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Molecular characterization of *Cryphonectria parasitica* isolates from Basilicata region (Southern Italy) and mycovirus identification

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Abstract

Chestnut is a valuable species that grows widely throughout the Italian peninsula. It is susceptible to different diseases and among them chestnut blight caused by *Cryphonectria parasitica* is the most devastating one. This study aimed to molecularly characterize and

distinguish virulent and hypovirulent *C. parasitica* isolates from the Basilicata region. Pure fungal cultures were obtained from symptomatic plant material, their genomic DNA was extracted and amplified using rDNA Internal Transcribed Spacer (ITS), M13 minisatellite and $(GACA)_4$ microsatellite primers. ITS sequencing allowed the identification of the fungal species while minisatellite and microsatellite Polymerase Chain Reactions (PCRs) differentiate between virulent and hypovirulent isolates placing them in distinct clusters. Additionally, sequence analysis of hypovirus partial genome showed that all identified hypoviruses, belonged to Cryphonectria hypovirus 1 (CHV-1) subtype, sharing a 99% sequence identity with the Italian isolate Marche of CHV-1. To our knowledge, this is the first study to differentiate virulent and hypovirulent isolates of *C. parasitica* at molecular level and identify the hypovirus subtype associated with the hypovirulent isolates in the Basilicata region.

Introduction

Chestnut (*Castanea sativa* Mill.) is a tree with high economic value, which can be affected by various diseases and pests, some of them being particularly widespread and economically important. The most devastating disease is the chestnut blight caused by *Cryphonectria parasitica* (Murr.) Barr.[1] a fungus attacking mostly the chestnut species but also harming *Quercus* spp. and other hardwood hosts. The attack on chestnut of *C. parasitica*, caused by virulent forms, produces necrotic lesions (known as cankers) on the bark of the stem and branches in case of the susceptible hosts (European and American chestnut) leading to, in the young trees, wilting and death of the tree parts nearby the infection site within few months[1]. The virulent form of *C. parasitica* produces typical evolutionary/lethal cankers and there are hypovirulent strains that cause atypical, callusing/non-lethal cankers.[1] Hypovirulence is linked to the micovirus presence into the fungus. There is gap of knowledge regarding the

clear distinction between virulent and hypovirulent isolates of *C. parasitica* by straightforward molecular tools. Four species of hypoviruses, members of the Hypoviridae family have been described so far in *C. parasitica*: Cryphonectria hypovirus 1 (CHV-1); Cryphonectria hypovirus 2 (CHV-2); Cryphonectria hypovirus 3 (CHV-3) and Cryphonectria hypovirus 4 (CHV-4)[2]. Nonetheless, due to the high efficiency of inducing hypovirulence the most important remains the CHV-1 which is widely distributed all over Europe.[1-5] Its presence (CHV-1 Italian subtype) was also recently reported in Montenegro.[6] The Reverse Transcription Polymerase Chain Reaction (RT-PCR) restriction fragment length polymorphism method and viral genome sequencing allowed the identification of the CHV-1 hypoviruses.[7-10] A recent comparative methylome study through Whole Genome Bisulfite Sequencing (WGBS) analysis of *C. parasitica* with or without hypovirus infection provided valuable insights into the understanding of DNA methylation and hypovirus infection relationships, showing that DNA methylation in promoter was negatively correlated with transcript abundance and gene expression, and hypovirus can perturb DNA methylation of its host by the regulation of the metabolic state of *C. parasitica*.[11] The current knowledge about *C. parasitica* virosphere RNA was expanded when, in 2021, Forgia and co-authors[5] through virome investigations reported the presence in Azerbaijan of a new mycovirus named Cryphonectria parasitica sclerotimonavirus 1 (CpSV1) and a putative new RNA virus which was called Cryphonectria parasitica ambivirus 1 (CpaV1) unrelated to the already described viral sequences so, adding new data for the possibility to control chestnut blight disease by using other hypovirulent isolates. Suzuki *et al*.[12] studied in-tree behavior of diverse viruses present in *C. parasitica* testing seven different viruses for their hypovirulence induction capacity, biocontrol potential and transmission properties between two vegetatively compatible but molecularly distinguishable fungal strains in chestnut trees, observing that the tested viruses exhibited different in planta behavior being classified in four groups (I-IV). It

was demonstrated that canker development and biocontrol potential of two tested English CHV-1 strains (I-5 and L-18) of *C. parasitica* were dependent on the virus concentration and the compatibility with fungal inocula.[13]

The control measures against chestnut blight forecast: the elimination of the infected parts of the tree; the use of healthy propagation material; the use of biological control tools against chestnut bark canker disease through the utilization of highly locally efficient (and vegetatively compatible) hypovirulent strains; the protection of the grafts using cicatrizing mastics; the transformation of the chestnut orchards from fruit to crown; and the use of resistant varieties to chestnut blight disease.[1,14,15]

Given the economic, landscape, environmental and cultural importance of chestnut cultures in Basilicata, the present study was undertaken with the main objective to molecularly investigate the virulent and hypovirulent isolates of *C. parasitica* in Basilicata region (Southern Italy) and identify the hypovirulent species, all pointing to improve chestnut bark canker biocontrol. In particular, the current study aimed to; i) differentiate between virulent and hypovirulent *C. parasitica* isolates using the Internal Transcribed Spacer (ITS) region, the microsatellites and minisatellites markers and ii) determine the mycovirus associated with the *C. parasitica* hypovirulent isolates from Basilicata region.

Materials and Methods

Symptoms evaluation and sampling

A series of field observations, during 2018-2022, were carried out in orchards located in seven chestnut cultivation areas of the Basilicata Region: Rionero in Vulture, Melfi, Tramutola, Moliterno, Lagonegro, Paterno, and Trecchina in order to identify trees with typical chestnut blight disease symptoms (Figure 1).

Basilicata region has a Mediterranean type of climate and the chestnut cultivations are located between approx. 600-800 m altitude. Samples represented by suckers, branches, twigs, branches and cortical portions of stems were randomly taken from the virulent and hypovirulent symptomatic trees. They were numbered, positioned in sterile plastic bags and shortly transported to the laboratory for fungal isolation.

Fungal isolation

Plugs of cortical and woody tissue pieces (2 × 2 cm) were taken from each sample from the margins of the symptomatic parts. Under sterile conditions they were superficially disinfected, cut in small pieces and placed in Petri dishes containing Potato-Dextrose-Agar (PDA) media Oxoid™ (Thermo Fisher Scientific Inc., Waltham, MA, USA) amended with kanamycin (50 µg/mL) following a protocol fully described by Mang *et al.*[16,17] Inoculated plates were incubated at 25°C in dark and kept under observation until the appearance of the mycelium. All developed *C. parasitica* colonies were individually transferred to new Petri dishes containing the same media to obtain pure fungal cultures (PFCs). After 10 days, the PFCs were divided based on morphological features in virulent, characterized by a red-orange color aspect and by numerous pycnidia, and hypovirulent, which showed a whitish mycelium and an absence or a limited number of pycnidia.

Total DNA isolation

Total genomic DNA (gDNA) was extracted from PFCs following the method described by Mentana *et al.*[18] Briefly, approximately 200 mg of mycelium of each isolate, was powdered with liquid nitrogen and then the genomic DNA (gDNA) was isolated using the Macherey-Nagel (Düren, Germany) kit following the manufacturer's instructions. Genomic DNA concentration and purity were assessed by readings at NanoDrop ND-1000

spectrophotometer (Thermo Fisher Scientific Inc., Welmington, Delaware, USA). Finally, the gDNA was diluted to 50 ng/µL and stored at -24°C until further used.

PCR and sequencing

Five µL of the gDNA was utilized in PCR tests using the universal ITS primers[19] ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') following the protocol described in previous studies.[20,21] PCR results were visualized with a transilluminator after electrophoresis in 1.5% agarose gel and staining with SYBR® Safe DNA gel stain (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) in the proximity of a molecular weight marker (1-kb DNA ladder, BRL Life Technologies, Carlsbad, CA, USA). PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) as fully described by Mang and Figliuolo[22] and directly sequenced, in both directions, at the Bio Molecular Research (BMR) Biotechnology Center of Genomics (Padua, Italy). In order to identify the fungal species, nucleotide sequences of the ITS region were matched, with those available in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) database using the Basic Local Alignment Search Tool (BLAST) program.[23,24] All ITS sequences acquired in this study had been deposited in the European Molecular Biology Laboratory (EMBL) and NCBI nucleotide databases.

For the purpose of a possible differentiation among virulent and hypovirulent *C. parasitica* isolates from Basilicata region, two different molecular markers were used: the $(GACA)_4$ microsatellite (GACAGACAGACAGACA) and the M13 minisatellite, derived from the phage sequence M13, (5'-GAGGGTGGCGGTTCT-3').[25,26] For the PCR with M13 the following amplification protocol was used: a pre-denaturation at 94°C (3 min), followed by 40 cycles: 94°C (1 min); 45°C (1 min); 72°C (1 min) with a final extension at 72°C (7 min). In the PCR with the M13 primer, the DyNAzyme II DNA Polymerase (Thermo Fisher

Scientific Inc., Waltham, MA, USA) was used. In addition, the PCR with $(GACA)_4$ was performed using the Thermo Scientific Phire Hot Start II DNA polymerase enzyme (Thermo Fisher Scientific Inc., Waltham, MA, USA) with the following amplification protocol: a pre-denaturation at 98°C 3 min (1 cycle), followed by 40 cycles: 98°C (5 s); 45°C (5 s); 72°C (20 s) and a final extension at 72°C (1 min). All PCR reactions were done in duplicates and their results were run on a 1.2% agarose gel at 50 V in Tris-Acetate-EDTA (TAE) 1× buffer and visualized by a transilluminator (EuroClone, Milan, Italy).

Phylogenetic analyses of the ITS region

All ITS nucleotide sequences, obtained in this study and those downloaded from GenBank, were analyzed and aligned through the MEGA11 phylogeny package using the ClustalW method (https://www.ebi.ac.uk/clustalw).[27,28] The analysis on sequences alignment was done using the Sequence Data Explorer function in MEGA11.[27] Nucleotide sequences for the ITS of *C. parasitica* hypovirulent isolates from this study along with two American *C. parasitica* isolates and one isolate from each country (Ukraine, Macedonia, Romania, Hungary, Slovakia and Canada) were downloaded from GenBank NCBI. Two ITS sequences of *Diaporthe ambigua,* a closely related species belonging to the same order Diaportales (AF543817, AF543818) from South Africa, were used as outgroups in the phylogenetic analyses. The phylogenetic investigation was performed using the Maximum Likelihood (ML) statistical method and setting the following parameters: bootstrap, 1000 replicates,[29] Tamura-Nei substitution model,[30] with the ratio and uniform substitution pattern between sites.

RT-PCR and genomic analysis

Total RNA isolation

A total of 100 mg of *C. parasitica* PFC mycelium from the hypovirulent isolates was grinded in liquid nitrogen. The total RNA (tRNA) extraction was performed using the Nucleo Spin Plant II™ kit (Macherey-Nagel, Dürren, Germany) following manufacturer's instructions. The total RNA was finally resuspended in 50 µL sterile water and stored at −80°C until further used.

cDNA synthesis and PCR

One µL of the tRNA was checked for its purity and quantity by a Nanodrop ND-1000 spectrophotometer (Thermo Scientific Inc., Waltham, USA). Subsequently, cDNA was synthesized from 100 ng total RNA using the Omniscript® Reverse Trasncription kit (Qiagen, Hidlerberg, Germany) following manufacturer's instructions. PCRs were performed with virus specific primers, which amplify different subtypes of CHV. In particular, primer combination EP 713-6 and EP 713-7, for a part of ORF B, was used as described by Alleman *et al*.[7]. For all primers combinations, PCR amplifications were carried out in a total volume of 50 µL reaction mixture containing cDNA template (1 µL), primer pairs (1.0 µM each), 2 × Platinum Super RT Master Mix, 1U Platinum SuperFi DNA polymerase (Thermo Fisher Scientific, Carlsbad, CA, USA) and nuclease free water from the same kit until 50 µL final volume. RT-PCR reactions cycling conditions were set as follows: 10 min at 58°C (1 cycle); 2 min at 98°C (1 cycle); 40 cycles of 10 s at 98°C, 10 s at 61°C, 1 min at 72°C and 5 min at 72°C (final extension). Finally, PCR outcome was checked by electrophoresis (1.2% agarose gel), and gels were visualized by a transilluminator (Euroclone S.p.A., Milan, Italy). Subsequently, the amplicons of partial Open Reading Frame (ORF) B of *C. parasitica* genome were sequenced by BMR Genomics Srl (Padua, Italy), in both directions, using the same PCR primers. Nucleotide sequences of ORF B of the hypovirulent isolates obtained in

this study were analyzed with the BLAST program from the NCBI database[24] to identify the subtype of the hypovirus based on high sequence similarity (>99%).

Phylogenetic analysis of the CHV-1 hypovirus partial genome

All nucleotide sequences of the ORF B obtained in this study along with some other CHV-1 subtype representative sequences from the GenBank (Accession numbers: ON180812, ON180815, MF421721, MF421723, AF082191, DQ861913, and ON022057, respectively) included for comparison were aligned with other sequences of the ORF B gene of CHV-1 published at NCBI (www.ncbi.nih.gov) using ClustalX[28] within MEGA11 phylogeny package[27]. The two hypoviruses, Cryphonectria ambivirus 2 (MT45466) and Cryphonectria mitovirus 1 (L31849) have been used for comparison with our isolates.The alignments were used to reconstruct the phylogenetic relationships between the ORF B regions of the hypovirulent isolates obtained in this study also in MEGA11 using the same method earlier described for the ITS region. Maximum Likelihood (ML) method was used to estimate evolutionary relationships and bootstrap analyses with 1,000 replicates were performed[29] to estimate their support.

Results

Symptoms evaluation and sampling

Chestnut blight attack was revealed at all the 7 sites investigated. A total number of 56 samples with cankers caused by *C. parasitica* were evaluated, within them 21 were hypovirulent and 35 were virulent ones.

Fungal isolation

A total number of 54 *C. parasitica* pure cultures were isolated on PDA from the 56 chestnut blight cankers investigated, and all of them were further used for the morphological and molecular analyses. Among them, 20 fungal isolates displayed reduced pigmentation (white color) and sporulation, which indicates the eventual presence of the Cryphonectria hypovirus while the other 34 fungal isolates showed and orange pigmentation associated to numerous pycnidium presence being considered virulent.

DNA isolation, PCR and sequencing

Total DNAs were obtained from 22 representative *C. parasitica* pure cultures which after PCRs with ITS5 and ITS4 primers produced amplicons of about 650 bp (Table 1). Their direct sequencing and analysis produced 22 ITS nucleotide sequences. After further sequence analysis of the ITS region it was observed that the *C. parasitica* isolates with different virulence differ from each other by 2%. The ITS nucleotide sequences from this study were deposited into NCBI GenBank under the following accession numbers: LN869538; LN869539; LT97228-LT972337; LT976486; LT976487 and HG992968-HG992975 (Table 1). Furthermore, the BLAST analysis performed online (www.blast.ncbi.nlm.nih.gov) showed that they highly matched (>99% similarity) with other *C. parasitica* isolates from different European countries. In particular, with: isolate C from Switzerland (KP824756); isolate 122102158 from Andalusia, Spain (KF220299); strain CBS:129354 from Portugal (MH865225); strain CBS: 242.54 from Italy (MH857312) and with all other nucleotide sequences of the ITS region for which the accession numbers are reported in Table 1. Minisatellite and micro satellite–primed PCRs performed with M13 and $(GACA)_4$, respectively allowed to distinguish between *C. parasitica* isolates investigated in this study based on two distinct polymorphisms observed for the virulent vs. hypovirulent types of fungal isolates. All investigated *C. parasitica* isolates of the same virulence type shared the

same profile as seen after electrophoresis, showing different and specific size of bands (Table 2).

The minisatellite M13-primed PCR allowed to obtain a total of 10 polymorphic bands after agarose electrophoresis distinctively distributed between the virulent and hypovirulent isolates of investigated *C. parasitica*. In particular, the virulent type of *C. parasitica* isolates exhibited one specific electrophoretic profile composed of 5 bands with size between 280 bp and 1100 bp while the hypovirulent isolates showed the same number of polymorphic bands but of different and distinct size between 350 bp and 1400 bp, respectively (Table 2). In addition, the microsatellite $(GACA)_4$-primed PCR revealed a total of 9 polymorphic bands once again differently distributed among the virulent and hypovirulent *C. parasitica* isolates. Five polymorphic specific bands were registered for the virulent isolates of *C. parasitica* with size between 280 bp and 530 bp while for the hypovirulent isolates of the same fungus only 4 polymorphic bands were observed but they were different from those seen in the virulent isolates having size between 380 bp and 750 bp (Table 2).

Phylogenetic analysis based on ITS region

The results of the phylogenetic analysis based on the ITS region of the ribosomal DNA demonstrated that all *C. parasitica* isolates obtained in the present study grouped together and they were also closely placed nearby isolates of the same species originated from different countries worldwide (Figure 2). Furthermore, the outcomes of ITS region phylogenetic analysis confirmed the correct morphological and molecular identification of the fungal isolates as belonging to the *C. parasitica* fungus with an elevated bootstrap support value (98%) while the *D. ambigua* species were clearly separated from the *C. parasitica* group, as expected and this with high bootstrap support (99%). The hypovirulent *C. parasitica* isolates from this study clustered together with some other *C. parasitica* isolates from Slovakia,

Hungary, and Romania (Figure 2). In addition, the virulent isolates of *C. parasitica* from Basilicata region also grouped together and were placed nearby the *C. parasitica* isolate originated from Macedonia (Figure 2).

RNA extraction, RT-PCR and sequencing

Total RNAs were extracted from all 8 PFC of hypovirulent *C. parasitica* and cDNA had been created and successfully amplified by RT-PCR with *C. parasitica* specific primers for the ORF B of the CHV-1 genome for all fungal isolates. The ORF B amplicons of all hypovirus type isolates of *C. parasitica* from this study after direct sequencing and BLAST analysis showed a 99.52% similarity with the ORF B polyprotein gene of CHV-1 isolate Marche (KY471627). Furthermore, all hypovirulent isolates from this study, after alignment, showed also a high sequence (CHV-1 ORF B) identity with other European and non-European isolates of CHV-1. In particular, with the: isolate LS-O 05-1 at 99.47% (ON180815 from Montenegro); haplotype HB14 at 99.37% (Italy); isolate EP721 at 99.21% (China); isolate CR23 at 98.91% (Croatia) and also with the isolate Euro7 at 98,81% (Italy). The partial nucleotide sequences of the ORF B polyprotein (1,007 bp) CHV-1 analysis also showed that 5 nucleotide difference was observed between the CHV-1 isolates of *C. parasitica* from Basilicata and the CHV-1 isolate Marche. A total of 1,002 sites were conserved, none of them were parsimony informative and 5 sites were variable being also singletons. Moreover, ORF B partial gene analysis illustrated that the genetic distance between the hypovirulent isolates of *C. parasitica* from Basilicata and other isolates of the same subtype from Europe was zero. All nucleotide sequences of the ORF B genome of the hypovirulent isolates of *C. parasitica* obtained in this study have been deposited in the NCBI under the following accession numbers: PP532689-PP532696.

Phylogenetic analysis of the CHV-1 hypovirus partial genome

Phylogenetic analysis based on the ORF B of the CHV-1 genome outcomes showed that the isolates of *C. parasitica* from Basilicata grouped together with the Italian isolate CHV-1 Marche and were placed closer to the CHV-1 isolate LS-O 05-1. On top, the Basilicata CHV-1 isolates clustered together with other isolates of the same hypovirus, namely CHV-1, thus confirming the presence in Basilicata of the CHV-1 subtype in all the hypovirulent isolates of *C. parasitica*. (Figure 3). The two isolates of the Cryphonectria hypovirus 2 were placed one (NC003534) closer to the CHV-1 subtype isolates while the other isolate (AH003861) was separated from them and closer to the Cryphonectria sclerotimonavirus 1 (MT354565). The reference Cryphonectria hypovirus 3 isolates (AF188515 and NC000960) grouped together in a separate cluster with 100% bootstrap support and they were separated from the Cryphonectria hypovirus 4 strain (NC006431) which was also highly supported (bootstrap=100%). The Cryphonectria ambivirus 2 (MT45466) and the Cryphonectria mitovirus 1 (L31849)] clustered together in a separate group which was also very well supported (bootstrap=100%) as shown in Figure 3.

Discussion

This research has shown that chestnut blight disease is widespread in the most important chestnut areas of the Basilicata region, where often mainly ancient local ecotypes are present. Our results showed that in the most affected chestnuts the percentage of infected plants exceeded 80% also showing a clear prevalence of evolutionary or lethal cankers.

The discovery of the hypovirulent *C. parasitica* strains and their use in therapeutic treatments of individual chestnut bark cankers offered the possibility of biological control of this devastating chestnut disease.[1] However, disease management strategies focusing on hypovirulence and resistance breeding, pointed out that relying on hypovirulence in long term

is not sufficient and increasing the host resistance could also be required.[1] The availability of hypovirulent isolates of *C. parasitica* represents a potential biological control tool for the disease in question following the artificial introduction of hypovirulent strains in a specific territory.[2,31-33] Biological control of chestnut blight occurs naturally in some chestnut growing areas regardless of anthropic intervention.[1,2,31,34] However, in North America with the exception of some areas of Michigan as well as in some European areas, biological control by experimental inoculations of hypovirulent strains in chestnut areas with a high incidence of the disease has produced null or negligible results.[2] This failure is mainly due to the limitation of virus transmission by vegetative incompatibility, a process leading to the programmed cell death between incompatible individuals, which inhibits heterokaryon formation in *C. parasitica.*[2]

Marked differences between the isolates with different virulence that infect the chestnut in Basilicata were also found with the aid of molecular biology techniques. In particular, nuclear ribosomal DNA (rDNA) sequences have been successfully used as molecular markers. The rDNA includes both highly conserved regions that evolve slowly and can be used to study phylogenetically distant fungi and chromists as well as variable regions such as ITS regions. The ITS regions evolve more rapidly showing variations in their nucleotide sequences which are frequent enough to allow differentiation of closely related strains and identification of a given fungal species at genus and species level.[19,35] The fact that ITS region is a good barcoding gene to investigate the phylogenetic relationships between different *C. parasitica* isolates[36] was also confirmed by the outcomes from our study which showed that ITS region was suitable to identify the *C. parasitica* isolates from Basilicata region at species level. In addition, the phylogenetic analysis based on the ITS gene region of 22 isolates of *C. parasitica* originated from the most important chestnut areas of Basilicata showed that they were similar to other isolates of the same pathogen from Europe and other countries.

Outcomes from this study also confirmed the use of the phylogenetic analysis for understanding the genetic diversity within *C. parasitica.*[37]

Previous studies on *C. parasitica* diversity using micro and minisatellites markers demonstrated that these markers allowed to differentiate between various species of *Cryphonectria.*[38,39] Results from this study showed that M13 minisatellite-primed PCR and the $(GACA)_4$ microsatellite-primed PCR revealed a typical polymorphism profile for virulent isolates of *C. parasitica* which was distinct from the polymorphic profile of the hypovirulent ones. In summary, it was observed that both molecular makers performed well, exhibiting a similar polymorphism level (10 polymorphic electrophoretic bands *vs*. 9 polymorphic electrophoretic bands) providing a simple and rapid way to discriminate among the *C. parasitica* isolates analyzed in this study which were also correlated with their virulence type. Furthermore, the mycovirus implicated in the hypovirulence was identified and molecularly characterized for the first time in Basilicata and it was demonstrated that it belongs to the CHV-1 subtype being identical at 99.52% to the Marche CHV-1 isolate, already reported in Italy. All hypovirus isolates of *C. parasitica* from this study clustered together with other European isolates of the same type and they were clearly different from the other hypoviruses (CHV-2, CHV-3 and CHV-4).

Research on Cryphonectria hypovirus is of great interest for the scientific community as demonstrated by several very recent worldwide published studies on new detection tools of Cryphonectria hypovirus 1 (CHV-1) using Real-Time PCR and Loop Mediated (LAMP),[40] characterization of CHV-1 strains and their transmission to different *C. parasitica* vegetative compatibility types[41] and physiological variations in wild and model-long term laboratory strains of *C. parastica* infected by hypovirus.[42] In this context, our study provided new data about the presence and the type of Cryphonectria hypovirus in a Southern part of Italy

(Basilicata region) where secular chestnut trees affected by chestnut blight disease are of key importance.

Conclusions

The outcomes of this study showed that all hypovirulent isolates from Basilicata region belonged to the CHV-1 type and were highly similar to another isolate from Italy (Marche-1). Additionally, the results of this investigation provided a rapid and simple molecular tool to distinguish among the virulent and hypovirulent isolates of *C. parasitica* based on a specific electrophoretic profile closely linked to the virulence type. Overall, this knowledge is useful to improve biological control of chestnut blight disease in Basilicata region where in some localities the chestnut production still represents an important income for the local people.

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Figure 1. Basilicata region map (taken and modified from https://d-maps.com/carte.php?num_car=7976&lang=en) showing the sampling sites for *C. parasitica.* Green stars mark the sampling localities.

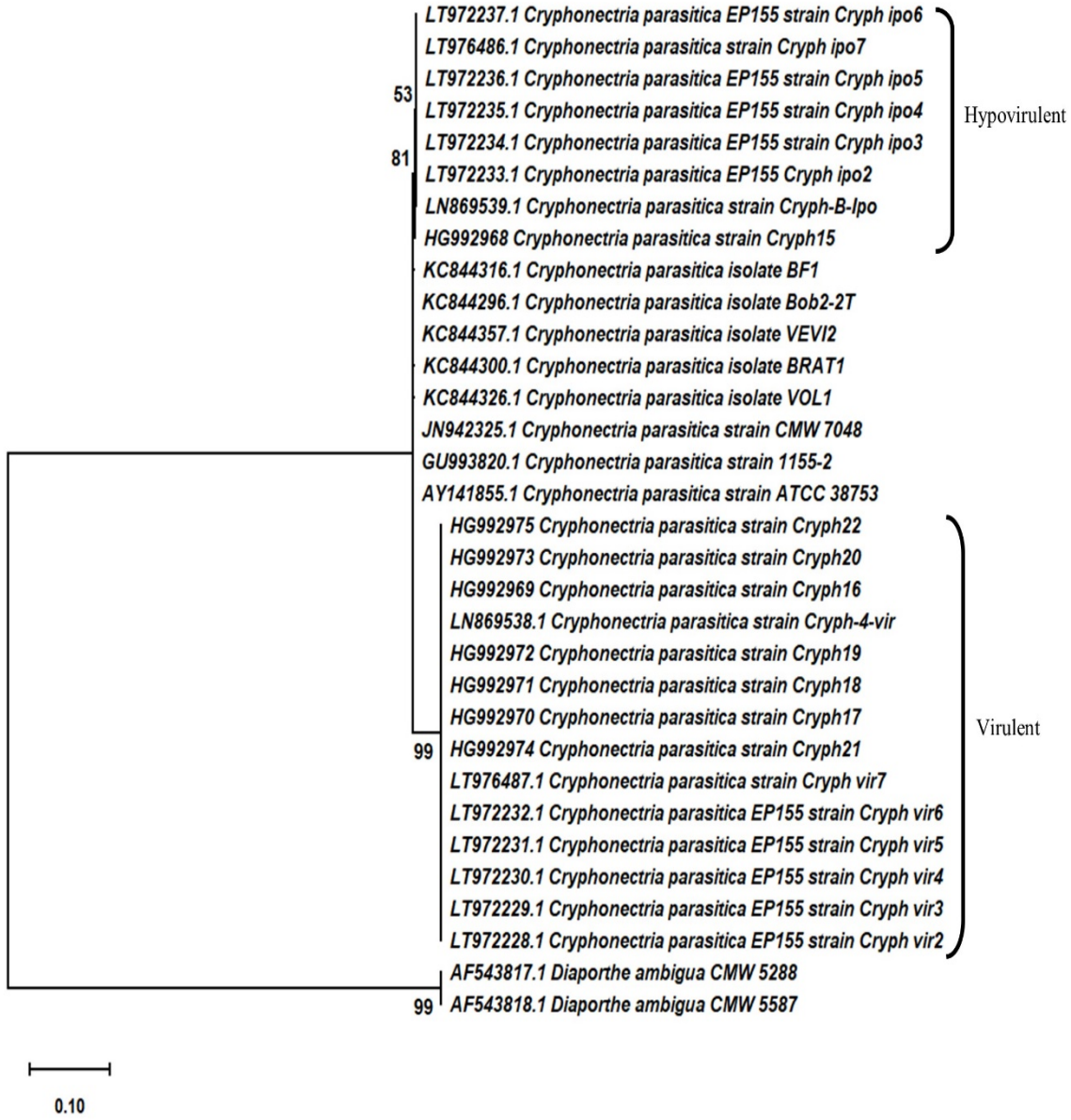

Figure 2. Maximum-Likelihood phylogenetic tree based on ITS gene region (606 bp) of 30 *Cryphonectria parasitica* isolates. Two ITS nucleotide sequences of *Diaporthe ambigua* isolates were used as outgroups. The percentage in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown below the nodes. Values less than 50% are not revealed. Scale = number of substitutions/site.

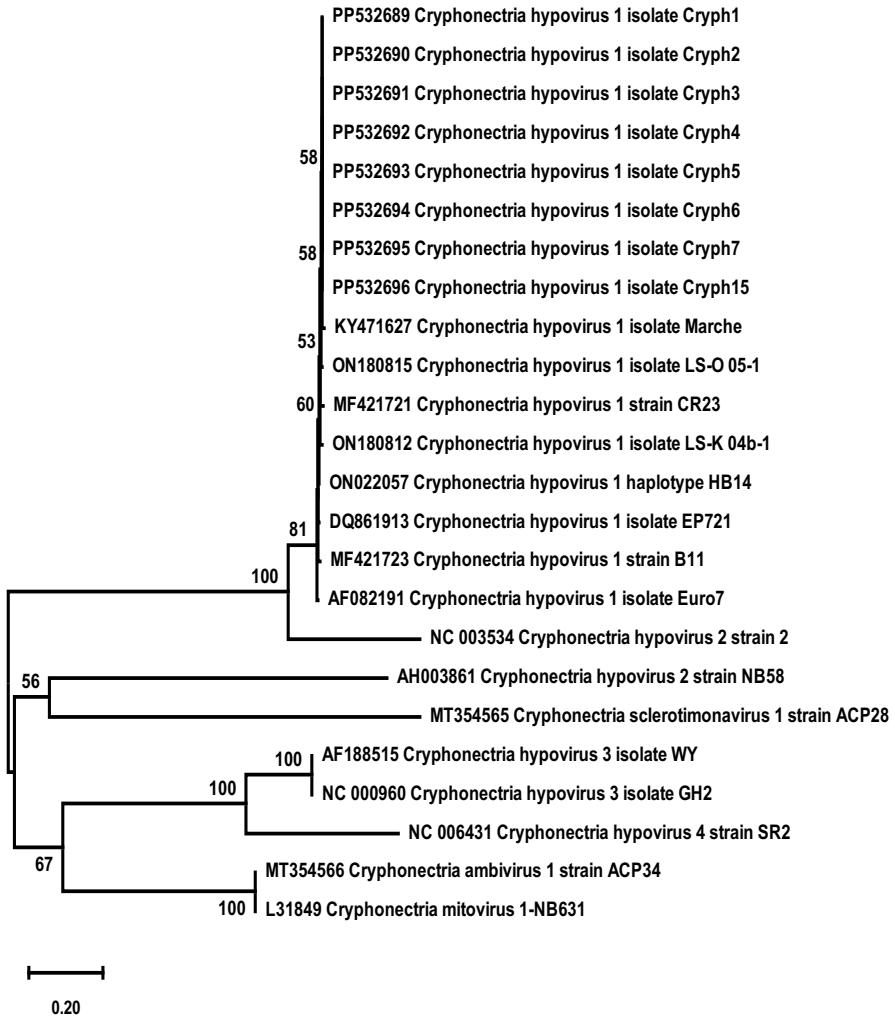

Figure 3. Maximum-Likelihood phylogenetic tree of 24 *Cryphonectria parasitica* isolates based on polyprotein of ORF B gene (950 bp) of the CHV-1. The percentage in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown below the nodes. Values less than 50% are not revealed. The tree is drawn to scale, with branch lengths measured in the number of substitutions/site.

Table 1. List of the fungal species used in this study.

Species	Isolate / Strain name	Origin (Country/Place)	Type*	Gene / GenBank ITS	Source
Cryphonectria parasitica	Cryph1	Italy / Moliterno	H	LN869539	This study
-″-	Cryph2	Italy / Moliterno	H	LT972233	-″-
-″-	Cryph3	Italy/Rionero in Vulture	H	LT972234	-″-
-″-	Cryph4	Italy/Rionero in Vulture	H	LT972235	-″-
-″-	Cryph5	Italy/Rionero in Vulture	H	LT972236	-″-
-″-	Cryph6	Italy/Rionero in Vulture	H	LT972237	-″-
-″-	Cryph7	Italy / Tramutola	H	LT976486	-″-
-″-	Cryph8_1	Italy / Moliterno	V	LN869538	-″-
-″-	Cryph9_2	Italy / Moliterno	V	LT972228	-″-
-″-	Cryph10_3	Italy / Tramutola	V	LT972229	-″-
-″-	Cryph11_4	Italy / Tramutola	V	LT972230	-″-
-″-	Cryph12_5	Italy / Paterno	V	LT972231	-″-
-″-	Cryph13_6	Italy / Tramutola	V	LT972232	-″-
-″-	Cryph14_7	Italy / Melfi	V	LT976487	-″-
-″-	Cryph15	Italy / Melfi	H	HG992968	-″-
-″-	Cryph16	Italy / Melfi	V	HG992969	-″-
-″-	Cryph17	Italy / Rionero in Vulture	V	HG992970	-″-
-″-	Cryph18	Italy / Trecchina	V	HG992971	-″-
-″-	Cryph19	Italy / Lagonegro	V	HG992972	-″-
-″-	Cryph20	Italy / Lagonegro	V	HG992973	-″-
-″-	Cryph21	Italy / Paterno	V	HG992974	-″-
-″-	Cryph22	Italy / Trecchina	V	HG992975	-″-
-″-	CMW 7048	U.S.A.	Not Specified	JN942325	GenBank
-″-	ATCC 38753	U.S.A.	Not Specified	AY141855	-″-
-″-	Bob2-2T	Ukraine	-″-	KC844296	-″-
-″-	VEVI2	Romania	-″-	KC844357	-″-

-″-	BF1	Hungary	-″-	KC844316	-″-
-″-	BRAT1	Slovakia	-″-	KC844300	-″-
-″-	VOL1	Macedonia	-″-	KC844326	-″-
-″-	1155-2	Canada	-″-	GU993820	-″-
Diaporthe ambigua	CMW 5288	South Africa	Outgroup	AF543817	-″-
Diaporthe ambigua	CMW 5288	South Africa	-″-	AF543818	-″-

Note: -″- = idem; *: H= hypovirulence; V= virulence.

Table 2. Outcomes of the electrophoresis profiles after PCRs with M13 and $(GACA)_4$ molecular markers.

	Polymorphic bands size (bp) after Minisatellite-Primed PCR*										Polymorphic bands size (bp) after Microsatellite-Primed PCR**								
	280	350	400	500	520	540	700	720	1100	1400	280	380	400	410	430	450	500	530	750
Virulent / Cryph8_1, Cryph9_2, Cryph10_3, Cryph11_4, Cryph12_5, Cryph13_6 Cryphy14_7, Cryph16, Cryph17, Cryph128, Cryph19, Cryph20, Cryph21 and Cryph22	+	-	+	-	+	-	+	-	+	-	+	-	+	+	-	-	+	+	-
Hypovirulent / Cryph1, Cryph2, Cryph3, Cryph4, Cryph5, Cryph6, Cryph7 and Cryph15	-	+	-	+	-	+	-	+	-	+	-	+	-	-	+	+	-	-	+

Note: Band present= "+"; Band absent ="-"; *= M13-primed PCR; **= $(GACA)_4$-primed PCR

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