Phenotypic and Molecular Investigations on Hypovirulent *Cryphonectria parasitica* in Italy

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Abstract

Chestnut blight is caused by the fungus *Cryphonectria parasitica*. As one of the most ecologically important diseases of *Castanea* spp., *C. parasitica* can rapidly kill trees. In Europe, mitigation of disease severity took place spontaneously through colonization of *C. parasitica* by mycoviruses, which reduced the virulence of the fungus. In the framework of a survey, 138 *C. parasitica* isolates were identified, and virulent/hypovirulent phenotypes were determined through morphological properties and pathogenicity tests. For a pool of four hypovirulent isolates, dsRNA was extracted, cDNA synthesized, and a library subjected to next-generation sequencing. The bioinformatics analysis allowed

detecting and reconstructing the complete genome of *Cryphonectria hypovirus 1* (CHV-1), denoted as CHV-1 Marche. When compared with the available genomes of other hypoviruses that affected the virulence of *C. parasitica*, available in databases, CHV-1 Marche showed some nucleotide diversity. The approach used in this study was effective to explore the virome inside a pool of hypovirulent *C. parasitica* isolates. Next-generation sequencing allowed us to exclude the presence of any other ssRNA and dsRNA viruses infecting the fungus and determine CHV-1 as the only responsible of hypovirulence of *C. parasitica* in the analyzed samples.

One of the most important and historical diseases of *Castanea* spp. is chestnut blight, which is caused by the Ascomycota fungus *Cryphonectria parasitica* (Murr) Barr (Rigling and Prospero 2017). This disease was first described in New York at the beginning of the last century. Then in Europe, *C. parasitica* was initially reported in 1938 in the Liguria region of Italy (Biraghi 1946), yet different introduction events were hypothesized to contribute to the current population structure as described by Dutech et al. (2010). Both in Italy and other areas in Europe, chestnut blight disease is less severe than for North America (Robin and Heiniger 2001), in part because the European chestnut (*C. sativa* Mill.) is less susceptible than *C. dentata*, and in part due to a reduction in the virulence of *C. parasitica*, a phenomenon deeply studied by several researchers (Grente 1965; Heiniger and Rigling 1994; MacDonald and Fullbright 1991; Milgroom and Cortesi 2004; Nuss 1992). Recovery from chestnut blight has been observed in many European countries and in some areas of the United States, such as in Michigan (Milgroom and Cortesi 2004). Particularly in Europe, this recovery was ascribed to the presence of hypovirulent strains of *C. parasitica*, which was infected by *Cryphonectria hypovirus 1* (CHV-1) (Robin and Heiniger 2001). This prevalence of CHV-1 and the consequent induced hypovirulence of *C. parasitica* have most likely prevented the occurrence of devastating chestnut blight epidemics and the destruction of the European chestnut forests to date.

CHV-1 is a non-encapsidated cytoplasmic virus of the *Hypoviridae* family (Ghabrial et al. 2015). The phylogeny and time estimates suggest that CHV-1 was introduced into Italy together with *C. parasitica*, and spread across southern-central Europe and then eastern Europe (Bryner et al. 2012), due to the low genetic diversity of the *C. parasitica* populations. Indeed, CHV-1 can be horizontally transmitted through hyphal anastomosis, which can occur efficiently between individual fungi that belong to the same vegetative compatibility group, but transmission can sometimes occur between genetically

close vegetative compatibility types (Liu and Milgroom 1996). In the European regions where this natural hypovirulence did not appear, rapid dissemination of CHV-1 was further aided by active bio-control efforts (Milgroom and Cortesi 2004). To date, chestnut blight incidence in Europe is high, but due to this hypovirulence, the disease is maintained at low severity in most regions (Bryner et al. 2012, 2014; Milgroom and Cortesi 2004).

CHV-1 infection leads to superficial swollen cankers (passive cankers), with reduced fungal growth and less sporulation compared with active cankers (Milgroom and Cortesi 2004). However, a lack of any clear relationship between canker morphology and virus infection for *C. parasitica* was recently reported (Bryner et al. 2014). Hypovirus-infected *C. parasitica* isolates are often white in color, and show reduced sporulation on potato dextrose agar (PDA) growth medium compared with noninfected isolates (Milgroom and Cortesi 2004). Recently, however, blight damage recurrence has been reported for different European chestnut areas that are highly infested by the chestnut gall wasp (*Dryocosmus kuriphilus*), even in the presence of hypovirulent cankers (Meyer et al. 2015).

Starting from the evaluation of hypovirulent isolates of *C. parasitica* recovered from chestnut areas in the Marche and Abruzzi regions of Italy, where the damage of chestnut blight has been particularly mitigated, we applied next generation sequencing to investigate ssRNA and dsRNA mycoviruses and in particular to reconstruct the CHV-1 full-length genome, mainly involved in the hypovirulence.

Materials and Methods

Isolation and morphological characterization. Within the framework of surveys carried out from 2013 to 2016 to determine the spread and severity of chestnut blight in the Marche and Abruzzi regions of Italy, cortical tissue from plants showing “active” (eight samples) and “passive” cankers (seven samples) were collected for each site, from plants approximately 10 m apart from each other.

Five bark samples per canker (i.e., one in the center, four along the margins) were removed with a cork borer (5 mm diameter) that was sterilized with 95% ethanol solution during canker sampling. The bark disks were divided into two parts, which were placed into Petri dishes (50 mm diameter; two pieces/plate) containing water agar (Duchefa Biochimie, Haarlem, The Netherlands), and incubated for 3 days at 25 ± 1°C in the dark. From the margin of each of the

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table is published online.

resulting fungal colonies, mycelial tips were collected and placed in Petri dishes containing PDA (Duchefa Biochimie), with further subculturing in the same medium to obtain pure colonies. Each fungal isolate was stored in a glass tube containing PDA at 4°C.

To characterize the morphological properties of each colony of *C. parasitica*, they were transferred to PDA Petri dishes (90 mm diameter) and incubated for 7 days at 25 ± 1°C in the dark. Fungal cultures were subsequently exposed to a photoperiod of 12 h for an additional 7 days, again at 25 ± 1°C. These conditions are suitable for morphological characterization of the *C. parasitica* isolates, and for distinguishing virulent colonies (orange mycelia, abundant pycnidia) from hypovirulent colonies (white mycelia, few or no pycnidia production) (Bissegger et al. 1997; Milgroom and Cortesi 2004).

Pathogenicity tests on chestnut cuttings. Based on the morphological characterization, 26 *C. parasitica* isolates (seven with orange and 19 with white mycelium) representative of the surveyed areas were selected and tested for pathogenicity on excised dormant chestnut stems. The virulence of each isolate was determined on stems (40 to 60 mm diameter) from healthy *C. sativa* plants. The stems were cut into 50-cm-long segments and surface disinfected with an aqueous solution of 70% ethanol. The bark of the chestnut cuttings was removed every 15 cm using a cork borer (5 mm diameter), and each wound was inoculated with a *C. parasitica* mycelial disc (5 mm diameter), then sealed with Parafilm. As a negative control, we used noninoculated PDA disks (5 mm diameter). Three isolates were assayed per cutting, with three replicates for each set of isolates tested. Both ends of each cutting were covered with cottonwool soaked with water, and incubated in a plastic box at 25°C under a 16-h photoperiod for 10 days. The length and width of the necrotic areas of the cortical tissue and the wood were then measured with digital calipers (model 500-196-30; Mitutoyo America Corporation, Kanagawa, Japan) and the elliptical necrotic areas were calculated.

The means and standard deviations (sd) were calculated. These data were analyzed statistically using one-way ANOVA, and the means were compared using Duncan's multiple range tests, at $P \leq 0.05$, using the Statistica package (Statsoft Inc., Tulsa, OK).

Reisolations of *C. parasitica* were carried out directly from necrotic lesions of cortical and wood tissue, according to the procedure already mentioned in the previous paragraph.

DNA extraction and molecular characterization of representative hypovirulent isolates. Based on morphological properties, pathogenicity, origin, and year of collection, four hypovirulent isolates (i.e., P16_2, P29B, L25A, L2A) were selected for total DNA extraction, according to Varanda et al. (2016). The DNA was used in PCR reactions with the ITS1/ITS4 primers (White et al. 1990), and sequenced at the Beckman Coulter Facility (Essex, U.K.). The raw sequences were analyzed by BLAST searches to look for sequence homology with other nucleotide sequences of *C. parasitica* available in GenBank (National Center for Biotechnology Information; NCBI). The vegetative compatibility of the selected isolates was also molecularly assessed according to Short et al. (2015).

dsRNA extraction. The same isolates of *C. parasitica* that were selected for total DNA extraction (i.e., P16_2, P29B, L25A, L2A) were tested for dsRNA. The isolates were inoculated in 40 ml potato dextrose broth (Duchefa Biochimie) at 25°C for 10 days under agitation (150 rpm). The mycelia were collected by centrifugation at 3,840 × *g* for 15 min, pressed between sterile sheets of paper for 24 h, and 10 g for each isolate were pulverized in liquid nitrogen. The dsRNA extraction was carried out according to Al Rwahnih et al. (2009). The dsRNA extracted was treated with 25 ng/ml RNase (Sigma Aldrich, Saint Louis, MO), 1 mg/ml DNase (Promega, Madison, WI), and 5 mg/ml proteinase K (Sigma Aldrich), to remove ssRNA, DNA, and protein, respectively. Aliquots of the purified dsRNA were run on 1% agarose gels, and images were acquired (Gel Doc XR; Bio-Rad, Hercules, CA).

Next-generation sequencing. Deep-sequencing of the extracted dsRNA samples was performed using Illumina technology. For library preparation, 800 ng dsRNA was subjected to heat denaturation (94°C for 12 min), and flash cooled in iced water. The fragmentation,

conversion to cDNA, and preparation for sequencing was performed using TruSeq RNA Sample Prep kit v2 (Illumina, San Diego, CA).

After PCR enrichment (15 cycles), the adapter-ligated cDNA fragments were quantified, diluted to 12 pM, and hybridized to a flow cell for Illumina sequencing. Clusters on the flow cell were then generated using TruSeq SR Cluster Kit v3-cBot-HS (Illumina). The flow cells with the DNA clusters were subjected to sequencing using the Illumina platform (HiScanSQ System, SELGE Network Sequencing Service; <http://www.selge.uniba.it/>), using SBS Sequencing v3 kit to generate 50-bp single-end reads.

Bioinformatics analysis and reconstruction of the CHV genome. High-quality (quality score ≥30) sequenced reads were filtered using Illumina Real-Time Analysis (RTA 1.13.48) and de novo assembled using CLC Genomics Workbench 7.0.3 (CLC Bio, Qiagen, Hilden, Germany) with standard parameters, and Velvet 1.1.06, followed by Oases 0.2.8 with a minimum contig length of 100 nt, and a multiple K-mer approach (Zhao et al. 2011). SeqMan Pro (Lasergene 10.1; DNASTAR, Inc., Madison, WI) was used for merging the resulting contigs. To search for homology to viral sequences, consensus sequences were subjected to BLASTN and BLASTX analysis against the NCBI database (www.ncbi.nlm.nih.gov), with the default parameters and the E-value cut-off of 10⁻³. The contigs were then classified according to the sequences with the highest bit score. Reference sequences were identified based on the BLAST results, and used in the subsequent analyses.

Transdecoder (<https://transdecoder.github.io/>) was used to identify open reading frames (ORFs) and to select the best assembly for the putative mycoviral sequences. Subsequently, sequencing reads were mapped back to the reference sequences of CHV-1 and the de novo assembled viral contigs, using SeqMan NGen and SeqMan Pro (Lasergene v.10.1; DNASTAR), to determine and correct miss-assembly errors, and to detect single nucleotide polymorphisms (SNPs).

Phylogenetic analysis. The consensus sequence identified as CHV-1 Marche was set up in FASTA format and aligned with the Clustal X software (Thompson et al. 1997), together with the nucleotide sequences of isolates CHV-1/EP-721 (GenBank accession no. DQ861913), CHV-1/EP713 (NC_001492), CHV-1/Euro7 (AF082191), and CHV-1/CN280 (KT726153). The phylogenetic tree was built using neighbor-joining in Molecular Evolution Genetic Analysis version 5 (MEGA5) (Tamura et al. 2011). The robustness of the tree topology was verified considering the bootstrap analysis (1,000 replicates). In the phylogenetic analysis, other hypoviruses that can infect *C. parasitica* were considered as outgroups, in particular CHV-2 (NC_003534), CHV-3 (NC_000960), and CHV-4 (NC_006431), the complete genomes of which are available in the NCBI database.

Results

Morphological characterization. From 150 samples of bark showing symptoms and signs of chestnut blight and collected in the framework of the surveys, 138 colonies of *C. parasitica* were recovered (Table 1). Using PDA medium with a 12-h photoperiod at 25°C, the majority of these isolates (85 out of 138) had orange mycelia and abundant pycnidia, and were thus recognized as virulent colonies (Fig. 1A). Fifty-three colonies grew with white mycelia and only sporadic pycnidia in the central part of the colonies, and were thus identified as hypovirulent (Fig. 1B).

Pathogenicity tests on chestnut cuttings. Significant differences were recorded between the *C. parasitica* virulent (orange) and hypovirulent isolates (white). The virulent isolates P4C4, P21A, P35D, and PD1 produced the widest elliptical necrotic areas on both the bark and the wood (Table 2). Conversely, the hypovirulent isolates showed generally slow growth and smaller necrotic areas (Table 2). After 7 days, from the reisolations of necrotic areas we obtained the corresponding virulent or hypovirulent isolates previously inoculated on the cuttings.

Molecular characterization of representative hypovirulent isolates. The DNA extraction of the four selected hypovirulent *C. parasitica* isolates, P16_2, P29B, L25A, and L2A, and the following

amplification with the ITS1/ITS4 primer pair yielded a specific band of ~600 bp, which showed high nucleotide homology with *C. parasitica* (GenBank accession no. KP824756). These four *C. parasitica* isolates belong to four different vegetative compatibility groups. Using multilocus PCR assays to assess *vic* genotype, P16_2 was confirmed molecularly as the vegetative compatibility genotype 1112-11 (corresponding to EU12), P29B as 2112-11 (EU17), L25A as 2211-22 (EU5), and L2A as 2112-22 (EU2).

dsRNA extraction. The dsRNA was extracted from a pool of the same four *C. parasitica* isolates, P16_2, P29B, L25A, and L2A, which showed similar morphologies and virulence but that were isolated from different locations. Only one band of 12,000 bp in size corresponding to L-dsRNA was obtained. No bands were visualized on the gels for P4C4, P21A, P35D, and PD1, representative virulent isolates of *C. parasitica* that were analyzed.

Sequencing and bioinformatics analysis of mycoviruses. The dsRNA was prepared for cDNA synthesis and library construction, followed by sequencing. A total of 19,068,188 short reads were initially generated in this study, which resulted in ~1 Gb of raw sequencing data (submitted in the NCBI Sequence Read Archive [SRA] database with submission number SUB2316937, under the accession number SRR5179114). There were 626,078 nonredundant reads. After filtering, only the high-quality reads (quality score ≥ 30) were considered for further analysis. To detect and identify putative mycoviral sequences in the selected isolates of *C. parasitica*, the reads were submitted to de novo assembly and analysis of the assembled contigs. CLC Genomics Workbench assembler produced 239 contigs, which

included many small sequences of <1 kb in length. Among the largest contigs, four that were 619, 2,015, 3,251, and 6,233 nt in length together contained 98.6% of the assembled reads and were identified as partial nonoverlapping sequences of the *Cryphonectria hypovirus 1* (CHV-1) genome (12,724 nts; GenBank accession no. DQ861913). Velvet, followed by Oases for the de novo RNA-Seq assembly were used to assemble the complete viral genome from the reads into a single contig. Most of the contigs produced at different k-mer values (i.e., 37-21), which ranged between 451 and 591, were clearly overlapping and showing only small differences in their sequences.

The largest assembled sequence (13,013 bp) that shared the highest sequence identity with the CHV-1 genome and the best candidate coding regions within its sequence was selected as the template for realignment of the reads and for manual curation. The complete viral genome of the CHV-1 that infects *C. parasitica* isolates from the Marche region in Italy (CHV-1_Marche) was conclusively obtained and has been submitted to GenBank (accession no. KY471627). This is 12,735 nt in length, has a sequence identity of 98% with CHV-1 (i.e., DQ861913, AF082191) (E-value = 0.0), and contains two putative ORFs of 622 and 3,164 amino acids, both with 99% amino acid identity to the two polyproteins identified as ORFA (ABI64295.1) and ORFB (ABI64296.1), respectively, in CHV-1. A total of 18,664,706 viral reads (97.9% of the total reads) was mapped to the genome consensus sequence, with an average coverage of 82,223x. The read depth was enough to obtain some preliminary data on the viral diversity in the analyzed CHV-1 population from this pool of four *C. parasitica* isolates. Single nucleotide variants were

Table 1. Morphological features associated with virulent (V) and hypovirulent (H) *Cryphonectria parasitica* isolates collected during the surveys from 2013 to 2016

Region	Location	Isolates	No. of isolates	Morphological features associated with V and H <i>C. parasitica</i>	Year of collection
Marche	Montemonaco (AP)	P1, P2, P3, P4, P4C4, P7, P8, P9, P11, P13b	12	V	2013
		PA2, PD1			
	Rotella (AP)	P3B, P4C2, P5, P6D, P6C, P10C, P11C, P12, P13A, PA1, PC	11	H	
		P14, P15, P16, P17, P18, P19C	6	V	2013
	Colle San Marco (AP)	P14-1, P16_2, P19A, P20	4	H	
		P40, P41, P42, P44	4	V	2013
	Colle, Ascoli Piceno	P40B, P43	2	H	
		L24b, L24a, L26A	3	V	2015
	San Gregorio, Ascoli Piceno	L25A, L26B	2	H	
		D3A, D4A	2	V	2016
	Montefortino (AP)	D1A, D2B, D6B, D9B, D4B, L2A	6	H	
		P29c, P30, P31A	3	V	2013
	Collefalciano, Acquasanta Terme (AP)	P28b, P29B, P30D	3	H	
		P21A, P22B, P23, P25B, P26, P27	6	V	2013
	Pozza, Acquasanta Terme (AP)	P21C, P22A, P24, P25	4	H	
		L16A, L16b, L17A, L17b, L18b, L18	9	V	2015
	Umito, Acquasanta Terme (AP)	E4A, E6A, E8 B			2016
		E2B, E3B, E7B bis	3	H	2016
		L19a, L19B, L20, L21a	9	V	2015
		A3A, A4B, A8A, A9A, A15B			2016
Abruzzi	Colle Frattale, Acquasanta Terme (AP)	L21b, L22A, L22b	8	H	2015
		A2A, A5C, A6A, A7A, A10B			2016
	Rocca di Montecalvo, Acquasanta Terme (AP)	L27a, L28A, L29a	3	V	2015
		L28c	1	H	
	Teramo (TE)	C5B, C6A	2	V	2016
		C3B, C4A	2	H	
	Pianaccio, Valle Castellana (TE)	P33, P34, P35D, P36, P38, P39	6	V	2013
		P32, P37A1, P38A2	3	H	
	Morrice, Valle Castellana (TE)	L1a, L1c, L3a, L4, L5a, L5b, L6A, L6b, L7	9	V	2014
		L8, L9A, L9b, L10c, L11, L12a, L13a, L13b, L15a, L15b	10	V	2014
	Valle Castellana (TE)	L14A	1	H	
		B3B	1	V	2016
		B5B, B6B, B9B	3	H	

identified by applying a read depth threshold of 50, with these recognized as putative SNPs when present in at least 20% of the reads (Supplementary Table S1). Out of the 125 nucleotide substitutions detected throughout the CHV-1 genome, 106 (84.8%) were conservative (pyrimidine to pyrimidine, or purine to purine). In detail, four SNPs were identified outside the coding regions, and of the remaining SNPs, 77 (61.6%) were synonymous or silent substitutions that did not affect the protein sequence, while 44 resulted in amino acid changes in the ORFA (11) and ORFB (33) polyproteins.

Phylogenetic analysis. The phylogenetic analysis carried out using the MEGA 5 software included the CHV-1 Marche genome (KY471627) in the cluster with the other CHV-1 genomes of European and Asiatic origin, which are phylogenetically different (as demonstrated by the bootstrap values) from the other hypoviruses (i.e., CHV-2, CHV-3, CHV4) that can infect *C. parasitica* (Fig. 2).

Discussion

Chestnut blight is common in the Marche region, where its incidence ranges from 20 to 60%. The severity of the symptoms appears to be related to the age of the plants, and mostly to the silvicultural

practices adopted (Amorini et al. 2001). In some situations, the severity of decline in chestnut is related to the infestation of the chestnut gall wasp (Meyer et al. 2015). During these surveys carried out from 2013 to 2016, *C. parasitica* was isolated and identified in about 90% of the bark samples.

Most of these isolates (i.e., 85 out of 138) were virulent, showing typical morphological features and resulting in wide necrotic areas during the pathogenicity tests. These were isolated from active cankers, on which perithecia were sporadically identified. In Europe, the production of perithecia has been observed in several countries, including Italy, although at medium to low frequency (Prospero and Rigling 2013). On the other hand, 53 hypovirulent *C. parasitica* isolates were obtained and characterized by white mycelia, reduced colony growth, and differentiation of few pycnidia. As demonstrated in previous investigations, these anomalies are caused by viruses that can infect *C. parasitica*, which can induce several phenotypic changes in this fungus, such as a reduction in asexual sporulation, inhibition of sexual reproduction, altered colony morphology, and reduced pigmentation (Nuss 2005). Mycovirus-induced hypovirulence has been reported not only in *C. parasitica*, but also in *Ophiostoma ulmi*, *O. novo-ulmi*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *S. homoeocarpa*, and *Rosellinia necatrix* (Ghabrial et al. 2015).

The *C. parasitica* hypovirulent isolates P16_2, P29B, L25A, and L2A were chosen as representative isolates and next submitted to more detailed molecular characterization. Molecular identification according to the analysis of internal transcribed spacer (ITS) nucleotide sequences confirmed the identity of these fungal isolates. In particular, the ITS region appeared appropriate for molecular identification and to establish the phylogenetic relationships, even with closely related fungal species (Myburg et al. 2004).

To determine the vegetative incompatibility gene profiles of the selected isolates of *C. parasitica*, multilocus PCR assays were carried out as described by Short et al. (2015). This new approach is in agreement with the genetically determined vegetative incompatibility genotypes (Cortesi and Milgroom 1998), and it is generally equivalent to the culture-dependent assays. These culture-dependent assays are time consuming and require well-maintained in-house EU tester strains, and they are not always easy when interpreting

Fig. 1. Representative virulent (A) and hypovirulent (B) colonies of *Cryphonectria parasitica* isolated during the surveys, cultivated for 15 days with a 16-h photoperiod, at 25°C.

Table 2. *Cryphonectria parasitica* isolates used in the pathogenicity tests with chestnut cuttings^z

Isolate	Morphological features	Cortical necrotic area (mean $\pm$ sd; cm ²)	Wood necrotic area (mean $\pm$ sd; cm ²)
P4C4	Orange	7.3 $\pm$ 0.3 a	10.2 $\pm$ 0.6 a
P21A	Orange	6.5 $\pm$ 1.1 bc	7.0 $\pm$ 1.3 b
PD1	Orange	5.4 $\pm$ 0.5 de	6.8 $\pm$ 0.2 b
P35D	Orange	6.9 $\pm$ 2 b	6.3 $\pm$ 1.8 b
P31A	Orange	4.4 $\pm$ 1.1 e-g	5.1 $\pm$ 1.4 c
P22B	Orange	5.3 $\pm$ 0.7 cd	5.0 $\pm$ 0.5 c
P10C	White	4.4 $\pm$ 0.6 ef	4.0 $\pm$ 0.6 d
P14-1	White	3.4 $\pm$ 0.3 f-i	3.5 $\pm$ 0.5 de
P40B	White	3.7 $\pm$ 1.1 f-h	3.4 $\pm$ 1 d-f
P13A	White	2.8 $\pm$ 0.5 f-l	2.8 $\pm$ 0.8 d-g
PC	White	2.3 $\pm$ 0.5 l-n	2.7 $\pm$ 0.7 e-h
P16_2	White	2.9 $\pm$ 0.2 g-l	2.5 $\pm$ 0.1 e-h
P6C	White	2.7 $\pm$ 0.8 h-m	2.5 $\pm$ 0.7 e-h
P19A	White	2.6 $\pm$ 0.6 h-n	2.5 $\pm$ 0.7 e-h
P25	White	2.5 $\pm$ 0.2 h-n	2.5 $\pm$ 0.3 e-h
P9	Orange	2.6 $\pm$ 1 h-n	2.4 $\pm$ 0.8 e-h
P38A2	White	2.4 $\pm$ 0.5 h-n	2.3 $\pm$ 0.8 f-h
P43	White	2.2 $\pm$ 0.1 i-n	2.3 $\pm$ 0.5 e-h
P12	White	2.6 $\pm$ 0.2 h-n	2.2 $\pm$ 0.3 f-h
P30D	White	2.5 $\pm$ 0.7 h-n	2.2 $\pm$ 0.6 f-h
P29B	White	2.4 $\pm$ 0.5 h-n	2.1 $\pm$ 0.6 gh
P11C	White	2.3 $\pm$ 1 i-n	2.1 $\pm$ 0.9 gh
L25A	White	2.3 $\pm$ 0.7 i-n	2.0 $\pm$ 0.6 gh
PA1	White	1.8 $\pm$ 0.3 l-o	1.6 $\pm$ 0.2 hi
P41	White	1.4 $\pm$ 0.3 n-o	1.5 $\pm$ 0.1 hi
L2A	White	1.5 $\pm$ 0.2 m-o	1.4 $\pm$ 0.4 hi

^z Data followed by different letters are significantly different (Duncan's multiple range tests; $P < 0.05$).

barrage reactions (Short et al. 2015). These *C. parasitica* isolates that originated in Marche show four main vegetative incompatibility genotypes that correspond to the vegetative compatibility groups EU12, EU17, EU5, and EU2.

L-dsRNA (~12 kb) was detected in all the four hypovirulent *C. parasitica* isolates, which is complete replicative form. M-dsRNA (8 to 10 kb) and S-dsRNA (0.6 to 1.7 kb), which represent deleted forms of L-dsRNA that can persist during replication (Montenegro et al. 2008), were not found. To date, mycoviruses have commonly been detected following the isolation of their dsRNA. Today, NGS has become feasible to use as whole-genome and metagenomics (Mokili et al. 2012) approaches, to screen fungi for the presence of potential novel viruses. Recently, Schoebel et al. (2014) detected mycoviruses in the plant pathogenic fungus *Hymenoscyphus fraxineus* through a combination of NGS with bioinformatics.

The approach used in the present study involved dsRNA isolation and fragmentation, and cDNA library preparation, with Illumina-based sequencing, de novo assembly, and identification of putative sequences of ssRNA and dsRNA virus. This was effective and accurate to determine the virome inside a pool of hypovirulent isolates of *C. parasitica*.

To the best of our knowledge, this is the first time that the CHV-1 genome has been reconstructed by NGS, and the quality is comparable to that of sequence genomes obtained by the Sanger method and available in the NCBI database. Indeed, Khalifa et al. (2016) recently demonstrated that the accuracy of the sequences obtained using the Illumina platform is comparable with Sanger sequencing analysis. Moreover, to enhance the accuracy of the Illumina analysis, we started from purified dsRNA to maximize the proportion of viral sequences in the data, while using sufficiently rigorous quality controls and maintaining the level of depth and coverage of the sequencing data.

In this situation, we started from a pool of four isolates that shared morphological characteristics and virulence, but were collected from different locations within the Marche region, and only the viral genome of CHV-1 was detected. It is known that *C. parasitica* can be the host of several hypoviruses, reoviruses, and crysovirus (Ghabrial et al. 2015). From the phylogenetic analysis, it was clear that CHV-1 Marche is in the same cluster with the other CHV-1 genomes available in the NCBI database. In particular, CHV-1 Marche belongs to subtype I, as well as CHV-1/EP721 and CHV-1/Euro 7, whose origin is Italy. It is slightly divergent from CHV-1/EP713 belonging to subtype F1, whose origin is France, and more consistently different from CHV-1/CN280 belonging to subtype CN6, whose origin is China (Du et al. 2017). On the other hand, CHV-1 Marche is clearly different from other hypoviruses that can infect *C. parasitica*, particularly CHV-2, CHV-3, and CHV-4. It was interesting to observe that CHV-2 is the hypovirus closest to CHV-1 and they have similar genome lengths. Conversely, CHV-3 and CHV-4 are genetically more distant, because they have shorter genomes (by about 9 kbp) and cause lower degrees of hypovirulence (Ghabrial et al. 2015).

The simplicity of the sample composition here allowed more detailed sequence analysis, which revealed interesting genetic diversity

Fig. 2. Phylogenetic tree constructed from the analysis of the complete genomes of CHV-1, CHV-2, CHV-3, and CHV-4, as available from GenBank.

inside the analyzed pool, although the origin is restricted. A wider genetic diversity was recorded by Feau et al. (2014), who analyzed several samples belonging to different CHV-1 subtypes and whose origins were different. Our study confirmed that in Italy, and in particular the Marche region, CHV-1 subtype I is the only one recorded, as well as in other European countries (Croatia, Greece, Hungary, Macedonia, Slovenia, and Switzerland) (Allemann et al. 1999; Prospero and Rigling 2013). On the other hand, different CHV-1 subtypes were recorded in Spain (D, E, F1, I) (Montenegro et al. 2008; Trapiello et al. 2017) and France (F1, F2, I) (Feau et al. 2014), where a multiple introduction of hypovirulence was hypothesized.

Moreover, in the present study, the genetic diversity is more related to silent substitutions (81), although these also include SNPs (44) causing amino acid substitutions in the two coding regions of the CHV-1 genome (i.e., ORFA, ORFB). The intraspecific variability that was recorded for the pool of four isolates is very common for RNA viruses, which are characterized by complex evolutionary dynamics, high mutation rates, and rapid replication kinetics. Moreover, the presence of silent and non-silent substitutions in ORFA and ORFB might be the result of the interactions between the CHV-1 genome and *C. parasitica*. In particular, viruses have countermeasures to escape host antiviral responses. Many RNA silencing suppressors that target different silencing stages have been reported, and these are diverse in their amino acid sequences and protein structures (Segers et al. 2006). Like pathogenic viruses of mammals, insects, and plants, hypoviruses also encode protein suppressors of RNA interference. The papain-like protease p29 that is encoded by CHV-1 functions as a suppressor of RNA silencing in the natural fungal host (Segers et al. 2006).

In conclusion, the application of NGS is a valid and accurate tool to explore the virome of *C. parasitica* hypovirulent isolates, to analyze the full-length genome of CHV-1, and to exclude the presence of additional ssRNA and dsRNA mycoviruses potentially involved in the hypovirulence.

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