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ORIGINAL ARTICLE

Species reassignment and rapid molecular detection of *Colletotrichum* associated with persimmon anthracnose in Brazil

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Abstract

Anthracnose is the main fungal disease affecting persimmon (*Diospyros kaki* Thunb.) and causes significant economic losses. In Brazil, the disease is caused by *Colletotrichum* species belonging to the *Colletotrichum gloeosporioides* (CGSC) and *C. acutatum* (CASC) species complexes. Accurate species identification is essential for effective disease management strategies. However, phylogeny-based approaches are often costly and labor-intensive. This study aimed to clarify the identification of *C. fructicola* strains associated with persimmon anthracnose in Brazil using multigene phylogenetic analysis and to develop species-specific PCR primers for *C. horii*, *C. chrysophilum*, *C. nymphaeae*, and *C. melonis*, the causal agents of persimmon anthracnose. The primers were validated using isolates collected from four persimmon-producing regions in Brazil, and their applicability was also assessed using *Colletotrichum* isolates obtained from apple ($n=8$) and pear ($n=1$). Multigene phylogenetic analysis based on *ITS*, *GAPDH*, and *TUB2* sequences demonstrated that seven isolates previously identified as *C. fructicola* clustered within the *C. chrysophilum* clade, leading to their reassignment. Based on partial *TUB2* gene sequences, species-specific primers were developed and validated using a collection of 183 isolates from persimmon orchards across four Brazilian states. *Colletotrichum horii* was the predominant species, representing 76% of the isolates and accounting for more than 90% of the population in Paraná, Santa Catarina, and Rio Grande do Sul. In contrast, *C. nymphaeae* was more frequent in São Paulo (45%), followed by *C. horii* (41%) and *C. chrysophilum* (14%). The primers also correctly identified *Colletotrichum* isolates obtained from other hosts, although cross-amplification was observed between *C. melonis* and *C. limetticola*. The new primers developed in this study provide a rapid and reliable tool for detecting the main *Colletotrichum* species associated with persimmon anthracnose and may also support species identification in other hosts.

Keywords *Diospyros kaki* · Molecular diagnoses · *Colletotrichum horii* · *Colletotrichum chrysophilum* · *Colletotrichum nymphaeae* · *Colletotrichum melonis*

Introduction

Members of the *Colletotrichum acutatum* species complex (CASC) and *Colletotrichum gloeosporioides* species complex (CGSC) account for most disease-causing agents affecting subtropical fruit crops in Brazil (Carraro et al. 2022b). Within this context, persimmon production has been particularly impacted, especially in the state of Paraná, where yield losses associated with disease pressure have contributed to a production decline of approximately 50% between 2012 and 2022 (IBGE – Brazilian Institute of Geography and Statistics 2025). This marked reduction has led some growers to reconsider the economic viability of persimmon cultivation, as the effective management of major diseases,

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especially anthracnose, remains a significant challenge in this cropping system.

Anthracnose, caused by *Colletotrichum* species, is the main fungal disease affecting persimmon production worldwide (Blood et al. 2020; Carraro et al. 2022a). The pathogen overwinters in infected twigs, remaining latent until favorable conditions (21–25 °C, high humidity) in spring and early summer promote its development (Dolinski et al. 2022). Under unmanaged conditions, successive infection cycles may occur throughout the growing season. Anthracnose symptoms are observed on shoots, twigs, leaves, flowers, and fruit; however, fruit infections are particularly damaging, frequently resulting in premature fruit drop in orchards with high inoculum pressure (Dolinski et al. 2022). In China, the world's leading producer of persimmon, anthracnose epidemics have been associated with productivity reductions of up to 90%, highlighting the severe economic impact of this disease (Deng et al. 2020).

Colletotrichum horii is the main causal agent of persimmon anthracnose in Brazil and worldwide. However, recent studies have have demonstrated that other *Colletotrichum* species, including *C. fructicola*, *C. nymphaeae*, and *C. melonis*, are also associated with anthracnose symptoms in Brazilian persimmon orchards (Carraro et al. 2019). These species have also been reported in apple orchards (Moreira et al. 2019a), whereas *C. fructicola* and *C. nymphaeae* have been reported in grapevines (Santos et al. 2018; Echeverrigaray et al. 2020) and *C. nymphaeae* has been reported in pear orchards (Moreira et al. 2019b). These fruit crops are commonly cultivated in the same production regions as persimmon in Brazil, increasing the potential for shared inoculum sources and cross-host disease dynamics (Carraro et al. 2022b).

The management of diseases associated with *Colletotrichum* presents significant challenges in fruit crops, particularly in production systems where multiple host plants coexist. In such environments, interactions among different *Colletotrichum* species may occur within the same agroecosystem, increasing epidemiological complexity and hindering effective disease control (Silva et al. 2017a, b; da Silva et al. 2020; Dowling et al. 2020; Boufleur et al. 2021). Accurate species identification is therefore essential for successful disease management, as *Colletotrichum* species differ in their sensitivity to fungicides and exhibit distinct biological traits, including enzyme activity and pathogenicity (Velho et al. 2018; Dowling et al. 2020; Carraro et al. 2023).

Accurate identification of *Colletotrichum* species has historically been challenging, as it was largely based on host association, cultural practices, and morphological traits (Jayawardena et al. 2021). However, phenotypic variation driven by factors such as growth medium and temperature often complicates species delimitation and may result in misidentification. Molecular comparisons of genomic loci have therefore become essential for reliable fungal identification. Although the internal transcribed spacer (*ITS*) region is widely accepted as a universal fungal barcode, it provides insufficient resolution for species-level identification within *Colletotrichum*. Consequently, multilocus approaches incorporating additional genes, such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-tubulin (*TUB2*), calmodulin (*CAL*), actin (*ACT*), chitin synthase (*CHS-1*), glutamine synthetase (*GS*), DNA lyase (*APN2*), and the intergenic region between DNA lyase and the mating type (Mat1-2) gene (*ApMat*) have been widely used to resolve species boundaries within this genus (Damm et al. 2012; Weir et al. 2012; Liu et al. 2015; Vieira et al. 2017).

Although molecular techniques substantially improve the accuracy of *Colletotrichum* detection and identification, they still require several labor-intensive steps, including PCR amplification of multiple loci, DNA sequencing, sequence inspection, and multigene phylogenetic analysis. Consequently, the development of species-specific PCR primers represents a practical alternative for well-characterized taxa, reducing both time and analytical costs while supporting more rapid decision-making in disease management programs. In citrus, species-specific PCR primers targeting the partial *GAPDH* gene were successfully developed for *C. abscissum* and *C. gloeosporioides*, enabling efficient diagnosis of postbloom fruit drop (PFD) (Silva et al. 2017a, b). In persimmon, the *ITS* region combined with partial *GAPDH* and *TUB2* sequences has been shown to reliably assign *Colletotrichum* isolates to either the CASC or CGSC complexes (Carraro et al. 2022a). However, the identification of genomic regions exhibiting higher interspecific variability within these loci remains critical for the development of species-specific primers capable of discriminating among *Colletotrichum* species associated with persimmon anthracnose.

Accurate identification and rapid detection of causal agents have become critical research priorities in the *Colletotrichum*-persimmon pathosystem; however, several key questions remain unresolved, particularly in light of recent taxonomic reclassifications within the genus. For example, Astolfi et al. (2022) re-evaluated isolates previously identified as *C. fructicola* from apple orchards in Brazil and Uruguay and reassigned them to *C. chrysophilum*, a species belonging to the Musae clade of the *C. gloeosporioides* species complex (CGSC) that shares a close evolutionary relationship with *C. fructicola*. These findings raised uncertainty regarding whether Brazilian persimmon isolates previously identified as *C. fructicola* (Carraro et al. 2019, 2022b) truly belong to this species or should instead be assigned to *C. chrysophilum*. Therefore, the aimed of the present study were to: (i) clarify the taxonomic identity of *C. fructicola*

strains associated with persimmon anthracnose in Brazil using multigene phylogenetic analysis; and (ii) develop and validate species-specific PCR primers for the main *Colletotrichum* species associated with persimmon anthracnose, using isolates from persimmon orchards and assessing their applicability to closely related isolates obtained from other fruit crops.

Material and Methods

Fungal isolates

Colletotrichum isolates used in this study were selected from the mycological collection of Laboratório de Epidemiologia para Manejo Integrado de Doenças de Plantas (LEMID) at the Federal University of Paraná (UFPR), Curitiba, Paraná, Brazil. A total of 192 isolates were included, all recovered from plant tissues showing typical anthracnose symptoms. Of these, 183 isolates were obtained from persimmon, eight from apple, and one from pear (Table 1).

Persimmon isolates were collected from symptomatic flowers, young branches, and fruits in four major persimmon-producing regions of Brazil: São Paulo ($n=49$), Paraná ($n=55$), Santa Catarina ($n=35$), and Rio Grande do Sul ($n=44$) (Table 1). All isolates were purified as single-spore cultures and preserved on sterile filter paper in microtubes containing silica gel at −20 °C, following the method described by Nakasone et al. (2004).

Table 1 List of *Colletotrichum* spp. Isolates from persimmon (*Diospyros kaki*), Apple (*Malus domestica*) and Pear (*Pyrus pyrifolia*) trees tested in this study

Colletotrichum species or complexes previously identified[a]	Number of isolates tested			Total of isolates tested
	Persimmon	Apple	Pear	
CGSC or CASC	165	-	-	
C. horii	7	-	-	
C. fructicola	6	1	-	
C. nymphaeae	4	1	1	
C. melonis	1	3	-	
C siamense	-	2	-	
C. limetticola	-	1	-	
Total	183	8	1	192

[a] Isolates were obtained from previous studies of Bragança et al. (2016); Moreira et al. 2019b); Carraro et al. (2022a). Abbreviations of *Colletotrichum gloeosporioides* species complex (CGSC) and *Colletotrichum acutatum* species complex (CASC). Isolate codes tested: *C. horii* (*DkC*PR 18−03, *DkC*PR 18−05, *DkC*PR 18−06, *DkC*PR 18−08, *DkC*PR 18−09, *DkC*PR 18−10, *DkC*SP 18−01); *C. fructiola* (from persimmon trees - *DkC*PR 18–52, *DkC*SP 18−02, *DkC*SP 18−03, *DkC*SP 18−04, *DkC*SP 18−05, *DkC*SP 18−06; and from apple trees - Col 33=CPC 20914); *C. nymphaeae* (from persimmon trees – *DkC*PR 18−01, *DkC*PR 18−02, *DkC*PR 18−04, *DkC*PR 18−07; from apple trees - Col 15=CPC 20899; from pear trees - PpC-nPR 17−02); *C. melonis* (from persimmon trees – *DkC*PR 18−11; from apple trees - Col 31=CPC 20912, MdCm 06, MdCm 09); *C. siamense* (MdCs 1R2E e MdCs 1R3J); and *C. limetticola* (MdCl 180)

Brazilian isolates from persimmons anthracnose symptoms previously identified as *Colletotrichum fructicola* belong to *Colletotrichum chrysophilum* species

Genomic DNA was extracted from all strains on potato dextrose agar (PDA) for 7 days after inoculation at 25 °C under a 12 h photoperiod, following the protocol described by Carraro et al. (2022a). Six isolates obtained from persimmon trees (*DkC*PR18-52, *DkC*SP18-02, *DkC*SP18-03, *DkC*SP18-04, *DkC*SP18-05, *DkC*SP18-06) and one isolate obtained from apple tree (Col 33, CPC 20914), all previously identified as *C. fructicola*, were used in the phylogenetic analyses (Table S1). Primer pairs and the PCR conditions used for gene amplification were as described by Carraro et al. (2022a, b). Sequences from three genomic loci were included in the analysis: the ITS region, *GAPDH*, and *TUB2*. For preliminary identification at the species complex level, sequences were compared with those of ex-type and reference strains available in the NCBI GenBank database using the BLASTn search tool (http://www.ncbi.nlm.nih.gov/BLAST/).

Based on the identified species complex, reference sequences were selected from previous studies (Marin-Felix et al. 2017; Khodadadi et al. 2020; Astolfi et al. 2022) and used for multiple sequence alignment and subsequent phylogenetic analyses using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. Sequence alignments were generated using MAFFT online platform (https://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2019) and, when necessary, manually adjusted in MEGA version 7 (Kumar et al. 2016).

Bayesian Inference was performed using MrBayes v. 3.2.6 (Ronquist and Huelsenbeck 2003) and Maximum Likelihood in IQ-TREE v. 2 (Hoang et al. 2018; Minh et al. 2020), both as plugins for the PhyloSuite software (Zhang et al. 2019). ModelFinder (Kalyaanamoorthy et al. 2017) was used to assess the evolutionary models, with the best-fit substitution model chosen based on the corrected Akaike Information Criterion for each gene. For the ML analysis, evolutionary simulations continued until likelihood scores converged, followed by an ultrafast bootstrap (UFBoot) approximation involving 1000 pseudoreplicates to estimate statistical branch support. Nodes with zero branch lengths were collapsed. In the BI analysis, two concurrent runs were conducted, one cold and three heated chains each, sampling every 10,000 generations. The initial 25% of the generated trees were discarded as burn-in before computing 50%

majority consensus trees for ML and posterior probability values for BI.

Visualization and comparison of tree topologies were performed using FigTree v 1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/), and final figures were edited using Inkscape software (https://inkscape.org/pt-br/). In the phylogenetic analysis, clades were considered well supported when posterior probability values exceeded 0.9 for BI analysis and when bootstrap values were greater than 80% in ML analysis.

Design and validation of Species-Specific primers for conventional PCR

Reference isolates belonging to the *Colletotrichum gloeosporioides* species complex (CGSC) and the *C. acutatum* species complex (CASC) were selected for primer development (Table 2). Primer design targeted the four *Colletotrichum* species associated with persimmon anthracnose, namely *C. horii*, *C. chrysophilum*, *C. nymphaeae*, and *C. melonis*. Individual analyses of the loci used in the multilocus phylogeny were initially performed to identify regions exhibiting high interspecific variability among the target species while maintaining low intraspecific variation. These regions were prioritized to maximize species-level discrimination.

Primer pairs were designed using Clone Manager software (Sci Ed Software) according to standard parameters for primer length, GC content, and melting temperature. Potential secondary structures, including hairpins and heterodimers, were evaluated using the OligoAnalyzer tool (Integrated DNA Technologies; https://www.idtdna.com/calc/analyzer). Primer specificity was further assessed in silico using Primer-BLASTn against nucleotide sequences available in GenBank (Altschul et al. 1997), with particular emphasis on closely related species within the CGSC and CASC. Annealing temperatures were estimated using the NEB Tm Calculator (https://tmcalculator.neb.com).

PCR amplifications were performed in an Applied Biosystems Veriti Thermal Cycler using a final reaction volume of 12.5 µL. Each reaction contained 6.25 µL of GoTaq® G2 Colorless Master Mix (Promega®, Madison, WI, USA), 0.5 µL of each primer (10 µM), 1 µL of genomic DNA (50–100 ng), and nuclease-free water to volume. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C (CGSC species) or 56 °C (CASC species) for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min.

Primer validation was conducted by amplifying all 192 isolates included in this study using the four species-specific primer pairs under the conditions described above. Each PCR assay included positive controls (reference isolates for each target species), negative controls (non-target *Colletotrichum* species), and a no-template control containing nuclease-free water. Amplification products were separated by electrophoresis on 1% agarose gels stained with GelRed™ (Invitrogen®, USA) and visualized under ultraviolet transillumination using a 100 bp DNA ladder (Invitrogen®, USA) as a molecular size marker. Primer specificity was evaluated using a panel of non-target *Colletotrichum* isolates in addition to the reference isolates of each target species. The specificity assays included isolates of *Colletotrichum* from other fruit hosts and from different geographic regions of Brazil.

Table 2 Isolates selected for the design of specific primer pairs with their respective codes, GenBank accession numbers, and plant tissue of origin

Isolates code	*Colletotrichum* species (Complex)[a]	Number of accesses in GenBank[b]		
		ITS	*GAPDH*	TUB2
*DKC*PR18-03	*C. horii* (CGSC)	MN959853	MN982436	MN982451
*DKC*SP18-06*	*C. chrysophilum* (CGSC)	MH750230	MH750233	MK209630
*DKC*PR18-01	*C. nymphaeae* (CASC)	MH750229	MH750232	MK209631
*DKC*PR18-11	*C. melonis* (CASC)	MK209080	MK216322	MK216323

[a] Abbreviation for *Colletotrichum gloeosporioides* species complex (CGSC) and *Colletotrichum acutatum* species complex (CASC). The isolate marked with (*) was reassigned in this study as *C. chrysophilum*

[b] Isolates selected from a previous study (Carraro et al. 2022a)

Results

Brazilian isolates from persimmons anthracnose symptoms previously identified as *Colletotrichum fructicola* belong to *Colletotrichum chrysophilum* species

Based on the NCBI BLAST search for each locus (*ITS*, *TUB2* and *GAPDH)*, the seven selected isolates (*DkCf*PR 18–52, *DkCf*SP 18−2, *DkCf*SP 18−3, *DkCf*SP 18−4, *DkCf*SP 18−5, *DkCf*SP 18−6 and CPC 20899=Col 33) belong to the *C. gloeosporioides* species complex (CGSC). Subsequently, the concatenated data set was used in a multigene phylogenetic analysis to identify the strains at the species level. The multigene alignment comprised DNA sequences from 77 isolates (including our 7 isolates and reference sequences) and *C. boninense* CBS 123,755 as

outgroup, resulting in 1141 bp (including gaps). The lengths of the gene partitions were: *ITS*=1-506, *GAPDH*=507–704, *TUB2*=705–1141. The GTR+F+G4 evolutionary model was selected for the *ITS* region and the *TUB2* partial gene, while the K2P evolutionary model was selected for the *GAPDH* partial gene (Fig. 1).

The consensus tree obtained in the Maximum Likelihood analysis was the same topology as that obtained in the Bayesian Inference analysis. Our seven isolates (previously identified as *C. fructicola*) were placed in the *C. chrysophilum* type strain clade (CMM 4268), with significant clade support (PP=0.93; UFBoot=98). Therefore, we reclassified them as *C. chrysophilum* (Fig. 1).

Validation of species-specific primers for conventional PCR

After reassignment of strains previously identified as *C. fructicola* to *C. chrysophilum*, species-specific primers were designed to identify the four causal agents of persimmon

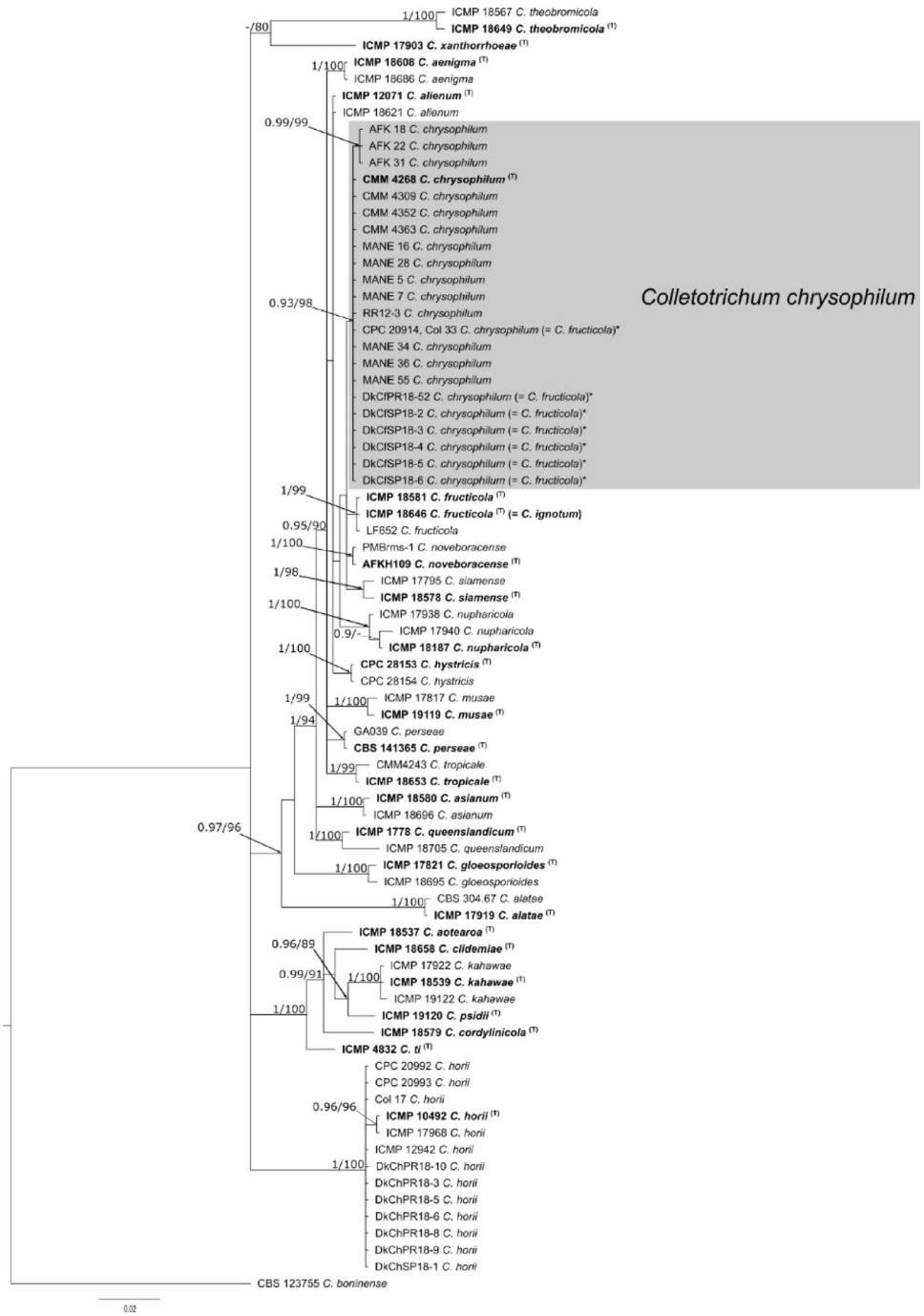

Fig. 1 Bayesian Inference phylogenetic tree of the *Colletotrichum gloeosporioides* species complex (CGSC) based on concatenated sequences of the ITS-rDNA region and the partial genes *GAPDH* and *TUB2*. *Colletotrichum* isolates re-evaluated in this study are marked with (*) and identified as *C. chrysophilum* (previous identification in parentheses – *C. fructicola*). Other sequences were obtained from the NCBI database, with type strains highlighted in bold and indicated with superscript (T). Posterior probability (PP) values greater than 0.9 and ultrafast bootstrap support (UFBoot) values greater than 80 are shown at branch nodes (PP/UFBoot). A hyphen (-) indicates support values below the predefined thresholds for the analyses. The scale bar represents the estimated number of substitutions per nucleotide. The phylogenetic tree is rooted with *C. boninense* CBS 123,755, which was used as the outgroup for the CGSC

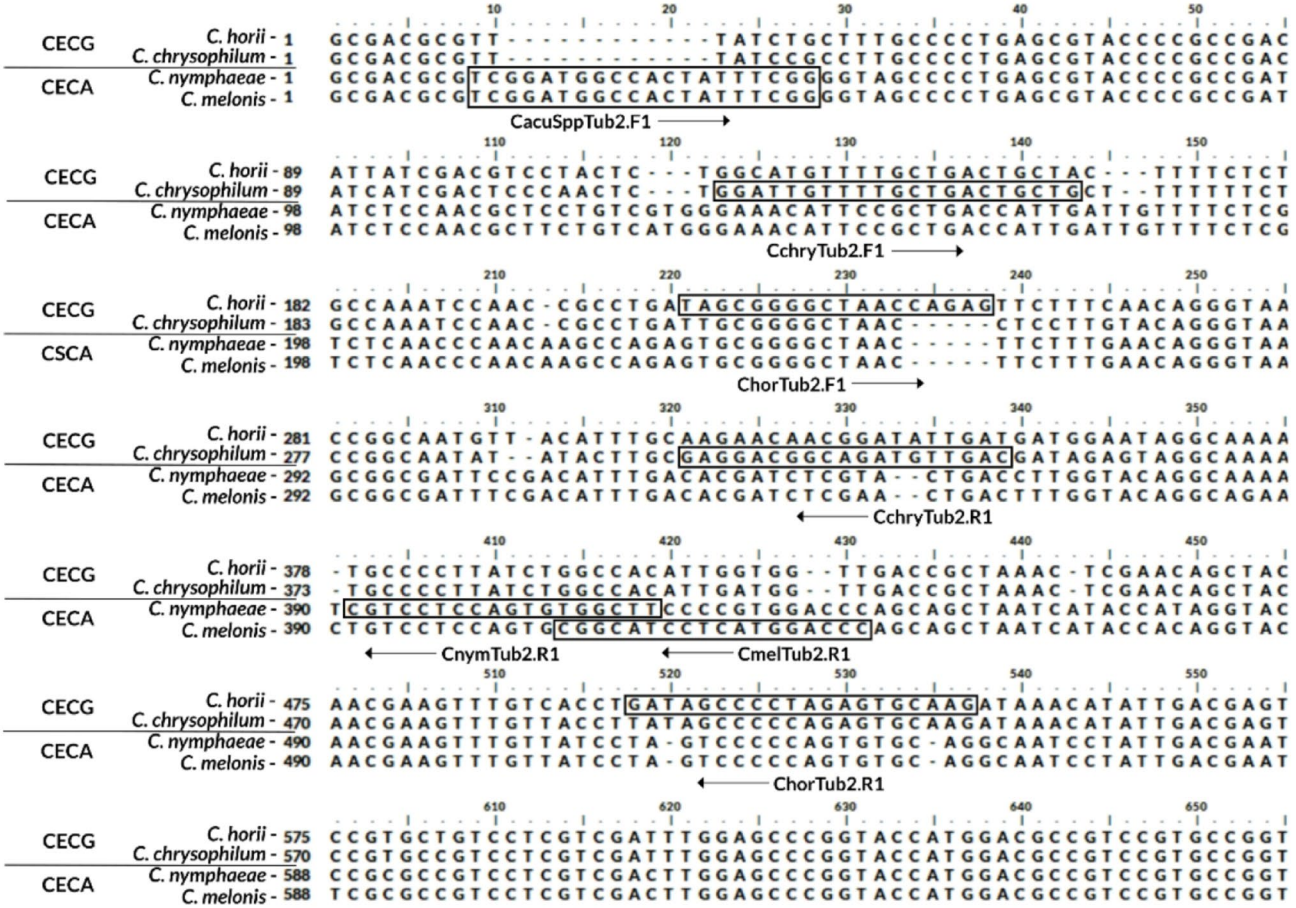

Fig. 2 Comparison of genomic DNA sequences of the partial *TUB2* gene from isolates belonging to the *Colletotrichum gloeosporioides* species complex (CGSC): *C. horii* (*DkC*PR18-03, MN982451) and *C. chrysophilum* (*DkC*SP18-06, MK209630); and to the *Colletotrichum acutatum* species complex (CASC): *C. nymphaeae* (*DkC*PR18-01, MK209631) and *C. melonis* (*DkC*PR18-11, MK216323). Primer sequence locations are highlighted by rectangles

Table 3 List of specific primers developed in this study for each *Colletotrichum* species, including sequences, amplification fragment sizes obtained via polymerase chain reaction (PCR), and annealing temperatures

Species	Primer Verse		Primer Reverse		Amp. (pb)	Tm Taq (400 nm)
	Name	Sequence (5'-3')	Name	Sequence (5'-3')		
C. horii	ChorTub2.F1	TAGCGGGGCTAACCAGAG	ChorTub2.R1	CTTGCACTCTAGGGGCTATC	311	53 °C
C. chrysophilum	CchryTub2.F1	GGATTGTTTTGCTGACTGCTG	CchryTub2.R1	GTCAACATCTGCCGTCCTC	206	53 °C
C. nymphaeae	CacuSppTub2.F1	TCGGATGGCCACTATTTCGG	CnymTub2.R1	AAGCCACACTGGAGGACG	400	56 °C
C. melonis			CmelTub2.R1	GGGTCCATGAGGATGCCG	412	56 °C

anthracnose in Brazil: *C. horii*, *C. chrysophilum*, *C. nymphaeae* and *C. melonis*. Genomic DNA sequences of the partial *TUB2* gene were compared among isolates belonging to the CGSC, including *C. horii* and *C. chrysophilum*; and the CASC, including *C. nymphaeae* and *C. melonis* (Fig. 2). Based on these comparisons, primer pairs were designed and are listed in Table 3.

In silico analyses, primers designed for *C. horii* showed species-level specificity. When assessed against other CGSC and CASC species associated with different fruit hosts using the NCBI Primer-BLAST tool, primers targeting CASC species exhibited 100% sequence similarity with other tested species within the complex. In addition, sequence similarity was observed between *C. chrysophilum* and *C. fructicola*, both members of the CGSC. A summary of the in silico specificity analyses is provided in Table 4.

Primer specificity was validated by amplifying isolates representing the four *Colletotrichum* species causing persimmon anthracnose. In in vitro assays, the primer pair ChorTub2.F1/R1 amplified exclusively the *C. horii* isolate (*DkC*PR18-03), producing a PCR band size of 311 bp. The primer pair CchryTub2.F1/R1 amplified the *C. chrysophilum*

Table 4 Reference isolates from this work for the design of species-specific primers and the *Colletotrichum* species showing 100% match with forward and reverse primers found in Silico using the NCBI Primer-BLASTn tool

Reference isolate	Forward primer	Reverse primer
C. horii (*DkC*PR 18−03)	*C. horii*	*C. horii*
C. chrysophilum (*DkC*SP 18−06)	*C. fructicola*, *C. chrysophilum*	*C. fructicola*, *C. chrysophilum*
C. nymphaeae (*DkC*PR 18−01)	*C. nymphaeae*, *C. scovillei*, *C. simmondsii*, *C. fioriniae*, *C. laticiphilium*, *C. lupini*	*C. nymphaeae*, *C. simmondsii*
C. melonis (*DkC*PR 18−11)	*C. melonis*, *C. nymphaeae*, *C. scovillei*, *C. simmondsii*, *C. fioriniae*, *C. laticiphilium*, *C. lupini*	*C. melonis*, *C. lupini*

isolate (*DkC*SP18-06), producing a band size of 206 bp. The primer pair CacuSppTub2.F1/CnymTub2.R1 amplified the *C. nymphaeae* isolate (*DkC*PR18-01), generating a band size of 400 bp, whereas the primer pair CacuSppTub2.F1/CmelTub2.R1 amplified the *C. melonis* isolate (*DkC*PR18-11), producing a band size of 412 bp (Fig. 3).

Primer performance was subsequently assessed across the entire culture collection. All isolates were successfully amplified and identified. Among the 183 persimmon isolates, 139 isolates were identified as *C. horii*, corresponding to 76% of the collection, whereas 6% were identified as *C. chrysophilum*, 17% as *C. nymphaeae*, and 1% as *C. melonis* (Table S1; Fig. 4). In relation to the Brazilian persimmon production states, isolates from Paraná, Santa Catarina and Rio Grande do Sul were predominantly identified as *C. horii*, accounting for more than 90% of the isolates in each state (Fig. 4). Paraná was the only state in which all four *Colletotrichum* species were detected. In contrast, in São Paulo, *C. nymphaeae* was the most frequent species (45%), followed by *C. horii* (41%) and *C. chrysophilum* (14%) (Fig. 4).

The applicability of the primer pairs to isolates obtained from hosts other than persimmon was assessed using isolates of *C. chrysophilum* (Col 33, CPC 20914), *C. nymphaeae* (Col 15) and *C. melonis* (Col 31) from apple trees, and one isolate of *C. nymphaeae* (*PpCn*PR 17−02) from pear. All these isolates were correctly identified, producing bands of expected sizes (Fig. 5). No amplification was observed for isolates belonging to *C. siamense* (MdCs 1R2E and MdCs 1R3J) (Fig. 5a). However, the *C. limetticola* isolate (MdCl 180) was amplified by the *C. melonis* primer pair (CacuSppTub2.F1/CmelTub2.R1), indicating cross-amplification between these closely related species (Table S1).

Discussion

This study assessed 192 *Colletotrichum* isolates, including 183 collected from persimmon orchards across four major producing regions in Brazil (São Paulo, Paraná, Santa Catarina and Rio Grande do Sul), as well as isolates obtained from apple and pear orchards. Multigene phylogenetic analyses demonstrated that seven isolates previously identified as *C. fructicola* should be reassigned to *C.*

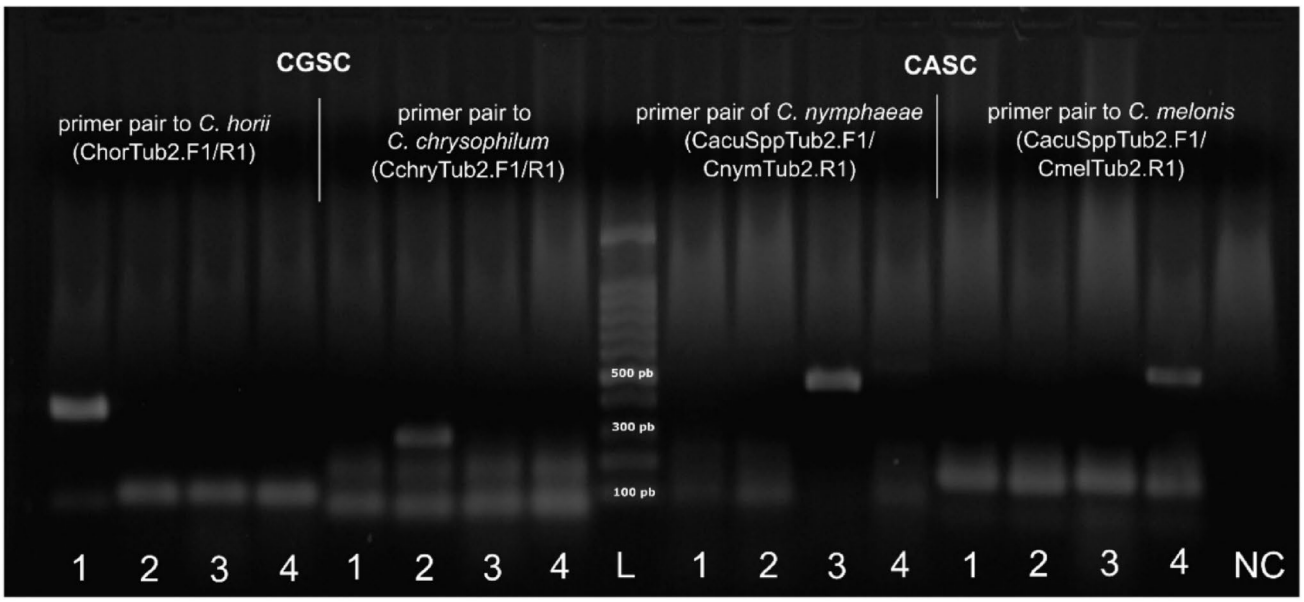

Fig. 3 Agarose gel electrophoresis of PCR products from isolates of *Colletotrichum* spp., causal agents of persimmon anthracnose, using specific primers. Samples 1 to 4 represent isolates *DkC*PR18-03 (*C. horii* isolate), *DkC*SP18-06 (*C. chrysophilum* isolate), *DkC*PR18-01 (*C. nymphaeae* isolate) and *DkC*PR18-11 (*C. melonis* isolate), respectively. L − 100-bp Ladder and NC - negative control without DNA

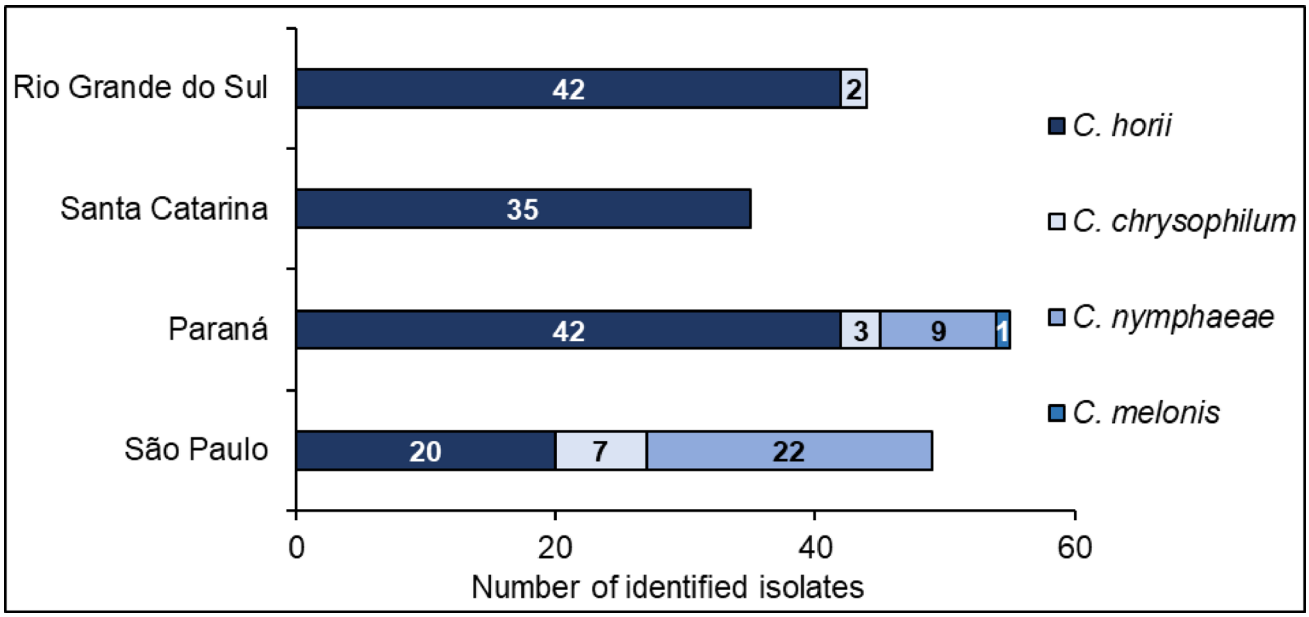

Fig. 4 Number of isolates identified in the four persimmon-producing states in Brazil with species-specific primer pairs for *Colletotrichum horii*, *C. chrysophilum*, *C. nymphaeae* and *C. melonis*

chrysophilum, clarifying the species composition associated with persimmon anthracnose in Brazil. Based on these findings, species-specific primers for conventional PCR were developed to enable the detection and differentiation of the four *Colletotrichum* species associated with persimmon anthracnose, namely *C. horii*, *C. chrysophilum*, *C. nymphaeae*, and *C. melonis*. Notably, the primer pair designed for *C. horii*, the predominant species in Brazilian persimmon orchards, showed the highest level of specificity and consistent amplification across all tested isolates. The primers also exhibited high specificity for *C. chrysophilum* and *C. nymphaeae*, including isolates obtained from hosts other than persimmon. In contrast, cross-amplification was observed between *C. melonis* and *C. limetticola*, likely reflecting the high sequence similarity within the targeted TUB2 region shared by these closely related species.

Over the last decade, substantial taxonomic revisions within the genus *Colletotrichum* have led to the recognition of approximately 340 species currently organized into 20 distinct species complexes (Talhinhas and Baroncelli 2023). Within this framework, the *GAPDH* and *TUB2* genes have been identified as highly informative molecular markers for discriminating closely related species within individual complexes (Vieira et al. 2020). In the present study, analyses of these loci, combined with the *ITS* rDNA region, enabled reliable species-level identification within the CGSC and CASC. However, in cropping systems characterized by high *Colletotrichum* diversity and the coexistence of multiple species complexes, multilocus-based identification remains challenging, as the number and combination of loci required for accurate species delimitation vary among complexes (Jayawardena et al. 2021). Moreover, such approaches are inherently time-consuming and costly, limiting their applicability for routine diagnostics and large-scale disease monitoring.

The development of species-specific PCR primers has been widely explored as a practical alternative to multilocus sequencing for the diagnosis of *Colletotrichum* spp. in different crop systems. For example, Silva et al. (2017a, b) developed *GAPDH*-based primers to discriminate *C. abscissum* and *C. gloeosporioides* sensu stricto in citrus, providing a rapid and cost-effective diagnostic tool tailored to a specific pathosystem. Similar endpoint PCR approaches have been reported for strawberry anthracnose, targeting individual species such as *C. nymphaeae*, *C. siamense*, and *C. fructicola* (Karimi et al. 2020; Chung et al. 2022), with assays designed to address host- and region-specific diagnostic demands. More recently, McHenry and Aćimović (2024) developed hydrolysis probe-based real-time PCR assays for multiple *Colletotrichum* species causing bitter rot of apple, achieving high analytical sensitivity and enabling quantitative applications. While these qPCR-based assays offer superior sensitivity, they require specialized equipment and are primarily intended for epidemiological studies and large-scale monitoring programs. In contrast, the primers developed in the present study were designed using partial *TUB2* gene sequences under an updated multilocus phylogenetic framework and validated across a large and geographically diverse population of persimmon-associated isolates.

In the present study, species-specific primers were developed based on partial *TUB2* gene sequences, which

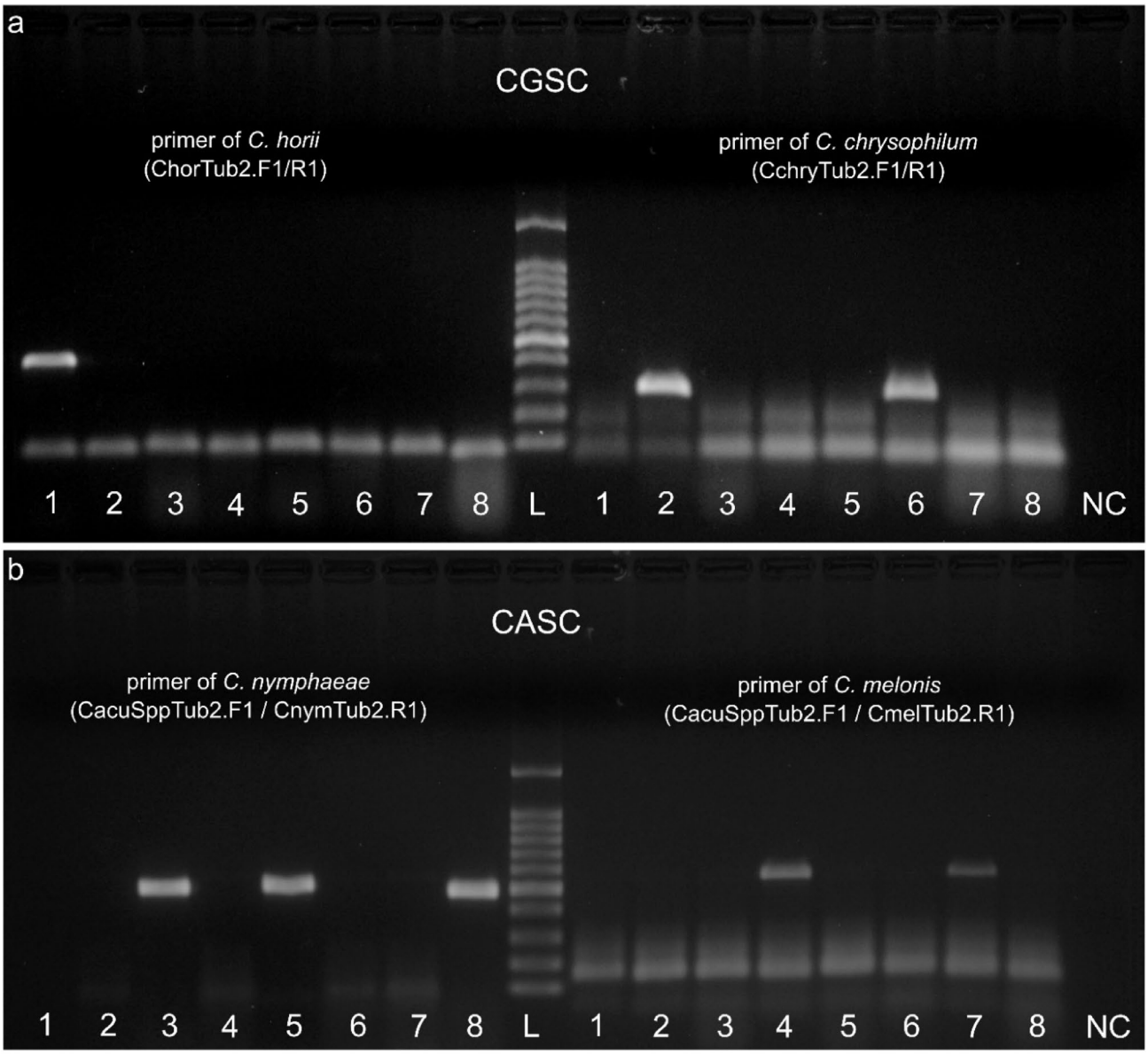

Fig. 5 Agarose gel electrophoresis of PCR products from *Colletotrichum* spp. isolates. of persimmon, apple and pear trees. Samples 1 to 8 represent isolates *DkC*PR18-03 (*C. horii* from persimmon tree), *DkC*SP18-06 (*C. chrysophilum* from persimmon tree), *DkC*PR18-01 (*C. nymphaeae* from persimmon tree), *DkC*PR18-11 (*C. melonis* from persimmon tree), Col 15 (*C. nymphaeae* from apple tree), Col 33 (*C. chrysophilum* from apple tree), Col 31 (*C. melonis* from apple tree), *PpCn*PR 17−02 (*C. nymphaeae* from pear tree), respectively. L −100-bp marker and NC – negative control without DNA. A, amplification with primer pairs from the *C. gloeosporioides* species complex (CGSC) and B, amplification with primer pairs from the *C. acutatum* species complex (CASC)

exhibited high interspecific variability among the target *Colletotrichum* species in in silico analyses. The primer pair ChorTUB2 F1/R1 showed a high level of specificity for *C. horii*, displaying a 100% match with this species in Primer-BLAST analyses. This result supports the reliability of the primer for accurate detection of *C. horii*. Importantly, previous studies have also reported a strong host association between *C. horii* and persimmon, with no confirmed records of this species causing disease in other hosts (Jayawardena et al. 2021; Talhinhas and Baroncelli 2021), further reinforcing the diagnostic specificity of the primer within the persimmon pathosystem. The analysis of the persimmon isolate collection revealed that more than 75% of the isolates belonged to *C. horii*, confirming its predominance and reinforcing the relevance of this study for accelerating species-level diagnosis within *Colletotrichum* collections, particularly for the identification of *C. horii* as the primary causal agent of persimmon anthracnose.

A broader phylogenetic context was considered by examining sequences previously reported as *C. fructicola*

in Brazil (unpublished data). In addition, an additional validation step was conducted using twelve *Colletotrichum* isolates obtained from mango and cashew orchards in northeastern Brazil, which had been previously identified as *C. fructicola* (Fig. S1). These isolates were included exclusively to further assess the specificity of the primers developed in the present study. A separate multigene phylogenetic analysis, performed to contextualize these isolates under the current taxonomic framework, indicated that only five of the twelve isolates correspond to *C. fructicola*, whereas the majority clustered within the *C. chrysophilum* clade (unpublished data). Consistent with this result, the *C. chrysophilum*-specific primer pair amplified all isolates identified as *C. chrysophilum*, whereas only one of the isolates corresponding to *C. fructicola* was amplified (Fig. S1). This suggests that in situations where both species are present, the primer developed in this study may not be sufficiently specific. However, these results demonstrate that the primer developed in this study exhibits greater specificity for *C. chrysophilum*. These results do not indicate misidentification in previous studies, but rather reflect taxonomic updates that occurred after those identifications were made. Therefore, it is recommended that studies reporting the presence of *C. fructicola* without including *C. chrysophilum* in their phylogenetic analyses be re-evaluated to achieve a more accurate understanding of the prevalence of these species in Brazil.

Primer design for *C. nymphaeae* and *C. melonis* was constrained by limited interspecific variation in the forward strand of the *TUB2* sequences, which resulted in the use of a shared forward primer (CacuSppTub2.F1) for both species. In contrast, sequence divergence in the reverse strand enabled the development of species-specific reverse primers, namely CnymTub2.R1 for *C. nymphaeae* and CmelTub2.R1 for *C. melonis*. During experimental validation, these primer combinations exhibited high specificity and allowed reliable discrimination between the two species. All tested isolates of *C. nymphaeae* and *C. melonis* were successfully amplified, with a single exception: the primer pair designed for *C. melonis* also amplified the *C. limetticola* isolate (MdCl180) obtained from apple. This result underscores the importance of confirming species identity through multigene phylogenetic analyses, particularly in pathosystems where closely related species coexist and primer specificity may be compromised. Although *C. limetticola* is not associated with persimmon and therefore does not affect diagnostic applications in this crop, this cross-amplification indicates that the primers are not suitable for distinguishing *C. melonis* in apple. In such cases, sequencing of the *ITS* region in combination with partial *GAPDH* and *TUB2* genes is recommended, as these markers provide reliable resolution among species within the *C. acutatum* species complex (Vieira et al. 2020).

Validation and additional testing of the *C. melonis* primer pair are recommended for crops in which phylogenetically closely related causal agents may occur, in order to ensure adequate specificity. Within the persimmon isolate collection evaluated in this study, only a single isolate was identified as *C. melonis*, indicating a low frequency of this species in the persimmon pathosystem. Moreover, previous studies have shown that *C. melonis* exhibits lower virulence compared with other *Colletotrichum* species associated with persimmon anthracnose (Carraro et al. 2022b), suggesting a limited epidemiological relevance in this crop. Although cross-amplification with *C. limetticola* was observed, the primary objective of differentiating the anthracnose causal agents belonging to the *C. acutatum* species complex in persimmon, *C. nymphaeae* and *C. melonis*, was successfully achieved.

In summary, a small proportion of *Colletotrichum chrysophilum*, *C. nymphaeae*, and *C. melonis* isolates was identified in the collection analyzed in this study. Unlike *C. horii*, these species are polyphagous, as they have been reported as causal agents of diseases in various crops, including apple (*Malus domestica*) (Moreira et al. 2019a; Astolfi et al. 2022), pear (*Pyrus communis*) (Moreira et al. 2021), grapevine (*Vitis vinifera*), among others previously mentioned. It is noteworthy that these hosts frequently coexist in persimmon cultivation areas (Carraro et al. 2022b). Numerous factors may influence this variability, such as cultivar susceptibility, climatic conditions, and region-specific management strategies, all of which should be considered (Dowling et al. 2020).

This study provides relevant insights into the prevalence *Colletotrichum* species in four major persimmon production regions in Brazil, highlighting differences in the population of isolates between the state of São Paulo and the states of Paraná, Santa Catarina, and Rio Grande do Sul. This is the first study to report the development of specific primers for the rapid detection of *C. horii*, *C. chrysophilum*, *C. nymphaeae*, and *C. melonis* using conventional PCR. Although formal limit-of-detection assays based on serial DNA dilutions were not performed, all primer pairs consistently amplified their target fragments using genomic DNA concentrations (50–100 ng) commonly employed in routine diagnostic PCR assays. Thus, the primers demonstrated robust analytical performance under standardized diagnostic conditions. The determination of detection limits will be addressed in future studies focused on assay optimization and quantitative applications. Overall, the primer pairs developed here represent a practical and efficient tool for rapid species-level diagnosis within the *Colletotrichum*-persimmon pathosystem and may support improved epidemiological studies and

disease management strategies. Future research should further validate these primers using alternative PCR platforms, including real-time PCR and multiplex PCR assays.

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Author contributions Thiago de Aguiar Carraro contributed to the experimental work, collected the infected material and their isolation, analyzed the data and wrote/edited the manuscript. Alan de Oliveira Silva performed the design of species-specific primers and wrote/edited the manuscript. Chirlei Glienke reviewed and wrote/edited the manuscript. Louise Larissa May De Mio conceptualized the study, acquired funds, revised and wrote/edited the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This manuscript does not involve research on humans or animals, nor does it include vulnerable populations.

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