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Development of a TaqMan qPCR assay for the detection and quantification of Gnomoniopsis castaneae in chestnut tissues

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Abstract

A novel real-time PCR assay based on the TaqMan probe was developed for the detection of *Gnomoniopsis castaneae*, causal agent of brown rot of chestnut kernels, and responsible for leaf necrosis, shoot blight and bark canker. A part of the pathogen life cycle is endophytic, colonizing all tissues of chestnut and additional hosts, which is suspected to play a key role in its epidemiology. Thus, a molecular tool for sensitive detection and quantification of *G. castaneae* in symptomatic and asymptomatic host tissues is urgently required to better understand *G. castaneae* ecology, biology and epidemiology. Primers and a species-specific probe for *G. castaneae* were designed based on the sequence of the single-copy elongation factor 1 alpha (EF1α) gene. The amplification efficiency of target DNA was 105.3% and the limit of detection of the assay was calculated at approximately 40 fg of pure fungal DNA. The pathogen was consistently detected in artificial mixtures of plant and pathogen DNAs with the same Limit of Detection (LOD) as pure fungal DNA. In naturally infected samples, the assay rapidly revealed the presence of the pathogen in all symptomatic specimens, as well as in asymptomatic tissues. Notably, a significant relationship between the results of a metagenomic HTS analysis and the qPCR assay on DNAs extracted from bulk fruit was found. This molecular tool will be of substantial aid in detecting and quantifying *G. castaneae*, even in the endophytic state, and in different host tissues.

1 | INTRODUCTION

Gnomoniopsis castaneae G. Tamietti (syn. *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest) (Crous et al., 2012; Tamietti, 2016; Visentin et al., 2012) is an emerging fungal pathogen causing brown rot of *Castanea sativa*, *C. mollissima* and the hybrids *C. sativa x C. crenata* (Sakalidis et al., 2019). The disease severely impacts fruit production and marketing in Europe with very high incidence, as for Switzerland with 91% of infected fruits (Dennert et al., 2015) and Italy, up to 93.5% (Lione et al., 2015). Brown rot of kernels is also reported in Australia (Shuttleworth et al., 2013), North America (Sakalidis et al., 2019) and Chile (Vannini, unpublished). *Gnomoniopsis castaneae* can also incite shoot blight and leaf necroses on chestnut as well as bark cankers on both chestnut (Dar & Rai, 2015) and hazelnut (*Corylus avellana*), as exhaustively reviewed by Lione et al., (2019). The ecology, biology and epidemiology of this fungus are particularly complex. *Gnomoniopsis castaneae* is a cryptic species commonly found as an endophyte in all tissues of chestnut and additional hosts such as *Quercus* spp., *Fraxinus ornus* L. and *Pinus pinaster* Ait. (Lione et al., 2019). The severe impact of the pathogen in the last decade was associated with a massive presence of inoculum in the environment boosted by climate change

(Lione et al., 2015), in synergy with infestation by the Chinese Gall Wasp, *Dryocosmus kuriphilus* Yasumatsu (Fernández et al., 2018; Magro et al., 2010; Vannini et al., 2017). The colonization and necroses caused by Chinese Wasp galls are believed to start from the endophytic inoculum (Vannini et al., 2018), while indirect evidence supports floral infection by external inoculum as the main pathway of fruit colonization and rot (Shuttleworth & Guest, 2017). Although artificial inoculations reproduced the symptoms, it is not yet clear how the pathogen infection process leads to the development of bark cankers (Pasche et al., 2016). In this context, the endophytic behaviour of *G. castaneae* might play a key role in the biology of the fungus and its epidemiology. The possibility of monitoring the distribution of the inoculum in the different tissues and the patterns of endophytic inoculum accumulation as a function of host phenology and environmental parameters is a paramount requirement in clarifying the biology of the fungus and understanding the ability of this organism to shift from an endophytic to a pathogenic phase. At the moment, no protocols are available to monitor the presence of *G. castaneae* in chestnut tissues other than the classical biological detection through isolation in pure culture, highly specific but of low sensitivity (Manias et al., 2020). Quantitative PCR (qPCR) represents a powerful, accessible tool to address this issue. Thus, the aim of this work was to design and develop a highly sensitive and specific TaqMan assay for the detection of *G. castaneae* and submit it to a robust set of validation tests, including a comparison with parallel metagenomic data.

2 | MATERIALS AND METHODS

2.1 | Primer and probe design

A preliminary screening was carried out to select the most informative DNA regions for *G. castaneae* in fungal barcoding genes (i.e. ITS rDNA, partial β-tubulin, nuclear ribosomal RNA gene large subunit [LSU] and translation elongation factor 1 alpha EF1α). Available sequences of *G. castaneae* and additional *Gnomoniopsis* spp. were downloaded from the NCBI database and aligned with MUSCLE, implemented in Unipro UGENE v.37 (Okonechnikov et al., 2012). The most favourable regions for the primers and probe design were manually selected and Primer3, implemented in the same UGENE software, was used with the default search criteria to precisely identify the regions. Primers and probe were synthesized by Eurofins Genomics. The TaqMan probe was labelled with the reporter dye FAM (6 -carboxyfluorescein) on the 5′ end, and the quencher BHQ1 (Black Hole Quencher 1) on the 3′ end.

2.2 | Fungal strains and DNA extraction

To validate the assay, 17 isolates of *G. castaneae* from different sampling sites and years, 3 strains of a different *Gnomoniopsis* species and 13 additional fungal taxa commonly isolated from chestnut tissues, were obtained from the DIBAF fungal collection of the Regional project Sancast (www.sancast.it). Taxonomic details of isolates and GeneBank accession numbers of ITS rDNA sequences for representative strains are listed in Table 1.

Pure isolates were subcultured to PDA and incubated at 27°C in the dark for 7 days before scraping the mycelium from the agar surface and extracting the DNA using the NucleoSpin Plant II mini kit (Macherey Nagel), following the manufacturer's instructions. DNA concentration was measured with Qubit (Thermo Fisher) using the High Sensitivity dsDNA Assay kit. Extracted DNA was stored at −20°C until further analysis.

2.3 | Field samples and DNA extraction

A series of 26 samples including 17 individual fruits (10 symptomatic and seven asymptomatic), five leaves and four twigs were collected from chestnut trees in the Monti Cimini area in September/October 2020 (Table 2). Tissues from these samples were divided into two parts after surface sterilization: one half was used in a standard isolation procedure on PDA, whereas the remaining part was ground in a TissueLyser II (Qiagen, Hilden) and 200 mg of the powder used for total DNA extraction with the same kit described above. To obtain pure and putative endophytes-free chestnut DNA, the same method was applied to *in vitro* plantlets of *C. sativa*, kindly provided by Dr. Beatriz Cuenca Valera (Grupo TRAGSA–Sepi). Finally, for comparative metagenomic analysis DNA was extracted from 15 bulk samples, obtained by separating the endocarp and pericarp from 500 g fresh chestnut fruit, and grinding them independently, as above. All extracted DNA was stored at −20°C until further analysis.

2.4 | Tuning qPCR assay

After a series of optimization experiments aimed to determine the best performing concentration for primers and probe (data not shown), the qPCR reaction mix comprised 10 μl 2× GoTaq Probe qPCR Master Mix (Promega), 0.5 μM each primer, 0.3 μM probe, and variable aliquots of DNA according to the aim of the experiment; ultrapure water was added to a final volume of 20 μl and also used as negative control (No Template Control–NTC). Amplifications were performed in a RotorGeneQ (Qiagen, Hilden) under the following conditions: initial denaturation step at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 45 s. Fluorescence was measured once per cycle at the end of the 60°C segment and the Cq values automatically determined by the device.

2.5 | qPCR performance testing

Primers and probe were designed to provide the best discrimination at species level, that is maximizing the number of mismatches in

TABLE 1 List of fungal taxa used in the present study. Their source tissue, sampling site and accession numbers (when available) of ITS rDNA sequences in NCBI database are indicated

Code	Fungal species	Plant tissue	Sample origin (site, region, country, year)	Accession number	qPCR detection
GN01	*Gnomoniopsis castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	MW494885	+
AM	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
3c	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
M7	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
5s	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
9s	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
1m	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
3m	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
7m	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
8m	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2020	n.a.	+
G68	*G. castaneae*	wood	Zagarise, Calabria, Italy, 2012	n.a.	+
G69	*G. castaneae*	wood	Zagarise, Calabria, Italy, 2012	n.a.	+
M6PerA	*G. castaneae*	fruit	Monti Cimini, Latium, Italy, 2010	n.a.	+
BIA	*G. castaneae*	fruit	Switzerland	n.a.	+
TAV	*G. castaneae*	fruit	Switzerland	n.a.	+
CAS	*G. castaneae*	fruit	Switzerland	n.a.	+
1a	*G. castaneae*	fruit	Chile, 2018	n.a.	+
Gia2	*G. sp.*	fruit	Japan, 2012	n.a.	-
Gia1M	*G. sp.*	fruit	Japan, 2012	n.a.	-
Gia1S	*G. sp.*	fruit	Japan, 2012	n.a.	-
ct01	*Aureobasidium pullulans*	twig	Monti Cimini, VT, Italy	MW494940	-
ct02	*Cladosporium cladosporoides*	twig	Monti Cimini, VT, Italy	MW494941	-
ct04	*Alternaria tenuissima*	leaf	Monti Cimini, VT, Italy	MW497610	-
ct06	*Penicillium glabrum*	fruit	Monti Cimini, VT, Italy	MW497642	-
ct07	*Penicillium thomii*	fruit	Monti Cimini, VT, Italy	MW497611	-
ct08	*Cladosporium perangustum*	twig	Monti Cimini, VT, Italy	MW497612	-
ct09	*Colletrichum fioriniae*	leaf	Monti Cimini, VT, Italy	MW494943	-
ct14	*Alternaria alternata*	leaf	Monti Cimini, VT, Italy	MW494944	-
ct15	*Epicoccum nigrum*	leaf	Monti Cimini, VT, Italy	MW494945	-
ct17	*Sordaria fimicola*	leaf	Monti Cimini, VT, Italy	MW494947	-
ct18	*Nigrospora sphaerica*	leaf	Monti Cimini, VT, Italy	MW494948	-
ct22	*Didymella pomorum*	leaf	Monti Cimini, VT, Italy	MW494949	-
ct0gr	*Ceratocystis adiposa*	leaf	Monti Cimini, VT, Italy	MW494951	-

Abbreviation: n.a, not available.

primers and, more importantly, in the probe. To test these factors, a preliminary *in silico* check of the specificity of the whole assay was carried out by NCBI BLAST analysis on the NCBI nucleotide collection (nr/nt) database. Subsequently, this analytical feature of the assay was wet-lab tested using the panels of DNA extracted from the fungal taxa listed in Table 1.

To assess the efficacy of the assay for detecting the pathogen, the efficiency was evaluated through the proportionality of Cq values in respect to the amount of target template DNA and sensitivity measured according to the limit of detection (LOD) and limit of quantification (LOQ) of target DNA, respectively defined as the lowest concentration of target DNA at which 95% of the positive samples can be detected or quantified. Analytical parameters were tested on a set of 10-fold serially diluted DNA from *G. castaneae* strain GN01, ranging in concentration from 10 ng μl^{-1} to 10 fg μl^{-1}. Five replications were amplified for each dilution to prepare the standard curve. LOD and LOQ were estimated using a curve-fitting modelling approach (Merkes et al., 2019) with the R script code available at https://github.com/cmerkes/qPCR_LOD_Calc.

Repeatability was determined on 10 replicates of 4 standard DNA concentrations (10 ng, 1 ng, 100 pg and 10 pg per PCR), while reproducibility was assessed on 3 replicates of standard DNA at the

TABLE 2 Results of qPCR assay on DNA extracted directly from different chestnut tissues. symptomatic and not. Kernel samples were incubated alongside on PDA for fungal isolation

Sample code	Plant tissue	Presence of symptoms	*G. castaneae* isolation	Cq value (mean ± SD)
K1M	kernel embryo	yes	yes	21.37 ± 0.12
K3M	kernel embryo	yes	yes	19.04 ± 0.25
K7M	kernel embryo	yes	yes	23.52 ± 0.33
K8M	kernel embryo	yes	yes	22.01 ± 0.40
KF24	kernel embryo	yes	yes	23.92 ± 0.16
K7N1	kernel embryo	yes	yes	21.84 ± 0.15
K7N3	kernel embryo	yes	yes	28.16 ± 0.19
K8R3	kernel embryo	yes	yes	23.71 ± 0.19
K8R9	kernel embryo	yes	yes	24.41 ± 0.07
K9N4	kernel embryo	yes	yes	25.01 ± 0.16
KF12	kernel embryo	no	yes	28.56 ± 0.08
K2S	kernel embryo	no	no	-
K4S	kernel embryo	no	no	-
K5S	kernel embryo	no	yes	21.43 ± 0.16
K7S	kernel embryo	no	no	33.56 ± 0.52
K8S	kernel embryo	no	no	35.32 ± 0.47
K9S	kernel embryo	no	yes	23.74 ± 0.38
F1	leaf	no	n.a.	-
F2	leaf	no	n.a.	-
F3	leaf	no	n.a.	35.25 ± 0.20
F4	leaf	no	n.a.	31.57 ± 0.03
F5	leaf	no	n.a.	-
R1	twig	no	n.a.	-
R2	twig	no	n.a.	33.33 ± 0.50
R3	twig	no	n.a.	35.28 ± 0.53
R4	twig	no	n.a.	31.34 ± 0.32

same concentrations in four different PCR runs performed by different operators on different days with different primers and probe preparations.

The effect of inhibitors potentially contained in chestnut tissues was tested using DNA extracted from endophyte-free *in vitro* plantlets of *Castanea sativa*. Fifty nanograms of plant DNA was spiked with increasing DNA concentrations of the pathogen (10 ng, 1 ng, 100 pg, 10 pg and 1 pg per reaction, 3 replicates each).

The overall performance of the qPCR assay was assessed based on the criteria described by Broeders et al., (2014) for efficiency (90–110%), linearity ($R^2 \geq 0.98$), repeatability (relative standard deviation ≤25%) and reproducibility (relative standard deviation ≤25%).

2.6 | qPCR validation on naturally infected samples

The qPCR assay was finally tested on the DNA extracted from 26 samples of fruit, leaves and twigs collected from chestnut trees exposed to natural infection by *G. castaneae* in the Monti Cimini area described above.

Validation was also attempted on the DNA of enriched fungal libraries from 15 bulk samples of fruit, double checked for the presence of *G. castaneae* with High Throughput Sequencing (HTS). Briefly, the ITS1 region was amplified with a dual indexing primer using the tagged primer pair ITS1F (5′-xxxxCTYGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-xxxxGCHRCGTTCTTCATCGDTGC-3′), where xxxx represents the barcoding key (Morales-Rodriguez et al., 2019). Amplicons were purified using MagJET NGS Cleanup (Thermo Scientific), quantified with Qubit (Invitrogen, USA) and pooled at equal concentrations for sequencing. Paired-end sequencing (2 × 300 bp) was carried out on an Illumina MiSeq sequencer by Eurofins Genomics GmbH. Data sets were analysed and Operational Taxonomic Units (OTUs) assigned following the pipeline described by Morales-Rodriguez et al. (2021). Data on bulk samples from both qPCR and HTS were analysed through a simple linear regression using GraphPad Prism version 9.0.1 (GraphPad Software) to score possible correlations. For each number of HTS reads, five qPCR repetitions were considered, for a total of 75 points overall. The goodness of the regression was assessed by the coefficient of determination, R^2 and statistical probability calculated with a F-test.

3 | RESULTS

3.1 | Primer and probe design

Among the four barcoding genes initially trialled, the elongation factor 1 alpha (EF1α) was selected because of the presence of regions with significant differences between the available species in the genus *Gnomoniopsis*, but at the same time highly conserved in *G. castaneae*. Thirty-one sequences of *G. castaneae* and 34 of 13 other *Gnomoniopsis* species, together with six sequences of *Melanconis marginalis* subsp. *marginalis* (the closest species returned by the blast search apart from *Gnomoniopsis* spp.) were downloaded and aligned. Figure 1 shows the alignment of the regions corresponding to the selected primers and probe in those sequences. The amplicon of *G. castaneae* was estimated to be 193 bp, differing in length from most of the other *Gnomoniopsis* species that ranged from 183 bp of *G. macounii* to 199 bp of *G. clavulata*. However, the specificity of the assay relied on the unique nature of primers and probe sequences. First, the sequences of the primer pair and of the probe were perfectly conserved in all 31 strains of *G. castaneae*. Conversely, considering the 34 sequences of the 12 non-target *Gnomoniopsis* species, the sequence of the forward primer GC-F1 (5′-AAACGTGACCCACTTTACCG-3′) ranged from a minimum of four to a maximum of six mismatches, the reverse primer GC-R3 (5′-TCAGCCTTCAGCTTGTCCAG-3′) had 0 to 1 mismatches, whilst the probe GC-Probe (5′-CCACCCCATCTTCTTGTTGT-3′) mismatches ranged from 11 (*G. comari*) to 17 (*G. racemula*) out of 20 bp.

The *in silico* blast of the primer pair to the NCBI GenBank database returned no sequences without mismatches on the primers other than the target EF1α-gene fragment of *G. castaneae*. This finding, together with the highly specific probe sequence, suggested that the assay had the desired specificity.

3.2 | qPCR performance

3.2.1 | Specificity

The specificity of the assay was wet-lab validated by amplification of DNAs obtained from 17 isolates of *G. castaneae* from different sampling sites and years, three strains of a different *Gnomoniopsis* species, and 13 fungal species commonly isolated from chestnut tissues or organs. *Gnomoniopsis castaneae* DNAs were consistently amplified, whilst all the other species, including *Gnomoniopsis* sp., gave no amplification signal (Table 1).

3.2.2 | Sensitivity

Amplification of pure DNA from strain GN01 was always proportional to its concentration. The standard curve generated by plotting five repetitions of each log DNA concentration against the Cq value as determined by qPCR, resulted in a linear response over six logs, from 10 ng to 100 fg, with a high correlation coefficient ($r^2 = 0.991$; Figure 2). Reaction efficiency, determined from the slope of the log-linear portion of the standard curve, reached 105.3%, perfectly fitting the 90–110% range of acceptable values. LOD and LOQ were 114 fg and 39.30 fg, respectively (Figure 2).

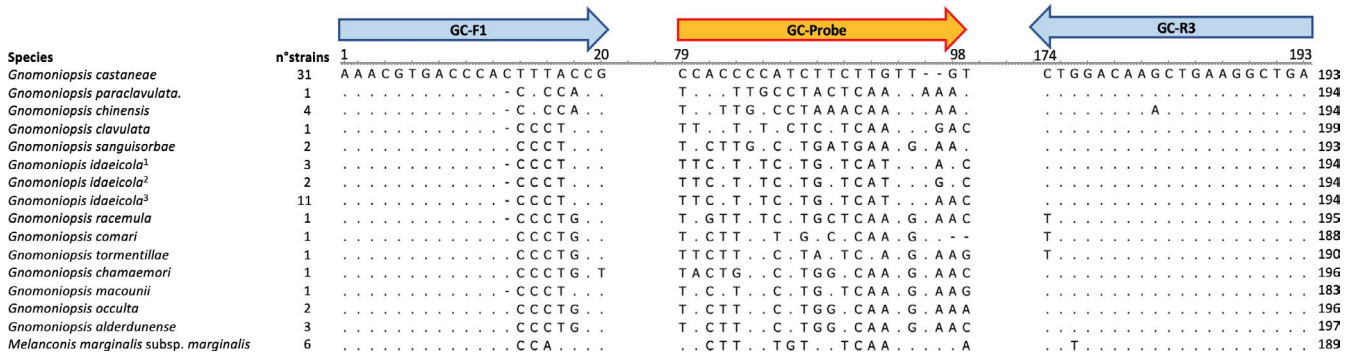
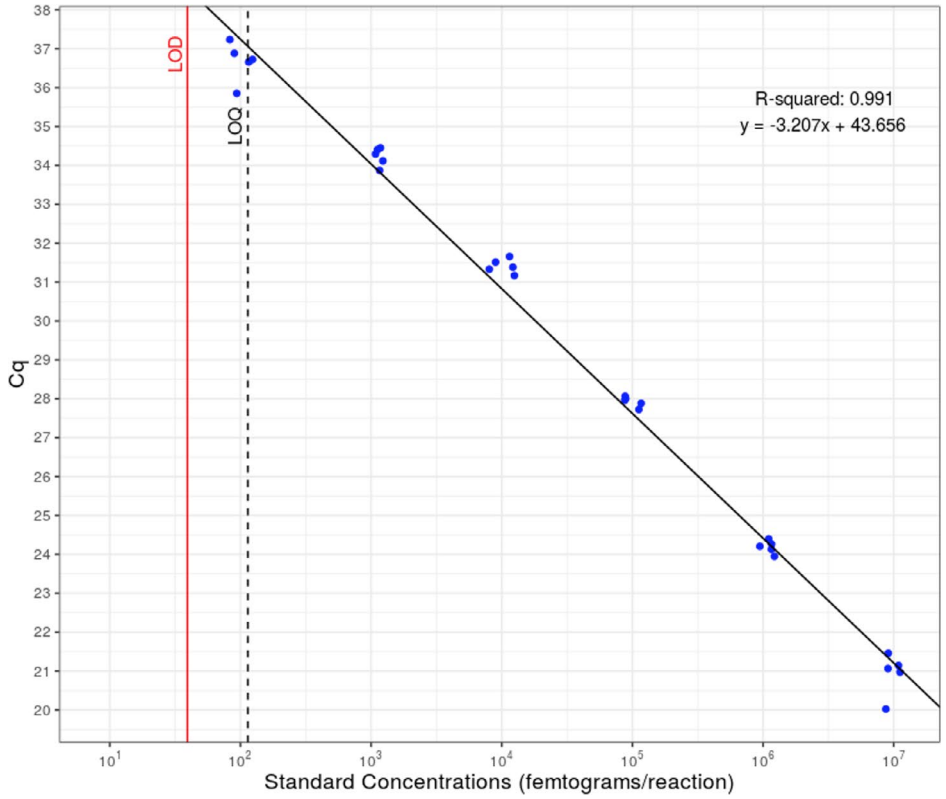


FIGURE 1 Alignment of primers and probe to elongation factor 1 alpha gene of all strains available of the genus *Gnomoniopsis* and the closest species in a different genus (*Melanconis marginalis* subsp. *marginalis*). The mismatching nucleotides of primers (blue arrows) and probe (orange arrows) to the sequences of *G. castaneae* (first line) are explained. The length of the entire aligned sequences for each species is reported to the right of each sequence; numbers above the alignment refer to positions in *G. castaneae* strains. The accession numbers of all the sequences are reported below. (*G. castaneae*: MH213482, MH213483, MH213484, MH213485, MH213486, LT593848, KX929733, KR072536, KP824758, KP824760, KP824762, JQ791201, JQ791198, JQ791204, JQ791202, JQ791200, MT435531, MT435530, KP824759, JQ791209, KR072538, KR072537, KR072534, KP824761, KR072535, JQ791206, JQ791211, JQ791199, JQ791208, JQ791210, JQ791203; *G. paraclavulata*: GU320815; *G. chinensis*: MH545370, MH545371, MH545372, MH545373; *G. clavulata* GU320807; *G. sanguisorbae*: GU320805, GU320806; *G. idaeicola*[1]: MG773589, MG773587, GU320796; *G. idaeicola*[2]: MG773593, MG773590; G. idaeicola[3]: MG773588, MG773585, MG755816, GU320799, MG773591, GU320811, MG878403, MG773592, MG773586, GU320798, GU320797; *G. racemula*: GU320803; *G. comari* GU320794; *G. tormentillae* GU320795; *G. chamaemori* GU320809; *G. macounii* GU320804; *G. occulta*: GU320812, GU320800; *G. alderdunense*: GU320813, GU320801, GU320802; *Melanconis marginalis* subsp. *marginalis*: MN780791, MN780792, MN780793, MN780795)



DNA concentration (ng/µl)	Cq value (mean ± SD)
10	21.07 ± 0.54
1	24.21 ± 0.16
0.1	27.96 ± 0.14
0.01	31.40 ± 0.17
0.001	33.85 ± 0.24
0.0001	36.72 ± 0.52
0.00001	-
LIMITS (femtograms/reaction)	
LOQ	114
LOD	39.30

FIGURE 2 Standard curve obtained with 10-fold dilutions of DNA extracted from *Gnomoniopsis castaneae* strain GN01 (5 replicates) and related statistics. The LOQ and LOD values were obtained with the R script code available at https://github.com/cmerkes/qPCR_LOD_Calc

Assay repeatability, assessed on 10 reps of standard DNA, showed a relative standard deviation of 18.1%, whereas reproducibility, measured through 4 different PCR runs, had a standard deviation of 21.7%.

The amplification of mixtures of redundant DNA from in vitro chestnut plantlets (50 ng) spiked with DNA from the pathogen (10 ng to 1 pg) gave results in agreement to those obtained with pure pathogen DNA, without affecting the amplification efficiency nor the sensitivity of the assay (Table 3).

3.3 | qPCR validation on naturally infected samples

Among the 26 DNA samples extracted from fruit, leaves and twigs of trees exposed to natural infection by *G. castaneae* in the Monti Cimini area all those showing symptoms of infection, that is 10 rotting kernels, were positive in the qPCR assay with a Cq ranging from 19.04 (±0.25) to 28.16 (±0.19). Of the 16 asymptomatic samples, 10 showed the presence of the pathogen according to qPCR; amongst which two, K5 and K9, gave positive isolations of *G. castaneae* and consistent qPCR results, with Cq of 21.43 (±0.16) and 23.74 (±0.38). In the remaining eight asymptomatic samples from which the pathogen was not isolated, Cq ranged from 31.34 ± 0.32 to 35.32 ± 0.47. Six asymptomatic samples, from which no isolates could be obtained, were confirmed by qPCR as exempt from *G. castaneae* infection (Table 2).

TABLE 3 Comparison of amplification results of decimal dilutions of *G. castaneae* DNA in presence of chestnut DNA and not. The mean and the standard deviation of 3 repetitions are reported

G. castaneae DNA (ng/µl)	Plant DNA (ng/µl)	Cq value (mean ± SD)
10	-	21.59 ± 0.21
	50	21.85 ± 0.18
1	-	23.91 ± 0.09
	50	24.00 ± 0.13
0.01	-	27.21 ± 0.22
	50	27.13 ± 0.18
0.001	-	29.98 ± 0.21
	50	29.91 ± 0.19
NTC	-	-
	50	-

Abbreviation: NTC, No Template Control.

In bulk fruit samples, HTS analysis showed a total of 4 786 589 reads after filtering, these reads were assigned to 538 OTUs, with an average of 82 OTUs per sample. Only OTUs assigned to *G. castaneae* (OTU2) were considered further in the present work. *Gnomoniopsis castaneae* was detected in all samples with read numbers varying from 124 to 115,141 (percentages ranging from 0.17% to 64.11%). Amplification of the same DNA samples by qPCR detected the

presence of *G. castaneae* with Cq ranging from 28.56 (±0.36) to 32.66 (±0.21) (Table 4).

The connection between data obtained by qPCR and HTS metagenomics was tested by linear regression analysis. The equation of the best fit line, together with its graphical representation, and the experimental data are reported in Figure 3. The R^2 is 0.62 and the F-test reported significance $p < 0.001$ (F = 115.4, NDF = 73).

4 | DISCUSSION

In this paper, we report the design, development and validation of a novel *G. castaneae* real-time PCR detection assay, to our knowledge the first tool of this type available for this threatening pathogen.

The assay reliably distinguished *G. castaneae* from other taxonomically related fungi and from other fungi usually recognized as endophytes or saprophytes of chestnut tissues that can putatively contaminate field samples. The specificity of the assay was strictly related to the choice of the EF1α-gene as the target for amplification. This gene is known to possess the necessary level of polymorphism to discriminate taxonomically related species (Roger et al., 1999; O'Donnell et al., 1998), is less sensitive to base composition artefacts (Hashimoto & Hasegawa, 1996) and provides robust phylogenies among major eukaryotic groups (Baldauf & Palmer, 1993), therefore representing an effective alternative to ITS ribosomal DNA markers.

Moreover, EF1α appears to be consistently present in genomes as a single-copy gene, a key feature that improves the likelihood of the assay giving precise quantification of the pathogen. Although the unique nature of the gene might slightly reduce the sensitivity of the assay in comparison with assays targeting multicopy genes (as with the ITS ribosomal DNA), it adds great accuracy and affordability to quantifying fungal infection in plant tissue because of the direct proportionality between the fluorescence measured and the number of fungal cells present (Guinet et al., 2016; Klymus et al., 2020; LaSalle et al., 2011). However, the assay proved to be exceptionally sensitive as demonstrated by the LOQ and LOD of genomic DNA, which are surrogate representations of the number of fungal genomes/cells detected by the assay. Assuming a genome size of approximately 52 Mb, based on that of *Ophiognomonia clavigignenti-juglandacearum* (Accession n°PYFS00000000—the only genome available from the Gnomoniaceae), then a single genome would weigh approximately 55 fg. This figure would indicate, for the assay developed here, a LOQ of approximately two genomes/fungal cells (and a LOD of 1), thus exceeding the theoretical minimum of three copies/reaction referred to as stochastic limits of low-copy PCRs (Bustin et al., 2009). The possibility of carefully measuring the number of pathogen cells is of great interest for epidemiological studies involving production of natural *G. castaneae* inoculum and dispersal, and its role and impact in floral infection and nut rot development (Lione et al., 2015).

The pathogen was detected definitively, without any evident impact attributable to DNA of the host plant, *Castanea sativa* or other inhibiting substances, at least with the DNA extraction method used in this work. Coupled with the results from naturally infected samples, the assay appears to be robust and affordable. Apart from the

TABLE 4 Results of qPCR assay and ITS metagenomics (HTS) on DNA extracted directly from bulk fruit samples

			Metagenomic analysis (HTS)		
Sample code	Fruit bulk (tissue)	Cq value (mean ± SD)	*G. castaneae* reads (n°)	total reads (n°)	*G. cast./* total reads (%)
BK3p	pericarp	28.56 ± 0.36	115 141	228 124	50.47%
BK1P	pericarp	28.63 ± 0.13	78 946	123 141	64.11%
BK18e	endocarp	29.22 ± 0.09	45 870	84 485	54.29%
BK13p	pericarp	29.44 ± 0.45	45 688	88 588	51.57%
BK8e	endocarp	29.58 ± 1.53	45 024	85 478	52.67%
BK42p	pericarp	30.70 ± 0.29	20 787	41 778	49.76%
BK41e	endocarp	30.98 ± 0.19	20 134	35 492	56.73%
BK22e	endocarp	30.11 ± 0.89	19 353	137 149	14.11%
BK11p	pericarp	31.38 ± 1.02	5 649	119 735	4.72%
BK36p	pericarp	31.31 ± 0.75	5 546	92 771	5.98%
BK27e	endocarp	32.59 ± 0.85	1 545	113 297	1.36%
BK34p	pericarp	31.65 ± 0.93	934	87 281	1.07%
BK31e	endocarp	32.61 ± 1.94	382	103 239	0.37%
BK35e	endocarp	32.47 ± 0.29	286	151 825	0.19%
BK33e	endocarp	32.66 ± 0.21	124	71 495	0.17%

Note: separating endocarps from pericarps. Cq means and SD values were obtained from 5 repetitions. For each sample. the number of *G. castaneae* reads. The number of total reads and the percentage of *G. castaneae* reads out of total are reported.

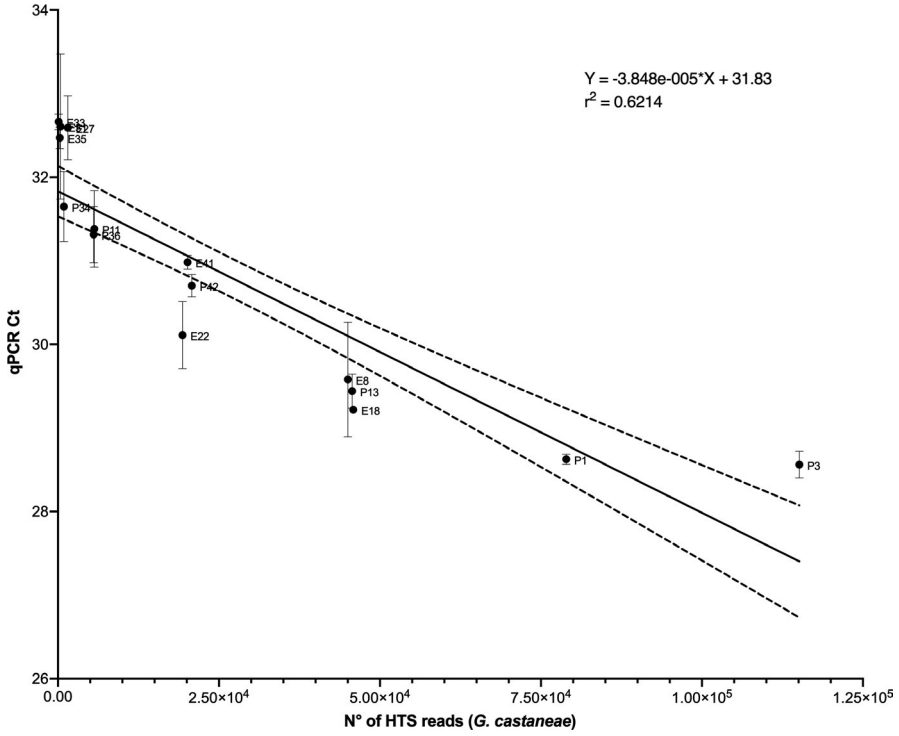


FIGURE 3 Linear regression analysis between HTS reads and qPCR results of bulk fruit samples. The best fit line, linear equation and R2 are reported. Black dots represent mean values (± SE) of five replicates. Dotted lines delineate the 95% confidence intervals

clear qPCR signal from analysing DNA extracted from symptomatic kernels, the assay also detected pathogen presence in asymptomatic kernels, twigs and leaves, evidently greatly surpassing the detection threshold of isolation methods. This facet is of particular significance when considering further research on the endophytic/pathogenic life strategy of the fungus. It is plausible that comparing the amount of the pathogen living latently in host tissues or when symptoms are evident would eventually enable the infection threshold that triggers the fungus to switch from an endophytic to a pathogenic lifestyle to be determined.

The correlation between qPCR results and the number of reads due to the pathogen obtained in parallel metagenomic HTS analysis of bulk fruit samples is worth emphasizing. In the linear regression analysis, even though some data were marginally outside the 95% confidence band, the F-test reported high significance ($p < 0.001$), confirming the reliability of the linear model in representing the data. Notably, deviation from linearity was mostly data points on the left side of the plot in Figure 3, which correspond to 1000 HTS reads or less, and to Cq values near 33 (about 1 pg/µl fungal DNA), thus approaching the LOQ and the LOD of the assay. The ability of HTS to provide quantification of taxa detected based on the resulting number of reads is a matter of debate. Morales-Rodríguez et al. (2021), using two mock communities ('even' and 'staggered'), highlighted that the number of reads did not correlate with the DNA concentration of each taxon in the mix, mainly because, when using ITS as the barcode, the number of copies per taxon might vary and affect the results. However, in the present study it was clear that the number of reads of the same taxon between different samples was consistent with the quantitative presence of that taxon measured in qPCR.

This proficiency in detecting the pathogen in very low quantities, irrespective of the presence of other fungal or plant DNA, demonstrates the significant advantages and great diagnostic value of this assay. When applied in the field, this assay will enable the movement of infected plant material, regardless of the infected part of the plant, fruit or plantlets, and its level of infection, to be curtailed, conceivably helping to reduce the spread of the pathogen on regional, national and international scales. It can also have a crucial impact in postharvest fruit storage, where control strategies aimed at reducing the impact of rot-causing fungi are crucial in reducing significant economic losses over time (Maresi et al., 2013; Ruocco et al., 2016); the opportunity to detect a latent infection could greatly help in optimizing the nature and the timing of treatments.

Another further goal for researchers is the development of strategies for the control of *G. castaneae* in the field. Even in this application, this qPCR assay will be a significant tool in tracking the presence and the amount of pathogen infection in order to evaluate the effectiveness of control measures.

In addition, this qPCR assay will help shed light on the numerous still unknown biological facets of this emerging pathogen, such as its detection in *Dryocosmus kuriphilus* and in the galls induced by this wasp (Morales-Rodriguez et al., 2019; Seddaiu et al., 2017; Vannini et al., 2017) to better understand the interactions between these organisms.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHOR CONTRIBUTIONS

A.M., C.M.R. and A.V. conceived the original idea and developed the theory. S.T., G.B. and C.M.R. contributed to sample preparation and carried out the experiment. C.M.R and S.T. verified the analytical methods. A.M. and A.V. supervised the project. A.M. wrote the manuscript in consultation with C.M.R and A.V. All authors discussed the results, provided critical feedback and contributed to the final manuscript.

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DATA AVAILABILITY STATEMENT

Sequence data created and analysed in this research are openly available from Genbank® (https://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers for each data are available in the paper. Other data supporting the findings of this study are provided in full in the results section of this paper and available from the corresponding author upon request.

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