been introduced to Europe independently (Gobbin et al. 2003). Differences in ecological fitness of the different subtypes were shown, indicating a superior fitness of the Italian subtype CHV-1/I (Bryner & Rigling 2011; Robin et al. 2010). This would explain its widespread distribution across Italy, Switzerland, south-eastern France, and south-eastern Europe.

The formation and stability of hyphal anastomosis between *C. parasitica* strains are governed by vegetative compatibility (vc), which is determined by at least six vic loci (Cortesi & Milgroom 1998). Hyphal fusions are stable and the hypovirus is easily transmitted between fungal strains of the same vc type, i.e. strains sharing the same alleles at all vic loci. Virus transmission may also occur between strains of different vc types, but generally at lower rates and depending on the nature and number of heteroallelic vic loci (Cortesi et al. 2001; Papazova-Anakieva et al. 2008). The vc type diversity is therefore thought to be a critical factor for the spread of the hypovirus within *C. parasitica* populations (Anagnostakis et al. 1986; Heiniger & Rigling 1994; MacDonald & Fulbright 1991). The diversity of vc types can increase due to the recombination of polymorphic vic loci during sexual reproduction (Cortesi & Milgroom 1998). As is typical for ascomycetes, sexual reproduction is controlled by two mating type alleles (MAT-1 and MAT-2) in *C. parasitica* (Marra & Milgroom 1999) and can only take place between two strains differing in mating type.

Hypovirus-infected ascospores have never been found, suggesting that sexual reproduction only contributes to dissemination of the virulent form of *C. parasitica* (Carbone et al. 2004; Prospero et al. 2006).

The native *C. parasitica* population in Asia is genetically very diverse compared to populations in North America and Europe (Liu & Milgroom 2007). However, a high variation in diversity is observed in some European populations. Established *C. parasitica* populations are generally more diverse (Cortesi et al. 1998; Dutech et al. 2010; Ježić et al. 2012; Robin et al. 2009) than populations at the expanding front of the disease (Hoegger et al. 2000; Milgroom et al. 2008). This situation has been depicted by typing collected European isolates either at the vic loci (e.g. Bragança et al. 2007; Montenegro et al. 2008; Sotirovski et al. 2004) or at neutral microsatellite loci (e.g. Dutech et al. 2010, 2012; Ježić et al. 2012; Milgroom et al. 2008; Prospero & Rigling 2012). Although vc type tests are easy to perform, vic loci may not be selectively neutral (Biella et al. 2002; Milgroom & Cortesi 1999) and some of them are still not genetically characterized (Robin et al. 2000). For this reason, neutral molecular markers have been used increasingly to study population diversity of *C. parasitica*.

In Germany, chestnut blight was officially discovered in 1992 (Seemann & Unger 1993), although dendrochronological data suggest that its presence dates back to at least 1985 (Seemann et al. 2001). Compared to other European countries, the disease can be considered as still recent and in expansion towards the northern distribution limits of *C. sativa*. Accordingly, a previous study revealed a low vc type diversity with only five vc types (EU-2, EU-5, EU-14, EU-28, EU-65) present in forest areas (Peters et al. 2012) and four vc types (EU-1, EU-12, EU-33, EU-X) on single trees in urban areas that were subsequently eradicated (Peters et al. 2012; Seemann et al. 2001). Based on the spatial and temporal distribution of vc types, several different introduction of *C. parasitica* into south-western Germany were assumed. Hypovirulence was only found in Baden-Württemberg at a very low incidence. Due to the lack of vc type diversity, biological control of chestnut blight by artificial introduction of CHV-1 in south-western Germany was considered to be a promising approach (Peters et al. 2012). Since this approach constitutes the introduction of a pathogen, albeit in its attenuated form and to areas already infected with chestnut blight, knowledge of the resident *C. parasitica* population is needed (Milgroom & Cortesi 2004; Turina & Rostagno 2007) to ensure hypovirus transmission and to prevent the diversification of the population due to the introduction. Therefore, the first objective of this study was to characterize the genetic diversity of *C. parasitica* in the distribution areas of *C. sativa* in both Baden-Württemberg and Rhineland-Palatinate. For this purpose, we resampled all known infected chestnut sites and conducted additional samplings in recently detected outbreak sites. Besides considering vc types to assess population diversity, we genotyped the obtained isolates at the microsatellite markers used by (Dutech et al. 2010; Prospero & Rigling 2012). Additionally, the mating type of the isolates was identified to determine the potential for sexual reproduction. The second objective was to determine the distribution and genetic relatedness of CHV-1 in the main study areas. Hypovirulence had been detected previously at very low incidence in the Ortenau district and seemed to be spreading slowly (Peters et al.

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