




A multiplexed genotyping assay to determine vegetative incompatibility and mating type in *Cryphonectria parasitica*

C. Cornejo  · B. Šever · Q. Kupper · S. Prospero · D. Rigling

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Abstract The highly destructive chestnut blight disease can be successfully controlled by infecting the virulent strain of *C. parasitica* with the hyperparasitic mycovirus CHV-1. The artificial application of the virus-induced hypovirulence, however, requires that the vegetative compatibility (vc) diversity in the target *C. parasitica* population be determined beforehand. Conventional vc type determination by pairing the unknown isolate with tester strains of known vc types is time consuming and not always reliable. A genotyping assay was developed that uses DNA from *C. parasitica* isolates and discriminates the six known di-allelic *vic* loci and the two mating idiomorphs in two fluorescence-labeled multiplex PCRs. Validation was conducted by successfully genotyping the 74 known European vc tester strains and a conventionally characterized natural population of *C. parasitica*. Cross-species amplification tests with three *Cryphonectria* species (*C. japonica*, *C. naterciae*, and *C. radicalis*) revealed a high amplification specificity for *C. parasitica*, while the analytical

sensitivity of the assay was established at 20 pg per reaction of genomic DNA. In conclusion, this fluorescent multiplex genotyping assay offers a simple and high-throughput tool for the characterization of the vegetative compatibility and mating type of *C. parasitica* at population level, which is particularly important for the application of mycovirus-mediated biological control of chestnut blight.

Keywords Chestnut blight · Genetic diversity · Diagnostics · Biocontrol · Plant pathogen · Ascomycete

Introduction

Cellular recognition processes play an important role in the sexual and vegetative life cycle of fungi. Sexual reproduction is typically initiated by mate recognition, which—in ascomycete fungi—is controlled by two alleles at a single mating-type locus (Ni et al. 2011). The recognition process between vegetative hyphae is governed by a vegetative incompatibility (*vic*) system that generally involves several *vic* loci (Paoletti 2016). Upon fusion of incompatible hyphae, the reaction between incompatible *vic* gene proteins induces a programmed cell death, thus preventing heterokaryon formation and cytoplasmic exchange. This non-self-recognition process assures the genetic integrity of fungal mycelium and restricts the transmission of deleterious cytoplasmic elements, such as mycoviruses. The fungal *vic* system has practical relevance if cytoplasmically-transmitted mycoviruses are being used

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C. Cornejo (✉) · B. Šever · Q. Kupper · S. Prospero · D. Rigling
Swiss Federal Research Institute WSL, Zuercherstr. 111,
8903 Birmensdorf, Switzerland
e-mail: carolina.cornejo@wsl.ch

B. Šever
Institute of Chemistry and Biotechnology, ZHAW, Gruentalstrasse
14, 8820 Waedenswil, Switzerland

for biological control of fungal pathogens (Ghabrial and Suzuki 2009). Moreover, sexual and vegetative compatibility testing has been widely used to study mode of reproduction and the diversity of fungal populations (e.g. Milgroom and Cortesi 1999; Hoegger et al. 2000; Chang et al. 2014).

Chestnut blight is a highly destructive tree disease involving three trophic levels: the tree host *Castanea* spp., the fungal pathogen *Cryphonectria parasitica* (Murr.) Barr., and hyperparasitic mycoviruses, e.g. *Cryphonectria hypovirus 1* (CHV-1) (Milgroom and Cortesi 2004; Rigling and Prospero 2018). *Cryphonectria parasitica* is native to East Asia where it causes minor damage to the local chestnut species, namely, the Chinese chestnut (*C. mollissima* Blume) and Japanese chestnut (*C. crenata* Siebold and Zucc.). The pathogen was introduced into North America and Europe, leading to severe disease epidemics on the susceptible American (*C. dentata* Marsh) and European (*C. sativa* Mill.) chestnut species (Anagnostakis 1987). Virulent strains of *C. parasitica* typically induce necrotic lesions (so-called cankers) on the bark of susceptible *Castanea* species. Perennial cankers are formed on thicker branches or on the trunk, and may enlarge rapidly, girdling the affected tree part. This leads to the death of stems or branches distal to the infection point. Because of chestnut blight, the American chestnut was almost entirely extinct as an important forest tree species in North America. The disease epidemic in Europe took a milder course, and recovery from chestnut blight has been observed in many chestnut growing areas (Heiniger and Rigling 1994). This recovery has been attributed to a viral disease in *C. parasitica* caused by mycoviruses of the family *Hypoviridae*, in particular CHV-1, that results in hypovirulence (Choi and Nuss 1992). *Cryphonectria parasitica* strains infected by CHV-1 exhibit reduced virulence and sporulation capacity compared to isogenic non-infected strains (Peever et al. 2000; Bryner and Rigling 2012). Hypovirulence turned out to be a particularly promising chestnut blight management system because it enables therapeutic treatments of infected chestnut trees (Choi and Nuss 1992; Heiniger and Rigling 1994).

CHV-1 is located in the cytoplasm of *C. parasitica*, and has no extracellular phase (Nuss 2005). It is transmitted horizontally from an infected to a non-infected fungal mycelium by somatic hyphal fusion (anastomosis) that results in the exchange of cytoplasmic components (Fig. 1). Transmission of CHV-1 via hyphal anastomosis

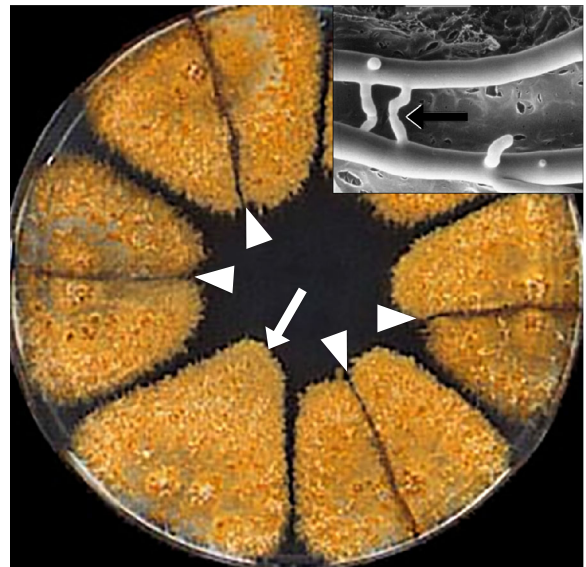


Fig. 1 Classical determination of the vegetative compatibility in *Cryphonectria parasitica*. Target isolates are pairwise co-cultured on potato dextrose agar with European tester strains of known vegetative compatibility types. Compatible isolates will merge into a single culture (white arrow), whereas a barrage line is formed along the contact zone between two incompatible isolates (white arrowheads). The hypovirus is transmitted horizontally from an infected to a non-infected fungal individual by somatic hyphal fusion that results in exchange of cytoplasmic components (fusion of two hyphae; black arrow in upper electron micrograph). Pictures: WSL/phytopathology

is controlled by a vegetative incompatibility (*vic*) system (Cortesi et al. 2001) involving several *vic* genes. To date, six di-allelic *vic* loci are known which define $2^6 = 64$ *vic* genotypes or vegetative compatibility (*vc*) types (Cortesi and Milgroom 1998). The six *vic* loci have recently been characterized at the molecular level, and studies have revealed complex allelic and non-allelic interactions involving polymorphic and idiomorphic genes (Choi et al. 2012; Zhang et al. 2014). *Cryphonectria parasitica* strains are vegetatively compatible if they have the same alleles at all *vic* loci. In this case, anastomoses are readily formed, and CHV-1 is rapidly transmitted between strains. If strains are heteroallelic at one or more *vic* loci, formation of stable anastomoses is prevented, and virus transmission is restricted (Cortesi et al. 2001). As an exception, one *vic* locus (*vic4*) was found not to affect virus transmission (Cortesi et al. 2001). The *vic* system in *C. parasitica* has practical consequences for biological control of chestnut blight using hypovirulence. At first, therapeutic canker treatments are only successful if hypovirus transmission is not blocked by vegetative incompatibility. Moreover, high *vc* type diversity prevents the spread of the hypovirus at the

population level, and is thought to be a main reason why hypovirulence could not become established among North American *C. parasitica* populations (Liu et al. 2000). Therefore, monitoring vc type composition and diversity in *C. parasitica* populations is crucial for successful biological control using hypovirulence.

Since the hypovirus is transmitted into asexual but not into sexual spores, the mode of reproduction of *C. parasitica* is another important factor affecting the spread of CHV-1 in a fungal population. Furthermore, sexual reproduction may negatively impact CHV-1 transmission by increasing vc type diversity through the genetic recombination of polymorphic *vic* loci. *Cryphonectria parasitica* has a single mating type (*MAT*) locus, which contains either the *MAT1-1* or *MAT1-2* idiomorph (Marra and Milgroom 2001). According to McGuire et al. (2004), *MAT1-1* encodes three genes (*MAT1-1-1*, *MAT1-1-2*, and *MAT1-1-3*) and *MAT1-2*, one gene (*MAT1-2-1*). Although *C. parasitica* seems to be a typical self-incompatible heterothallic ascomycete, in natural populations it is capable of selfing (Marra et al. 2004). Most self-fertile isolates are heterokaryotic for the mating type, i.e., they contain nuclei that are isogenic except at the mating type locus. The progeny of a self-fertile isolate segregates in a 1:1 ratio for mating type (McGuire et al. 2004, 2005). Asexual reproduction results in a clonal population structure, typically characterized by the dominance of a single vc type and mating type (Hoegger et al. 2000; Milgroom et al. 2008). Such clonal populations are particularly suitable for hypovirus invasions (Hoegger et al. 2003).

Despite the importance of accurately identifying vc types of *C. parasitica* strains for biological control of chestnut blight (Prospero and Rigling 2013), vc type testing is still a demanding task. First, *C. parasitica* has to be isolated in pure culture from infected bark tissue. Thereafter, isolates need to be co-cultured on a growing medium with European (EU) tester strains of known vegetative compatibility type. Finally, the vc type of the target isolate is determined based on colony morphological features. Compatible isolates will merge into a single culture, whereas a barrage line will be formed at the contact zone between two incompatible isolates (Biella et al. 2002) (Fig. 1). The barrage/merging response is, however, not always easy to assess and, thus, ambiguous vc type assignments are not uncommon. In addition, large series of pairings are needed for populations with high vc type diversity. As an alternative to pairing isolates with tester strains, Short et al. (2015) and Mlinarec et al.

(2017) developed PCR assays, which consist of six duplex reactions based on three different cycling protocols. Even though practical improvements have been achieved in this way, the proposed protocols suffer from limitations, such as the high number of individual PCR reactions and gel electrophoreses needed for each fungal individual. Furthermore, the mating type has to be assessed in two additional PCR reactions visualized by gel electrophoresis (Marra and Milgroom 2001).

The present study aims to develop a high-throughput genotyping assay for *vic* and *MAT* genes of *C. parasitica*. Genotyping has a firm place in forensic, diagnostic, and scientific applications, and is mainly performed by PCR with defined primers (e.g., analysis of simple sequence repeats, SSR) or by next generation sequencing techniques (e.g., analysis of single nucleotide polymorphism, SNP). In our specific case, we developed an assay that should be applicable as a multiplex in a 96-well plate format using fluorescent-labeled markers, which can be detected on a capillary DNA analyzer. To simplify procedures, resulting electropherograms should be scored automatically by a size-calling computer program. Our results demonstrate that genotyping *vic* loci and mating type in multiplex presents significant advantages over the traditional pairing assays or conventional PCR by providing accurate high-throughput results in a short time.

Material and methods

Tester strains and DNA samples

The *vic* genotypes of the vc types EU-1 to EU-64 at the six currently known *vic* loci (*vic1*, *vic2*, *vic3*, *vic4*, *vic6*, and *vic7*) were previously determined by Cortesi and Milgroom (1998). Ten additional vc types (EU-65 to EU-74) are officially recognized (Robin et al. 2000; Peters et al. 2014), although, the genetic basis for their incompatibility with the 64 EU vc types remains unknown. All European vc type tester isolates EU-1 to EU-74 are stored frozen at -80°C in glycerin stocks in the culture collection at WSL.

For primer testing, we selected eight EU tester strains (EU-1, EU-20, EU-33, EU-34, EU-35, EU-42, EU-43, and EU-44) that represent each *vic* allele and both *MAT* idiomorphs four-fold. Strains were grown on Potato Dextrose Agar (PDA; 39 g l^{-1} ; Difco™ Laboratories, Detroit, USA) overlaid with a cellophane sheet (BD Difco, Chemie Brunschwig AG, Basel, Switzerland)

for a period of 7 d at 25 °C in the dark. Thereafter, mycelia were harvested, transferred to 2 ml Eppendorf tubes, and lyophilized overnight. DNA was extracted using the MoBio PowerPlant DNA Isolation Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The DNA concentration was adjusted equimolar (2.0 ng/μl) for all eight samples. Ten additional DNA samples from our Swiss *C. parasitica* collection were selected to sequence the *vic7* locus, as well as DNA stocks of the *C. parasitica* population from Claro ($n = 58$), in Southern Switzerland. The *vc* type of all isolates from this population was previously determined in pairing assays (Hoegger et al. 2000). All DNA samples were stored at -40 °C in our DNA collection. DNA from stocks was diluted 1:10 in molecular grade water (Merck KGaA, Darmstadt, Germany) on a separate plate before using it for PCR amplifications. All specimen vouchers are listed in Table 1.

Sequence data and primer design

Sequence data from the *vic1–vic6* loci (Choi et al. 2012; Zhang et al. 2014), as well as from the *MAT* locus (Marra and Milgroom 2001; McGuire et al. 2004) were used as the basis for developing our genotyping assay. Two *vic7* sequences of the conserved exon region of *vic7* allele-1 are deposited in GenBank (KP743739; KP743840; Short et al. 2015), but introns or allele-2 sequences were not available. To ensure a high primer specificity able to distinguish the two *vic7* alleles, we amplified both *vic7* alleles using primers and protocols developed by Short et al. (2015). Amplicons were then sequenced in both directions at Microsynth AG (Balgach, Switzerland) based on the BigDye Terminator v3.1 cycle sequencing chemistry (Thermo Fisher Scientific corporation, Waltham, Massachusetts, USA).

Primers were designed with the CLC Main Workbench v7 software (QIAGEN) using the following criteria. First, sequences of both alleles per locus were aligned and exons and introns were annotated. Second, regions within conserved exon sequences were targeted to minimize allelic drop-out. The drop-out effect occurs if primer hybridization fails due to sequence polymorphism within the DNA primer-binding sequence, which is more likely to happen in variable intron regions. Third, exon regions with the greatest divergence between both alleles were targeted to increase the primer specificity. Fourth, the amplicon lengths had to differ sufficiently in order to distinguish fluorescence peaks from each another in the

electropherograms within the range of 100 bp to 500 bp. All primer sequences were synthesized by Microsynth AG.

Primer testing

For a more cost-effective primer testing, forward primers were initially labeled with the M13-tag 5'-TGTA AAACGACGGCCAGT-3' according to Schuelke (2000). All PCRs were performed on Veriti Thermal Cyclers (Thermo Fisher Scientific). The optimal annealing temperature was assessed in a temperature gradient increasing by one-degree from 57 to 62 °C under the following conditions: Initial denaturation for 5 min 95 °C; 30 cycles of 30 s 95 °C, 90 s 57–62 °C, and 30 s 72 °C, with a final extension of 10 min 72 °C. The 10 μl PCR mix contained: 1 μl of 2 ng/μl genomic DNA, 200 nM primer per reaction, 3.8 μl water, and 5 μl Type-it Microsatellite PCR kit (QIAGEN). The PCR fragments were verified on 1.5% agarose gels using 1x TBE buffer, 30 min at 80 V/cm for electrophoresis and ethidium bromide (Merck) as the DNA staining dye. From here on, all reactions were set at the optimal temperature of $T_a = 60$ °C. Testing of the newly designed primer pairs followed Schuelke's (2000) protocol, but differed in the duplex reactions that contained both primer pairs for one *vic* locus and, hence, double the quantity of the dye-labeled M13-primer. The PCR mix (10 μl) consisted of 1 μl DNA, each 2.5 μM primer (M13-tailed forward primer: 0.2 μl, reverse primer: 0.8 μl, and dye-labeled M13-primer: 1.6 μl), 0.4 μl water, and 5 μl Type-it. All obtained PCR products were diluted 1:10 in a 1% GeneScan 500 LIZ – Hi-Di formamide mixture (Thermo Fisher Scientific) and sized on the genetic analyzer ABI PRISM 3100 Avant (Thermo Fisher Scientific) using the G5 dye set for fragment analysis.

Primer specificity and sensitivity

The analytical specificity and selectivity of the primer pairs were evaluated (i) *in silico* using the Primer-BLAST option in the NCBI-suite (<https://www.ncbi.nlm.nih.gov>), (ii) testing the assay with three closely related *Cryphonectria* species, i.e., the Asian *C. japonica* (syn. *C. nitschkei*, Liu et al. 2003; three isolates), the European *C. naterciae* (Bragança et al. 2011; two isolates) and *C. radicalis* (Hoegger et al. 2002; three isolates), and (iii) testing the assay with the 74 EU tester strains and 58 isolates from Claro that were

Table 1 List of fungal species used for sequencing of the *vic7* locus, validation test, and cross-species amplification test

Application	Fungal species	Sample ID	Host	Origin	GenBank/Reference
Sequencing <i>vic7</i>	<i>C. parasitica</i>	M5109	<i>C. sativa</i>	Switzerland	MH836570
		M4020	<i>C. sativa</i>	Switzerland	MH836570
		VIN-B147k	<i>C. sativa</i>	Switzerland	MH836570
		M1704	<i>C. sativa</i>	Switzerland	MH836570
		M3967	<i>C. sativa</i>	Switzerland	MH836570
		M2295	<i>C. sativa</i>	Switzerland	MH836570
		M2304	<i>C. sativa</i>	Switzerland	MH836570
		M2334	<i>C. sativa</i>	Switzerland	MH836570
		GAY-B136K2	<i>C. sativa</i>	Switzerland	MH836570
		VIN-B107K	<i>C. sativa</i>	Switzerland	MH836570
Validation test	<i>C. parasitica</i>	EU-1 – EU-64	<i>C. sativa</i> /lab	Italy, Switzerland	Cortesi and Milgroom 1998
		EU-65 – EU-74	<i>C. sativa</i>	France, Switzerland	Robin et al. 2000
		Claro population (<i>n</i> = 58)	<i>C. sativa</i>	Switzerland	Hoegger et al. 2000
Cross-species test	<i>C. japonica</i>	M9249	<i>C. crenata</i>	Japan	M. Milgroom
		M9250	<i>C. crenata</i>	Japan	M. Milgroom
		M9251	<i>C. crenata</i>	Japan	M. Milgroom
	<i>C. naterciae</i>	M3659	<i>Q. suber</i>	Portugal	Bragança et al. 2011
		M3664	<i>Q. suber</i>	Portugal	Bragança et al. 2011
	<i>C. radicalis</i>	M2268	<i>C. sativa</i>	Switzerland	Hoegger et al. 2003
		M2269	<i>C. sativa</i>	Switzerland	Hoegger et al. 2003
		M2270	<i>C. sativa</i>	Switzerland	Hoegger et al. 2003

characterized in a former study using the conventional method (Hoegger et al. 2000). All specimen vouchers are listed in Table 1. Analytical sensitivity of the assay was determined using a 10-fold dilution series of *C. parasitica* DNA from 2.0 ng/μl up to 2.0 E-6 ng/μl concentration. All test assays were performed under the same PCR conditions established in the present study for *C. parasitica*. Previously genotyped controls and a non-template control consisting of DNase-free water were introduced within each array.

Results

Primer design and multiplexing

The alleles *vic7-1* and *vic7-2* were each sequenced successfully from five different isolates from our culture collection. The obtained sequences (GenBank accessions MH836570 – MH836579) were analyzed together with the coding region of *vic7-2* of the *C. parasitica* strain EP155 v2.0 (available from the reference genome

portal at the U.S. Department of Energy Joint Genome Institute, JGI; <https://jgi.doe.gov>) to determine the exon and intron regions in the alignment (Fig. 2). Primers for the *vic7* alleles were designed based on this alignment. Previously published sequences were used to design the primer for the other *vic* loci and for the mating types. Finally, all primer pairs could be placed within exon regions of *vic* and *MAT* genes, except Cp-*vic7-1-R*, which hybridizes for few nucleotides at the 5' end to an intron region. Cp-*vic1a-2* and Cp-*MAT1-2* primers were also placed in the exon regions, but each with an intron between them.

Since multiplex PCR methods can be prone to amplification failure such as primer dimer interactions, for primer multiplexing, we sorted primers in different combinations of six and eight pairs. Afterwards, we screened for potential primer interactions using the Thermo Fisher Scientific multiple primer analyzer tool (<https://www.thermofisher.com>). Finally, we sorted all primer pairs into two multiplex assays that showed little interaction in silico and labeled forward primers with fluorescent dyes (cf. Table 2). Since each primer pair exhibits



Fig. 2 Map of partial sequences of six di-allelic *vic* genes and two *MAT* idiomorphs of *Cryphonectria parasitica* with primer binding positions. Sequences are designated with their GenBank accession numbers. Black lines represent introns, while green bars denote exon regions and red bars primer sequences. Black box:

Alignment of *vic7* sequences. For the *vic7* locus, ten sequences were generated newly (MH836570 – MH836579; black lines) and analyzed together with KP743739, KP743740, and jgilCrypa2|231853| to determine the exon and intron regions in the alignment (green bars)

different binding and amplification kinetics, we optimized the DNA amplification efficiency by varying the amount of each primer pair used in multiplex assays. The optimized PCR protocol uses a total volume of 20 μ l containing 2.5 μ l (multiplex 1) or 1.9 μ l (multiplex 2) of genomic DNA (concentration as extracted and subsequently diluted 10-fold), varying primer quantity (Table 2), 10 μ l Type-it (QIAGEN) and requires the following conditions: Initial denaturation for 5 min at 95 $^{\circ}$ C; followed by 25 cycles of 30 s 95 $^{\circ}$ C, 90 s 60 $^{\circ}$ C, and 30 s 72 $^{\circ}$ C; with a final extension of 45 min 72 $^{\circ}$ C.

Specificity and sensitivity of the genotyping assay

Primer-BLAST in the NCBI-suite compared the primer pairs of the present study with fungal references in GenBank (fungi taxid:4751; April 17, 2018) and revealed that all primer pairs matched all nucleotides for the respective locus of *C. parasitica* only. The primers for the *vic7-2* allele, which was not available in GenBank, were confirmed as not matching any other fungal sequence by Primer-BLAST. All primer pairs

were tested on DNA samples from three *Cryphonectria* species (*C. japonica*, *C. naterciae*, *C. radicalis*). These tests revealed that none of the three species amplified at all possible *vic* loci, varying from no amplification at all in *C. japonica* to one amplicon detected in *C. naterciae* using the Cp-*vic3a-1* markers and two weak signals in *C. radicalis* (*vic2* and *vic4*) (data not shown). The analytical sensitivity test showed that 20 pg/ μ l initial concentration of input DNA—which equals a concentration of 2.0 pg/ μ l in the PCR mix—was just at the limit of detection (data not shown).

Allele-specific genotyping

Alleles were automatically sized on GeneMapper 5 using predefined sets of corresponding allele dyes and lengths. The detection threshold was set at 50 height for peak strength by default, i.e., the software cannot identify peaks that fall below this level. For example, analytical sensitivity test showed that a DNA concentration of 20 pg/ μ l was at the limit of detection for automatic size calling with peak heights of just over the threshold

Table 2 Composition of multiplex 1 and 2 developed in the present study

Gene	Allele	Primer name	Primer sequence 5'–3'	Fluorescent dye at 5' end	Volume [μ l] ^a	Fragment length [bp]
Multiplex 1						
<i>vic1a</i>	1	Cp- <i>vic1a</i> -1	F: ATAGCAAAAGCCACAACCAC R: AGCCAACATATACTACTCCG	FAM –	0.50 0.50	263
	2	Cp- <i>vic1a</i> -2	F: TGCGACAGTCAAGGAAGATG R: ACGAGTGTCTCTGCAAATTA	FAM –	0.50 0.50	461
<i>vic2</i>	1	Cp- <i>vic2</i> -1	F: TGCGACGGATAAGGTGACTA R: TCGGTTGGCCTCGATATTG	PET –	0.50 0.50	340
	2	Cp- <i>vic2</i> -2	F: TATACGGCAAAAGGGAGGG R: CTGGGAAGTCTTTGAGCAT	PET –	0.50 0.50	448
<i>vic3a</i>	1	Cp- <i>vic3a</i> -1	F: CTGCAGACAACAACCGGTC R: GGGCACGACAAATGGCACT	VIC –	0.25 0.25	368
	2	Cp- <i>vic3a</i> -2	F: ACCGAAGACCACATGCGCAA R: AGGTTCCCCACCAGCGTCA	VIC –	0.38 0.38	191
<i>MAT1-1-2</i>	1	Cp- <i>MAT1</i> -1	F: ACATCAGTCGTGGAAATGAAAA R: ACATCACAAGTCGGCTCCCA	NED –	0.50 0.50	145
<i>MAT1-2-1</i>	2	Cp- <i>MAT1</i> -2	F: AGGAATGTGATCTGTGGTGTCT R: AATTACTTTCACCGGATTATG	NED –	0.59 0.59	400
Multiplex 2						
<i>vic4</i>	1	Cp- <i>vic4</i> -1	F: CTTTCTTCTCCAACCTGCT R: TTCTTCTCCGGCTTGCTT	PET –	0.83 0.83	359
	2	Cp- <i>vic4</i> -2	F: GCCGAGTCATCCAAAGCAG R: GGGAGGCAGAATGGAAGAAA	PET –	0.77 0.77	257
<i>vic6</i>	1	Cp- <i>vic6</i> -1	F: CTTTTGCCTGCGAGGTCTTTCC R: TCAGTCTTCTTCTGTACGTG	VIC –	0.54 0.54	381
	2	Cp- <i>vic6</i> -2	F: ATCCATGACCCTCTGCCAA R: CCAGCCACCGACTACTTTG	VIC –	0.48 0.48	328
<i>vic7</i>	1	Cp- <i>vic7</i> -1	F: TTGTCGAAAGGAACGCGGCT R: TTCTTCTTACCATCATCTGTGG	FAM –	0.64 0.64	128
	2	Cp- <i>vic7</i> -2	F: ACTTCAAACACGAATTCTGGC R: ACCATCTCCGTCTCGATTCT	FAM –	0.77 0.77	225

F, forward primer; R, reverse primer

^a Volume used for each 2.5 μ M oligonucleotide in a 20 μ l-reaction

value of 50. Although DNA from the EU tester strains and that from strains from the Claro population was diluted ten-fold before use in the PCR, there were no samples or single loci that exhibited low signal intensity. Thus, each detected peak was displayed in a sizing table directly without lowering the detection threshold. A Microsoft Excel VBA (visual basic for application) macro recoded each allele combination into the corresponding EU and mating type for each isolate (data not shown). All detected *vic* allele combinations corresponded to the expected *vc* genotypes for EU-1 to EU-64. Since the *vic* loci that determine EU-65 to EU-74 are unknown to date, the analysis of these isolates gave the same *vic1*–*vic7* allele combinations as for

other EU types (cf. Table 3). Therefore, we verified the (in)compatibility of EU-65 to EU-74 with those EU types, which have the identical allele patterns at *vic1a* – *vic7*, by pairing tests according to Table 3. A barrage line developed along the contact zone between all co-cultured isolates indicated the vegetative incompatibility. Although the *vic* allele combinations have been described previously for all EU tester strains, the corresponding mating types have not. The *vic* and *MAT* genotypes for each EU tester strain can be found in Table S1, Supporting Information.

Among the 58 isolates from the natural population from Claro, there was only one discrepancy between the *vc* type results in Hoegger et al. (2000) and the present

Table 3 Allele patterns of the European *Cryphonectria parasitica* tester strains EU-65 to EU-74 for *vic* loci and mating type idiomorphs. EU-65 to EU-74 have identical allele patterns at *vic1a* – *vic7* with the EU types in brackets

Sample ID	Collector/origin	EU type	Allele pattern ^a	<i>MAT</i> type
M1717	P. Hoegger/Switzerland, Ollon	EU-65 (EU-41)	2 / 2 / 2 / 2 / 2 / 1	<i>MAT1-1</i>
M3901	C. Robin/France, Dordogne	EU-66 (EU-9)	2 / 1 / 1 / 1 / 1 / 1	<i>MAT1-2</i>
M3902	C. Robin/France, Ardèche	EU-67 (EU-4)	1 / 1 / 1 / 2 / 2 / 2	<i>MAT1-2</i>
M3903	C. Robin/France, Ardèche	EU-68 (EU-38)	2 / 1 / 2 / 2 / 2 / 2	<i>MAT1-2</i>
M3904	C. Robin/France, Ardèche	EU-69 (EU-19)	1 / 2 / 1 / 1 / 2 / 1	<i>MAT1-1</i>
M3905	C. Robin/France, Dordogne	EU-70 (EU-13)	1 / 2 / 1 / 1 / 1 / 1	<i>MAT1-2</i>
M3906	C. Robin/France, Dordogne	EU-71 (EU-46)	2 / 2 / 2 / 1 / 1 / 1	<i>MAT1-1</i>
M3907	C. Robin/France, Dordogne	EU-72 (EU-37)	1 / 2 / 2 / 1 / 1 / 1	<i>MAT1-1</i>
M3908	C. Robin/France, Dordogne	EU-73 (EU-33)	2 / 2 / 2 / 2 / 1 / 1	<i>MAT1-2</i>
M3909	C. Robin/France, Dordogne	EU-74 (EU-13)	1 / 2 / 1 / 1 / 1 / 1	<i>MAT1-2</i>

^a *vic1a* / *vic2* / *vic3a* / *vic4* / *vic6* / *vic7*, excluding *vic5*

study. One isolate identified as EU-6 by Hoegger et al. (2000) was found to be EU-2 in our study. Co-culturing the isolate in question with tester strains of type EU-2 and EU-6 confirmed the EU-2 type of this isolate. Our mating type assay gave the same results as in the

previous study, with the exception that we detected both mating types in four samples, i.e., two NED-labeled peaks were present in the same electropherogram (Fig. 3a). To verify this result, we amplified both *MAT* genes in separate PCRs using the methods described

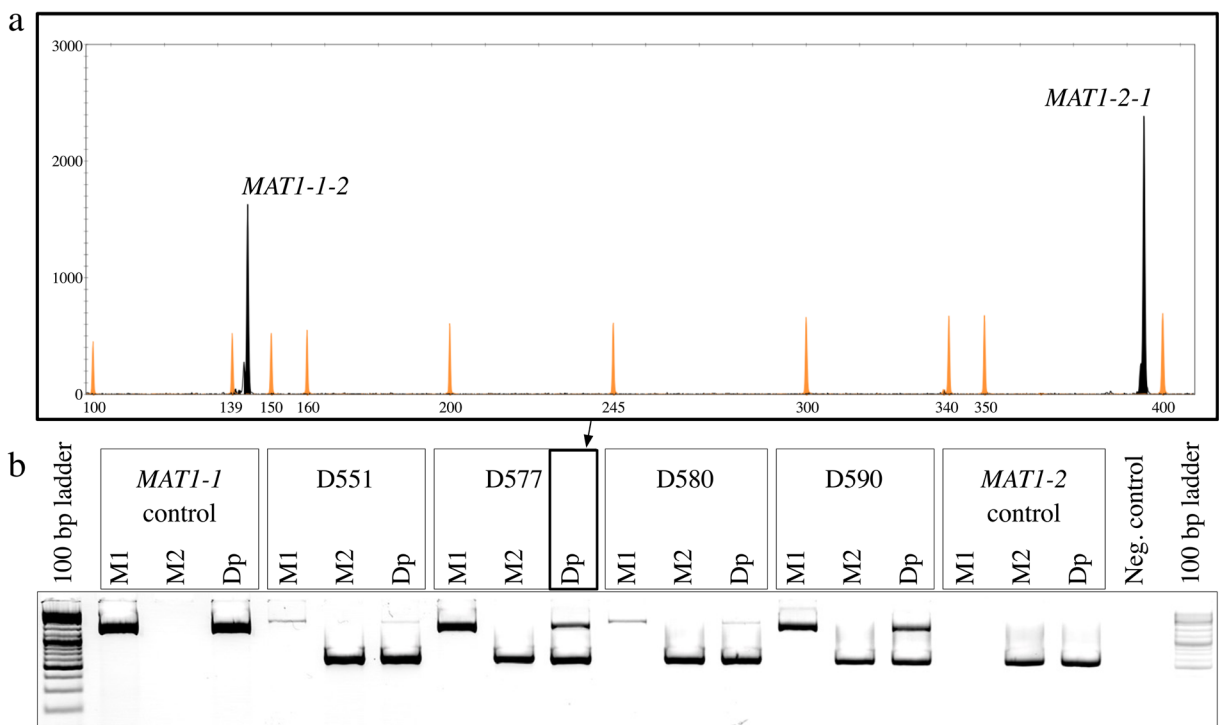


Fig. 3 Detection of both mating types in the same isolate. (a) Two NED-labeled peaks (black) were present in the same electropherogram of the isolate D577 of *Cryphonectria parasitica* (orange peaks: DNA size standard). (b) Verification of heterokaryotic strains applying two single PCR reactions according to Marra

and Milgroom (2001), which amplify the *MAT1-1* (M1) and *MAT1-2* (M2) loci specifically, and using the same protocol in a duplex (Dp). The concentration of the PCR product yielded varied considerably and the larger fragment (*MAT1-1*) from the duplex reaction was in a few cases practically invisible on the gel image

previously by Marra and Milgroom (2001). This confirmed the presence of both mating types in the samples D551, D577, D580, and D590 (Fig. 3b).

Discussion

Sexual and vegetative incompatibility provide useful genetic markers to study the population biology of fungi (e.g., mode of reproduction, genetic diversity or migration). These markers are also relevant from a practical point of view because both incompatibility systems affect the spread and persistence of biocontrol mycoviruses in populations of pathogenic fungi. The chestnut blight fungus (*C. parasitica*) and its hyperparasitic mycovirus (CHV-1) represent such a biological control system where sexual and vegetative compatibility testing has been intensively applied (Rigling and Prospero 2018). Conventional characterization of vegetative compatibility in *C. parasitica* is based on pairing assays involving tester strains of known vc types (Cortesi and Milgroom 1998) or, more recently, on conventional PCR (Short et al. 2015; Mlinarec et al. 2017). Due to the expertise required and difficulties associated with accurately distinguishing the barrage line in pairing assays, false vc type assignments are not uncommon. Additionally, conventional PCR has limitations if a large number of isolates need to be tested. Thus, one of the main challenges at present relates to an accurate molecular technique that becomes indispensable for the analysis of massive sampling. In the present study, genotyping was able to correctly determine each *vic* genotype of the genetically defined European tester strains EU-1 to EU-64 as well as the previously reported testers EU-65 to EU-74. The genetic basis of these additional EU types has not yet been resolved beyond the known *vic* loci. Presumably, they differ from EU-1 to EU-64 at additional *vic* loci. Alternatively, one or several of the known *vic* loci may have evolved more than two alleles. In any case, once available, new molecular information (additional *vic* loci or additional alleles) about the incompatibility mechanisms of EU-65 to EU-74 should be included in our assay. Until then, *vic* genotyping in populations where EU types other than EU-1 to EU-64 have to be expected, should be verified by pairing assays with EU types that share the same *vic* allele pattern (cf. Table 3).

Careful primer design is critical to ensure amplification of the correct fragment in PCR. In the present study, we

used genetically variable regions within exons to develop allele-specific primers in a way that the probability of finding that particular stretch more than once in the genome becomes small. Indeed, *in silico* primer testing in the NCBI-suite gave no matches with other loci neither in the *C. parasitica* genome nor in other fungal species. Nevertheless, *in vitro*, a *vic* marker (Cp-*vic*3a-2) amplified in *C. naterciae* and two in *C. radicalis* (Cp-*vic*2-1, and Cp-*vic*4-2), while no amplification was obtained with the mating type primer for all other *Cryphonectria* species. These amplifications were unexpected and, even if the amplification yield was low, the obtained fragments showed the expected length for the respective alleles. The present work does not address other species than *C. parasitica*, nevertheless this finding has important implications for diagnostics: allelic drop-out at most loci must be interpreted as misidentification of the specimen species. Loss of one or more alleles during PCR may be caused by variable mechanisms like single nucleotide polymorphisms in the primer binding sequence. In our specific case, this suggests that species identity requires verification, for instance, by sequencing the barcode locus ITS. *Cryphonectria naterciae*, for example, has been recovered from symptomatic bark of *C. sativa* in areas affected by chestnut blight in Portugal (Bragança et al. 2011), indicating that both *Cryphonectria* species can be present in the same chestnut stand.

Comparative testing of the newly developed genotyping assay with the classical vc and mating typing methods previously applied on the Claro population (Hoegger et al. 2000) showed our assay to have greater sensitivity. First, one isolate was previously classified as EU-6, but our analysis revealed that it clearly belongs to EU-2. These two vc types only differ at the *vic*4 locus (Cortesi et al. 2007), which is known to induce a weak incompatibility reaction with a faintly marked barrage line formed between co-cultured strains. Thus, false vc type assignments caused by *vic*4 are not uncommon. Second, the genotyping assay detected both mating types in some isolates. Using state-of-the-art methods at that time, Hoegger et al. (2000) amplified a short, conserved sequence of the HMG box of the *MAT1-2* locus and deduced from negative amplification which isolates should belong to the *MAT1-1* type. In the present study, we verified the detection of heterokaryotic isolates by applying two single PCR reactions that were developed after Hoegger's study (Marra and Milgroom 2001). We also tested the same protocol in a duplex reaction that is commonly used in many laboratories. The concentration of the PCR product yielded varied

considerably, and the largest fragment (*MAT1-1*) from the duplex reaction was practically invisible on the gel image in a few cases. Skewed nuclear ratios have been reported in heterokaryotic *C. parasitica* strains, and may result in underestimating the frequency of *MAT* heterokaryons in *C. parasitica* (McGuire et al. 2004). However, our genotyping assay—which uses short fragments for both *MAT* idiomorphs—was able to detect *MAT1-1* accurately, but still at a relatively low concentration level compared to *MAT1-2*. Detection of both mating types in one isolate is not surprising, and presumably is related to parasexual events (McGuire et al. 2004, 2005) or could indicate the presence of two different strains of *C. parasitica*.

It has been suggested that vegetative incompatibility can be sidestepped by genome editing *C. parasitica* strains (Stauder et al. 2019). However, since current European legislation does not allow the release into the environment of genetically modified organisms, such an approach is not applicable in European countries. The fluorescent multiplex genotyping assay presented here is a valuable tool for determining the vegetative compatibility and mating types in *C. parasitica* populations, which is a prerequisite for the artificial application of CHV-1 to control chestnut blight. In Europe, the mycovirus CHV-1 acts as a successful biological control agent of chestnut blight by reducing the parasitic growth and sporulation capacity of *C. parasitica*. Individual cankers can be therapeutically treated with hypovirus-infected *C. parasitica* strains and the hypovirus may subsequently spread to untreated cankers and become established in the *C. parasitica* population (Rigling and Prospero 2018). As the hypovirus is best transmitted between *C. parasitica* isolates belonging to the same vc type, the fungal strain carrying CHV-1 should match the vc type(s) present in the target cankers. Ideally, the carrier strain should also have the predominant mating type of the target population, in order to not promote sexual reproduction.

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Compliance with ethical standards

Conflict of interest This research does not contain any conflicts of interest, nor research involving humans or animals.

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