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

Silvia Turco¹ | Giorgia Bastianelli² | Carmen Morales-Rodriguez² | Andrea Vannini²,³ | Angelo Mazzaglia¹

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.

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is also reported in Australia (Shuttleworth et al., 2013), North America (Sakalidis et al., 2019) and Chile (Vannini, unpublished).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 phenomenology 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-Sep). 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
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
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 (‘xxxxCTYGGTCA’$^\text{T}T$TAGA $^\text{G}$GAAGTAA-3’) and ITS2 (‘xxxxGCHRGC’$^\text{T}$TCTTTCATGDTGC-3’), where xxx represent 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.

### 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 | yes                      | 21.37 ± 0.12            |
| K3M         | kernel embryo yes yes | yes                      | 19.04 ± 0.25            |
| K7M         | kernel embryo yes yes | yes                      | 23.52 ± 0.33            |
| K8M         | kernel embryo yes yes | yes                      | 22.01 ± 0.40            |
| KF24        | kernel embryo yes yes | yes                      | 23.92 ± 0.16            |
| K7N1        | kernel embryo yes yes | yes                      | 21.84 ± 0.15            |
| K7N3        | kernel embryo yes yes | yes                      | 28.16 ± 0.19            |
| K8R3        | kernel embryo yes yes | yes                      | 23.71 ± 0.19            |
| K8R9        | kernel embryo yes yes | yes                      | 24.41 ± 0.07            |
| K9N4        | kernel embryo yes yes | yes                      | 25.01 ± 0.16            |
| KF12        | kernel embryo no yes | yes                      | 28.56 ± 0.08            |
| K2S         | kernel embryo no no | -                        | -                      |
| K4S         | kernel embryo no no | -                        | -                      |
| K5S         | kernel embryo no yes | 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 | 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            |
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 183 bp of Gnomoniopsis length from most of the other species, together with six sequences of Gnomoniopsis 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′-AAACGTGACCCACTTCCAGC-3′) ranged from a minimum of four to a maximum of six mismatches, the reverse primer GC-R3 (5′-TCAGCCTTACGTTGAGCC-3′) had 0 to 1 mismatches, whilst the probe GC-Probe (5′-CCACCCCATCTTCTTGTTG-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 replications 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 (r² = 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. chiniensis: MH545370, MH545371, MH545372, MH545373; G. clavulata GU320807; G. sanguisorbae: GU320805, GU320806; G. ideaeicola1: MG773589, MG773587, GU320796; G. ideaeicola2: MG773593, MG773590, G. ideaeicola3: MG773588, MG773585, MG755816, MG720799, MG73591, 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. alderdense: GU320813, GU320801, GU320802; Melanconis marginalis subsp. marginalis: MN780791, MN780792, MN780793, MN780795)
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).

| 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         |

**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

**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/cmerk/qPCR_LOD_Calc

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 than other genes, and the experimental data are reported in Figure 3. The R$_2$ is 0.62 and the F-test reported significance $p < 0.001 (F = 115.4, NDF = 73)$.

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

| 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.
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.

PEER REVIEW
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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.

ORCID
Silvia Turco https://orcid.org/0000-0001-9041-6461
Giorgia Bastianelli https://orcid.org/0000-0002-2830-6840
Carmen Morales-Rodriguez https://orcid.org/0000-0002-2971-2840
Andrea Vannini https://orcid.org/0000-0003-4318-9088
Angelo Mazzaglia https://orcid.org/0000-0002-1295-2565

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