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ORIGINAL ARTICLE

Loop-mediated isothermal amplification and PCR combined assay to detect and distinguish latent *Colletotrichum* spp. infection on strawberry

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Abstract

Strawberry is economically important. Anthracnose caused by *Colletotrichum* spp. has been a serious threat to this crop globally. To detect and distinguish latent *Colletotrichum* spp. infection on asymptomatic plants at an affordable cost is crucial for this disease control and the sustainability of strawberry industry. Loop-mediated isothermal amplification (LAMP) assay is superior for the higher sensitiveness, efficiency, and lower cost than other methods for pathogen diagnosis. In this study, six previously reported barcodes were evaluated against DNA templates from strawberry fungal pathogens including six *Colletotrichum* species and five beyond this genus, and further verified in 40 *Colletotrichum* isolates. Based on the discernibility revealed, a LAMP assay was developed for the diagnosis of *Colletotrichum* spp. by designing six primers recognizing the conserved regions in *β-tubulin 2* gene from 13 *Colletotrichum* species of *C. gloeosporioides* and *C. acutatum* complexes. The specificity and accuracy of LAMP assay was tested against six *Colletotrichum* species of two complexes, with a detection sensitivity of 100 pg/μL genomic DNA. Current LAMP assay beginning with a 10 min plant lysis enabled a direct and quick diagnosis of *Colletotrichum* spp. in strawberry within one hour. Followed by PCR using primers specific to ApMat, Marker 2, and Marker 1, this assay could specifically differentiate *Colletotrichum* species, at least for the dominant species *C. fructicola* and *C. siamense* in China, realizing a diagnosis of latent infection at a species level. Current LAMP-PCR combined protocol allows for a sensitive and efficient detection and differentiation of latent *Colletotrichum* infection on strawberry, which will be useful for disease management and monitoring pathogen population.

Keywords Strawberry · *Colletotrichum* spp. · DNA diagnosis · PCR · LAMP

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Introduction

Modern strawberry (*Fragaria* × *ananassa* Duchase) is of great economic importance and widely cultivated (Zhao et al. 2012; Edger et al. 2019). Anthracnose caused by *Colletotrichum* spp. is a serious threat to strawberry production worldwide (Buddie et al. 1999; Martínez-Culebras et al. 2000). All the tissues of strawberry plant could be infected with this disease. Sunken necrosis in strawberry leaves, petioles, and stolons often occurs after a rainfall in the field if no fungicides have been applied in advance. Plant wilting due to anthracnose crown rot constantly causes great losses in nursery and production field after transplanting (Freeman and Katan 1997). Many farmers share their experiences that, during seedling propagation about 90% fungicides are used for anthracnose controlling in the season of high temperature and humidity in Shanghai, where typhoons and heavy

rainfalls frequently occur in summer. A broad range of hosts and wide adaptation to temperature in *Colletotrichum* spp. increase the difficulty for disease management (da Silva et al. 2020). Anthracnose has long been one of the crucial limiting factors for strawberry production, especially hampering soil conservation and ecological cultivation.

Colletotrichum genus was among the top eight important groups of fungal pathogens in the world (Dean et al. 2012). The genetic limits among strawberry anthracnose pathogens of worldwide origins had long been clarified as three clades of *C. acutatum, C. fragariae,* and *C. gloeosporioides* (Martínez-Culebras et al. 2000, 2003). Many biologists contributed to understanding *Colletotrichum* systematics related to strawberry (Cannon et al. 2012), although some synonymies proposed in their work were later considered to be inaccurate (Damm et al. 2012). The emergency of molecular data and multi-loci phylogeny finally revolutionized *Colletotrichum* species identification.

Currently, a total of 14 species complexes have been proposed in *Colletotrichum* genus (Damm et al. 2019). Of them, *C. gloeosporioides*, *C. acutatum* and *C. boninense* species complexes including at least 17 species have been reported in relation to strawberry anthracnose: seven species of *C. acutatum* complex including *C. acutatum*, *C. foriniae*, *C. godetiae*, *C. miaoliense*, *C. nymphaeae*, *C. salicis*, and *C. simmondsii*, eight of *C. gloeosporioides* complex including *C. aenigma*, *C. changpingense*, *C. fructicola*, *C. gloeosporioides*, *C. kahawae*, *C. siamense*, *C. theobromicola* (syn. *C. fragariae*), and *C. viniferum*, as well as two species *C. boninense* and *C. karstii* of *C. boninense* complex (Weir et al. 2012; Han et al. 2016; Wang et al. 2019; Chung et al. 2020). The former two complexes *C. gloeosporioides* and *C. acutatum* were widely reported in relation to strawberry anthracnose co-existing in North America and East Asia.

The spread of anthracnose in strawberry field is very rapid under appropriate weather conditions, aggravated by ordinary agricultural practice such as transplanting, watering, and trimming old leaves (Ren et al. 2008). To detect and eliminate asymptomatic latent infection is vital for controlling the damage of anthracnose and avoiding a severe outbreak of this disease in strawberry field. Polymerase chain reaction (PCR) has been successfully used for identifying *C. acutatum* in strawberry (Parikka and Lemmetty 2004). Specific detection of *Colletotrichum* spp. has been developed for some species (Tao et al. 2013; Azevedo-Nogueira et al. 2021). A barcode named Marker 2 was reported to differentiate among *C. fructicola*, *C. siamense*, and *C. aenigma* virulent to strawberry (Gan et al. 2017). A DNA macroarray was developed for the diagnosis of latent infection of *C. acutatum* in strawberry plants, based on a PCR using primers specific to *MAT1-2* gene (encoding a factor determining mating type) and a subsequent microtube hybridization (MTH) (Furuta et al. 2017). In another report, a loop-mediated isothermal amplification (LAMP) assay was developed for detecting *C. gloeosporioides sensu lato* in strawberry (Wu et al. 2019). Up to date, there is no method reported for detecting both *C. acutatum* and *C. gloeosporioides* species aggregates simultaneously.

Both real-time PCR and digital PCR (dPCR) allow for accurate quantitation of pathogen molecules and have been widely used in disease diagnosis (Yang et al. 2015; Quan et al. 2018). But these methods rely on special instruments, and the cost for diagnosis often is too expensive to be practically applied in strawberry industry. Loop-mediated isothermal amplification (LAMP) is a rapid nucleic acid amplification technique at a constant temperature with a relatively low cost (Notomi et al. 2000). A LAMP assay is more sensitive and specific than PCR (Hara-Kudo et al. 2007). Hydroxy naphthol blue added into a LAMP reaction tube provides a convenient and fast visual observation of DNA amplification via color changes with a low risk of contamination (Goto et al. 2009). LAMP enables the feasibility of point-of-care testing (POCT) on sites such as strawberry field without a need of special devices. Generally, both LAMP and conventional PCR are affordable for strawberry farmers.

Molecular diagnosis of anthracnose is very important for the sustainable development of strawberry industry. The basic question for strawberry health disease managers, government quarantine personnel and strawberry breeders is to know what *Colletotrichum* species infect strawberry, and what the range of fungal targets is. To meet this need, the present study intended to evaluate previously published barcodes for differentiating *Colletotrichum* spp. pathogenic to strawberry. Based on the discernibility revealed, a direct LAMP was developed to identify latent *Colletotrichum* infection on strawberry, and the LAMP combined with PCR further enabled a definitive resolution in species-specific diagnosis for the dominant species.

Materials and methods

Fungal isolates

This work was based on two previous efforts for characterizing *Colletotrichum* spp. from diseased strawberry in central and eastern China (Han et al. 2016; Zhang et al. 2020). Six isolates of *Colletotrichum* spp. representing distinct species were utilized as standard strains (Supplementary Table S1), including five strains of *C. gloeosporioides* species complex i.e., GQH124 (*C. aenigma*) and GQHZJ19 (*C. siamense*) (Zhang et al. 2020), CGMCC3.17371 (*C. fructicola*) (Ren et al. 2008), Jsh-7-1 (*C. gloeosporioides*), and Nj-2 (*C. murrayae*) (Han et al. 2016), as well as one of *C. acutatum* complex Lch5 (*C. nymphaeae*) (Han et al. 2016). Five additional pathogenic fungi beyond *Colletotrichum* genus were used as negative controls in evaluating the discernibility of

reported markers. These pathogens consisted of an isolate of *Nectria pseudotrichia* originating from Guangxi Province caused necrosis and brown to red crown rot (Zhang et al. 2018), an isolate of *Fusarium oxysporum* triggered wilting from Qingpu Shanghai (ITS rDNA barcode identical to MT453296), an isolate of *Pestalotiopsis clavispora*) (ITS rDNA barcode-MF399814) from northern Anhui Province causing red leaf disease (Ning et al. 2019), and an isolate of *Botrytis cinerea* from strawberry fruits causing gray mold (ITS rDNA barcode identical to MH860108). The genomic DNA of *Podosphaera aphanis* (isolate NWAU1, 2017 purified from Shaanxi) causing strawberry powdery mildew, was a gift from Dr. Jiayue Feng of Northwest A&F University (ITS rDNA barcode-KU207048).

Furthermore, a total of 40 isolates belonging to *C. gloeosporioides* species aggregate (including 20 *C. fructicola*, 18 *C. siamense*, and two *C. aenigma*, Supplementary Table S2) were used to validate the applicability of candidate barcodes for detecting *Colletotrichum* spp. These single-spore isolates were previously purified from diseased strawberry in the downstream regions of Yangtze River surrounding Shanghai during 2016 to 2018 (Zhang et al. 2020). All filamentous fungi were maintained on potato dextrose agar (PDA, Lot# 0091369, Becton, Dickinson and Company, USA) plates at 28 ℃.

DNA extraction and PCR amplification

Fungal DNA was extracted from about 100 mg fresh mycelia using the Quick-DNA^{TM} Fungal/Bacterial Miniprep Kit (ZYMO Research, USA, Cat. No. D6005) following the manufacturer's instruction with minor adaption. Specifically, fungal cells were homogenized at 55Hz for 7 min followed by a centrifugation at 10,000 x *g* for 2 min. When evaluating markers for *Colletotrichum* spp., strawberry genomic DNA was used as a negative control and purified from leaf tissues using a CTAB method as described previously (Liu et al. 2014). The quality of genomic DNA was evidenced on 1% agarose gel electrophoresis and quantified via a NanoDrop-1000 spectrophotometer (Beckman, USA). All DNA samples were diluted to 10 ng/μL and stored at -20 ℃ until use. For LAMP or PCR detection of *Colletotrichum* spp. in strawberry tissues either symptomatic or asymptomatic, a simple DNA extraction was accomplished in around 10 min using the lysis buffers of T5 Direct PCR Kit (Plant) (TsingKe Biological Technology, Beijing, China). Briefly, a piece of fresh tissue (leaf blade, petiole or crown) in an appropriate parameter of 2-3 mm was immersed into 50 μL buffer A at 95 ℃ for 10 min, and then mixed with 150 μL buffer B. The diluted lysate was directly used for PCR or LAMP assay.

Amplification with the fungal ITS *rDNA* (White et al. 1990) and/or strawberry *Actin* (gene 18570-v1.0-hybrid, *FvH4_1g23490*) specific primers (Yang et al. 2021) was performed to confirm the quality of DNA template. Six reported barcodes for *Colletotrichum* spp. were evaluated including *β-tubulin 2* (*TUB2*) (O'Donnell and Cigelnik 1997), *Glutamine synthetase* (*GS*) (Prihastuti et al. 2009), Marker1 and Marker 2 (Gan et al. 2017), Virulent-strain specific (Suzuki et al. 2008), and the Apn2-Mat1-2 intergenic spacer and partial mating type (Mat1-2) gene (*ApMat*) (Silva et al. 2012). All primer pairs used were shown in Supplementary Table S3.

PCR was performed on an ETC 811 DNA amplifier (Eastwin, Beijing, China) in a 20-μL reaction mixture containing 1 μL DNA template, 3 μM of each primer, 0.5 μL dNTP (2.5 mM), 1x PCR buffer with Mg^{2+}, 1 U *Taq* DNA polymerase (Biocolor, Shanghai, China). The PCR reaction consisted of an initial denaturation at 94 ℃ for 3 min, followed by 30 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 30 sec (for strawberry *Actin* specific primers) or 1 min (for fungal ITS, *ApMat*, *TUB2*, *GS*, Virulent-strain specific, Marker1 and Marker 2 specific primers), then a final extension of 72 ℃ for 5 min. When detecting with a coarse lysate DNA template from fresh strawberry or mycelium tissues, the same PCR system with 35 cycles was performed. PCR products were visualized on 1% (Marker 1 and ApMat specific products) or 1.5% (all the rest barcodes) agarose gel electrophoresis.

Primer design and optimization for LAMP assay to detect both *C. gloeosporioides* and *C. acutatum* aggregates

Full length DNA sequences for *TUB2* gene of 13 *Colletotrichum* species related to strawberry anthracnose were collected from GenBank based on a Blast search in NCBI. Sequence alignment revealed the conserved regions in *TUB2* gene of *C. gloeosporioides* and *C. acutatum* aggregates (Supplementary Fig. S1). A set of six LAMP primers (Table 1) comprising two outer primers F3/B3, two inner primers FIP (F1c-F2)/BIP (B1c-B2), and two loop primers LF/LB were designed in SNAPGENE software following the rules previously described (Notomi et al. 2000).

The components of LAMP assay were largely set as described by Li et al. (2018). The LAMP reaction was initially carried out at 64 ℃ for 60 min in a Digital Dry Bath (MIULAB, Hangzhou, China). DNA of CGMCC3.17371 (*C. fructicola*) was used as a positive control whereas *Botrytis cinerea* (*BC*1) and H_2O as negative controls. An optimization of Mg^{++} concentration ranging from 2 mM to 8 mM revealed that 4 mM $MgSO_4$ provided a sound differentiation, when the color in positive reaction tubes changed from violet to sky blue, whereas a negative one maintained violet. Visual color change was always confirmed by the gel electrophoresis on 2% agarose in 1 x TAE buffer at 95 V

Table 1 Primer sequences for loop-mediated isothermal amplification (LAMP) designed in current study

Primer Name	Location	Sequence (5'-3')[a]
Tub-F3	649-667	CTCGTCGAYTTGGAGCCCG
Tub-R3	878-896	TACCRGCACCGGTACCACC
Tub-FIP (F1c-F2)	745-764/669-687	TTGGCCCAGTTGTTGCCGGC TACCATGGACGCCGTCCGT
Tub-BIP (B1c-B2)	788-812/839-857	AGCTCGTYGAYCAGGTTC TCGATGT TGGAARCCC TGGAGGCAGT
Tub-LF	712-730	AGACGAAGTTGTCGGGGCG
Tub-LB	821-838	GCGAGGCYGAGGGMTGCG

[a]Letters in red represent degenerate nucleotide bases at certain positions

for 25 min. LAMP optimization was performed with three replicates in one assay, and independently repeated four times. After optimization, LAMP reaction system in current study was a 25-μL mixture of 2 μL DNA solution (10 ng/μL), 1 μL inner primers FIP/BIP (40 μmol/L), 0.5 μL outer primers F3/B3 (10 μmol/L), 1 μL loop primers LB/LF (40 μmol/L), 2.5 μL dNTPs (10 mmol/L), 1 μL$MgSO_4$ (100 mmol/L), 4 μL Betaine solution (5 mol/L, Sigma, Lot# SLCB2017), 1 μL Bst DNA polymerase Large Fragment (8U/ μL, Vazyme, P701-01), 2.5 μL Hydroxy naphthol blue (HNB) (3 mmol/ L, Sigma, Lot# SHBL8323), and 2.5 μL 10×ThermoPol Buffer. Visual color changes in positive tubes often appeared since 35 min at 64 ℃ and a 50 min bath was enough.

Determining the specificity and sensitivity of LAMP assay

For specificity analysis, the genomic DNAs of six *Colletotrichum* species and five fungi beyond *Colletotrichum* genus listed in Supplementary Table S1 were used as templates in LAMP assay adopting the optimal parameters. For sensitivity comparison, LAMP was performed against a serial dilution templates of genomic DNA from *C. aenigma*, namely, 100 nanogram (ng) /μL, 10 ng/μL, 1 ng/μL, 100 picogram (pg)/μL, 10 pg/μL, 1 pg/μL, 100 femtogram (fg)/μL, and 10 fg/μL. Side-by-side PCR always was carried out with LAMP assay for either specificity or sensitivity test. Both PCR and LAMP reaction products were illustrated by agarose gel electrophoresis. The experiments were independently carried out three times, and each DNA template was tested with three technical replicates one time.

LAMP-PCR combined assay for detecting *Colletotrichum* spp. in strawberry plants

To validate the detection method in real strawberry, two types of materials including symptomatic plants inoculated with *C. fructicola* and asymptomatic plants as suspicion of infections from field were used. A direct detection of *Colletotrichum* spp. in all strawberry tissues started with a 10 min lysis for plant materials using buffers from T5 Direct PCR kit and was followed by a regular LAMP assay as described above. The positive samples revealed by LAMP assay were further examined using a PCR analysis with three barcodes including Marker 1, Marker 2, and *ApMat*.

To validate the direct LAMP assay for symptomatic plants, five healthy cv. Shanghai Angel plants were inoculated with *C. fructicola* (CGMCC3.17371). Inoculum was prepared as a conidial suspension from 7 d culture in potato dextrose broth (Lot#0293648, Becton, Dickinson and Company, USA) at 180 rpm under 28 ℃. The resulting conidial suspension was filtered through three layers of lens wiping tissue and adjusted to a concentration of 2×10^6 per mL by diluting with 0.01% Tween 20 water solution. Plants were inoculated with conidial suspension by misting to runoff using a hand pump sprayer. Control plants were mock-treated with Tween 20 water solution. Plants were immediately placed in a growth incubator (A1000, Conviron, Canada) under 25 ℃ light/23 ℃ dark, and 90-100% relative humidity (100% RH for first two days). The diseased leaf blade, petiole, and crown tissues were sampled at 7-11 days post inoculation. Plant inoculation and sampling were independently repeated twice.

To detect latent infection on strawberry, the compound leaves with petioles were randomly sampled at a lower position from asymptomatic plants in a production field located at Zhuanghang trial station of SAAS, where several dead plants had been eliminated previously. For mycelia culture on diseased plant tissues, three pieces of leaf petioles in an approximate size of 3 x 10 mm were detached and twice sterilized using 70% ethanol for 30 s, followed by a culture at 28 ℃ for two days on PDA plate with 25 mg/L chloramphenicol. The positive samples revealed in LAMP assay were further examined using PCR against mycelia DNA for three barcodes including Marker 1, Marker 2, and ApMat. Plant sampling in field was independently performed three times, and similar results were obtained.

Results

PCR analysis of DNA markers for detecting *Colletotrichum* spp. pathogenic to strawberry

To identify marker(s) fit for molecular detection of *Colletotrichum* infection in strawberry, the discernibility of several previously reported barcodes was examined for differentiating between fungal species within and beyond *Colletotrichum* genus. Amplification of six barcodes displayed distinct profiles in strawberry fungal pathogens (Fig. 1). PCR with *ApMat* specific primers generated a uniform band in *C. fructicola*, *C. siamense*, *C. murrayae*, and *C. gloeosporioides sensu stricto*, but no band in *C. aenigma* while a relatively smaller band in *C. nymphaeae* of *C. acutatum* species complex. Amplicon of Marker 2 was uniform in *C. murrayae*, *C. siamense* and *C. gloeosporioides sensu stricto*, smaller in *C. aenigma*, larger in *C. fructicola*, whereas absent in *C. nymphaeae*. By contrast, PCR with *Glutamine synthetase* (*GS*) specific primers generated uniform bands in all five species of *C. gloeosporioides* species complex. Similar with Marker 2, *GS* related amplification was absent in *C. nymphaeae* of *C. acutatum* complex. PCR with *TUB2* specific primers produced a uniform band in herein six *Colletotrichum*

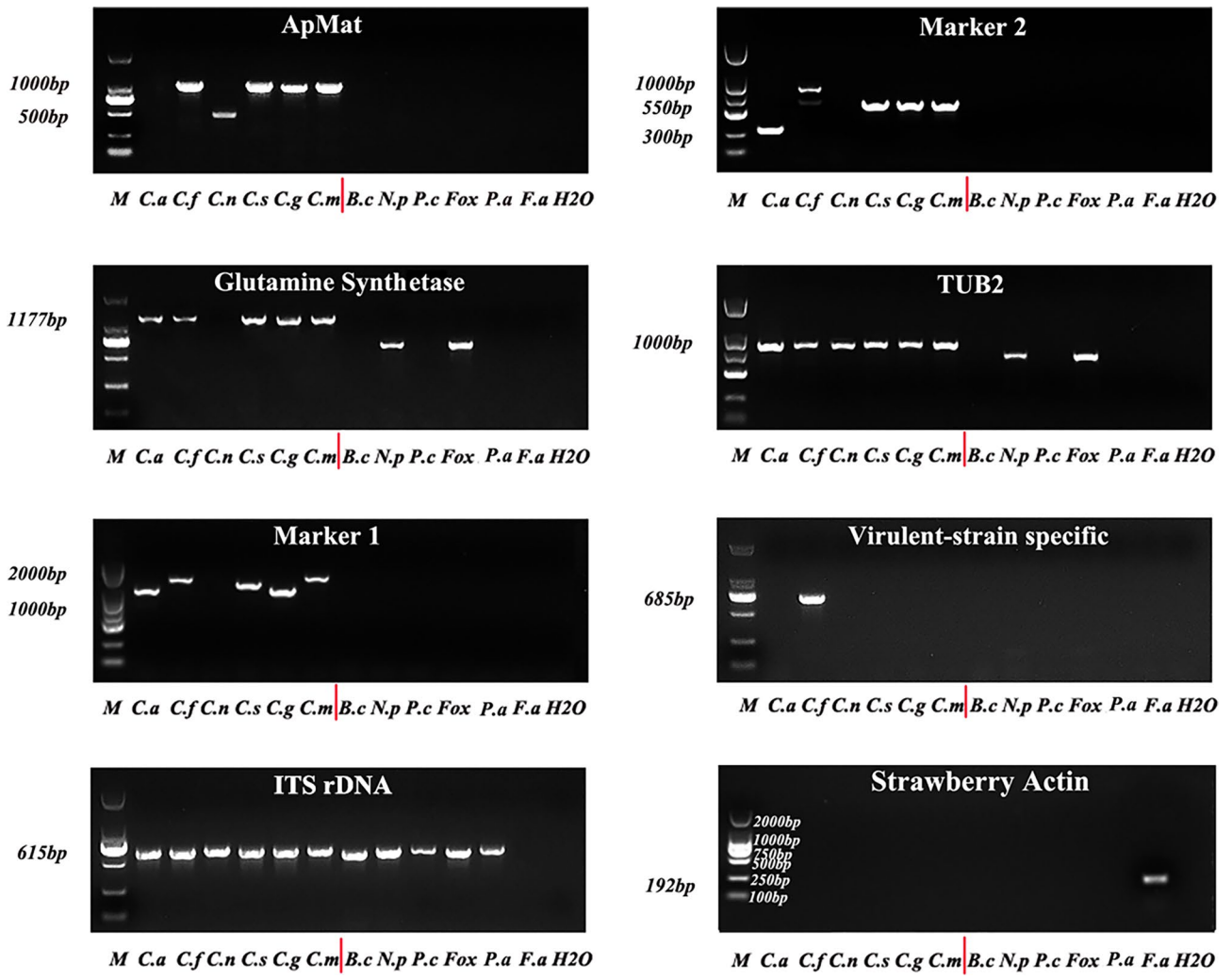

Fig. 1 PCR analysis of previous barcodes in differentiating *Colletotrichum* spp. endangering strawberry. Reported barcodes including the Apn2-Mat1-2 intergenic spacer and partial mating type (Mat1-2) gene (*ApMat*), Glutamine synthetase, Marker 1, Marker2, *β-tubulin 2* (*TUB2*), and Virulent-strain specific were amplified against the genomic DNAs of six *Colletotrichum* species from diseased strawberry. Five fungi pathogenic to strawberry beyond *Colletotrichum* genus were used as negative controls. Amplification of the fungal ITS rDNA and strawberry *Actin* was performed to verify the quality of DNA templates. M: DL2000-bp DNA size marker; C.a: *C. aenigma*, C.f: *C. fructicola*, C.n: C. *nymphaeae*, C.s: *C. siamense*, C.g: *C. gloeosporioides*, C.m: *C. murrayae*; B.c: *Botrytis cinerea*, N.p: *Nectria pseudotrichia*, P.c: *Pestalotiopsis clavispora*, F.ox: *Fusarium oxysporum*, P.a: *Podosphaera aphanis*; F.a: *Fragaria* × *ananassa* cv. Shanghai Angel, H2O: negative control. The approximate size of PCR amplicons in *Colletotrichum spp.* was indicated at the left side of each panel

species. Notably, the primers specific to *GS* and *TUB2* worked with DNA templates of *Nectria pseudotrichia* and *Fusarium oxysporum* too, although with obviously smaller bands. Amplification with the Virulent-strain specific primers seemed specific and limited to *C. fructicola*. Intriguingly, PCR with Marker 1 specific primers generated highly polymorphic profiles in distinct *Colletotrichum* spp. When we rearranged these PCR products on 1% agarose gel, a DNA ladder could be observed following the order as indicated in Supplementary Fig. S2.

Furthermore, the applicability of six barcodes in detecting *Colletotrichum* spp. was confirmed in a collection of 40 *Colletotrichum* isolates (Supplementary Table S2) belonging to two dominant species *C. fructicola* and *C. siamense* and the sporadically occurred *C. aenigma* previously isolated from diseased strawberry (Zhang et al. 2020). The results were largely consistent with above analysis in 11 distinct standard species of strawberry pathogens (Supplementary Fig. S3). Accordingly, the performance and discernibility of individual barcode specific primers in resolving *Colletotrichum* spp. was summarized (Table 2). *TUB2* specific primers generated a uniform band in all six *Colletotrichum* species, enabling a general diagnosis of *Colletotrichum* spp. infection, at least for *C. gloeosporioides* and *C. acutatum* species aggregates. *GS* specific primers could distinguish C. *gloeosporioides* species complex from other strawberry pathogens. PCR with Virulent-strain specific primers could be used to specifically recognize *C. fructicola*. Marker 2 can distinguish *C. aenigma* and *C. fructicola* from the rest *Colletotrichum* species here examined. Marker 1 seems promising in clarifying the genetic limits among six *Colletotrichum* spp, differentiating anthracnose fungi at a species level. A combined use of these primers in DNA diagnosis could provide a systematic and comprehensive diagnosis, meeting the needs of different levels.

TUB2-based LAMP assay for detecting *C. gloeosporioides* and *C. acutatum* species aggregates

TUB2 has been considered as one of the most popular diagnostic markers for *Colletotrichum* species (Damm et al. 2012). In current study, PCR with *TUB2* -specific primers produced uniform bands in *Colletotrichum* spp. and different bands in *Nectria pseudotrichia* and *Fusarium oxysporum.* Since LAMP based DNA amplification requires a set of four or six specific primers that recognize six distinct regions on the template DNA, this method has an extremely higher specificity than PCR (Notomi et al. 2000). Accordingly, *TUB2* gene was selected to establish a LAMP assay for a specific detection of both *C. gloeosporioides* and *C. acutatum* species aggregates.

There exist at least 15 species belonging to *C. gloeosporioides* and *C. acutatum* aggregates reported to be pathogenic to strawberry (Weir et al. 2012; Han et al. 2016; Wang et al. 2019; Chung et al. 2020). *TUB2* sequences in GenBank were not available for two species *C. changpingense* (Jayawardena et al. 2016) and *C. miaoliense* (Chung et al. 2020). Based on the *TUB2* sequences of the rest 13 *Colletotrichum* species belonging to two aggregates virulent to strawberry (Supplementary Fig. S1), a set of six primers consisting of two outer primers, two inner primers and two loop primers were designed for LAMP assay (Table 1). Side-by-side comparison between PCR and LAMP was always carried out to examine the efficiency of LAMP assay. Typical ladder-like bands were observed on gel electrophoresis of LAMP products for all six *Colletotrichum* species (Fig. 2). No amplification was present in host strawberry or in fungi beyond *Colletotrichum* genus. When HNB was used as a visual indicator, color change from violet to sky blue was observed as expected in the tubes of *Colletotrichum*

Table 2 Performance of individual gene specific primers at resolving *Colletotrichum* spp. within strawberry fungal pathogens using PCR and agarose electrophoresis

Organism	TUB2	GS	Marker1	Marker2	Virulent-specific	ApMat
Colletotrichum spp.[a]	Y[c]-D[d]	N-N	N-N	N-N	N-N	N-N
C. g.[b] *sensu lato*	Y-N	Y-D	Y-D	Y-D	N-N	N-N
C.g. sensu stricto	Y-N	Y-N	Y-D	Y-N	N-N	Y-N
C. fructicola	Y-N	Y-N	Y-D	Y-D	Y-D	Y-N
C. siamense	Y-N	Y-N	Y-D	Y-N	N-N	Y-N
C. murrayae	Y-N	Y-N	Y-D	Y-N	N-N	Y-N
C. aenigma	Y-N	Y-N	Y-D	Y-D	N-N	N-N
C. nymphaeae	Y-N	N-N	N-N	N-N	N-N	Y-D

[a]*Colletotrichum* spp. for *C. gloeosporioides* and *C. acutatum* complexes

[b]*C. g.* for *gloeosporioides;* sensu lato for species aggregate/complex

[c]PCR product present (Y) or absence (N)

[d]PCR with gene specific primers could differentiate between *Colletotrichum* species or separate *Colletotrichum* spp. from fungi of other species aggregate or genus successfully (D) or not (N)

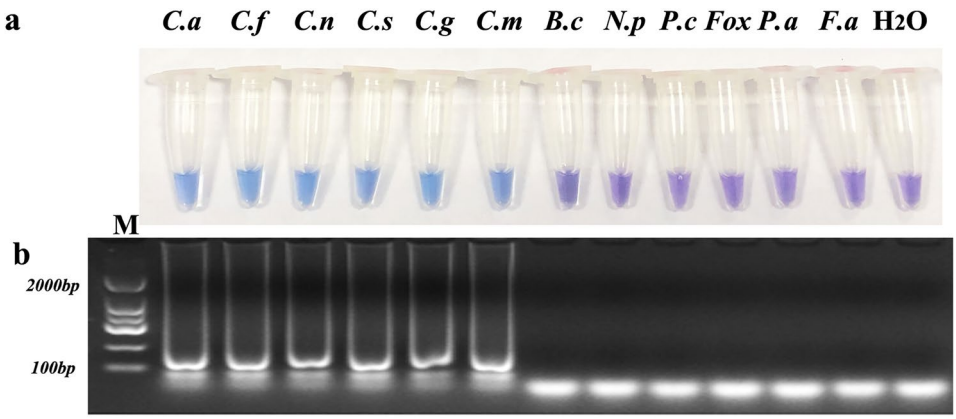

Fig. 2 LAMP assay using six primers specific to *β-tubulin 2* (*TUB2*) (Table 1) allowed a *Colletotrichum* spp. specific amplification. **a** Color changes evidencing the products of LAMP using hydroxynaphthol blue (HNB) as a visual indicator. **b** Detection of LAMP products via 2% agarose gel electrophoresis. The amplification templates were the same genomic DNAs (as in Fig. 1) of 11 fungal isolates infectious to strawberry including six *Colletotrichum* species (Lanes C.a, C.f, C.n, C.s, C.g, C.m) and five beyond *Colletotrichum* (Lanes B.c, N.p, P.c, Fox, P.a). Lanes F.a: strawberry, M: DL2000 DNA ladder, H2O: negative control

samples, while the color remained violet in other fungal or healthy strawberry sample tubes. Both gel electrophoresis and visual color change indicated that the LAMP reaction could specifically distinguish *Colletotrichum* spp. from host DNA and five other fungal pathogens of strawberry.

To reveal the sensitivity of the LAMP assay, a 10-fold serial dilution of the genomic DNA of *C. aenigma* (strain GQH124) from 100 ng to 10 fg was simultaneously tested in PCR and LAMP assay. Both the visual color changes (Fig. 3a) and gel electrophoresis (Fig. 3b) indicated that LAMP reaction successfully worked with DNA templates at concentrations ranging from 100 ng to 100 pg. Conventional PCR using the primers TUB2-T1 and TUB2-R2 generated a weak band in a reaction with 1 ng DNA template (Fig. 3c). Clearly, the LAMP assay exhibited a lower detection limit, with at least 10 times higher sensitivity in DNA diagnosis for strawberry anthracnose than PCR.

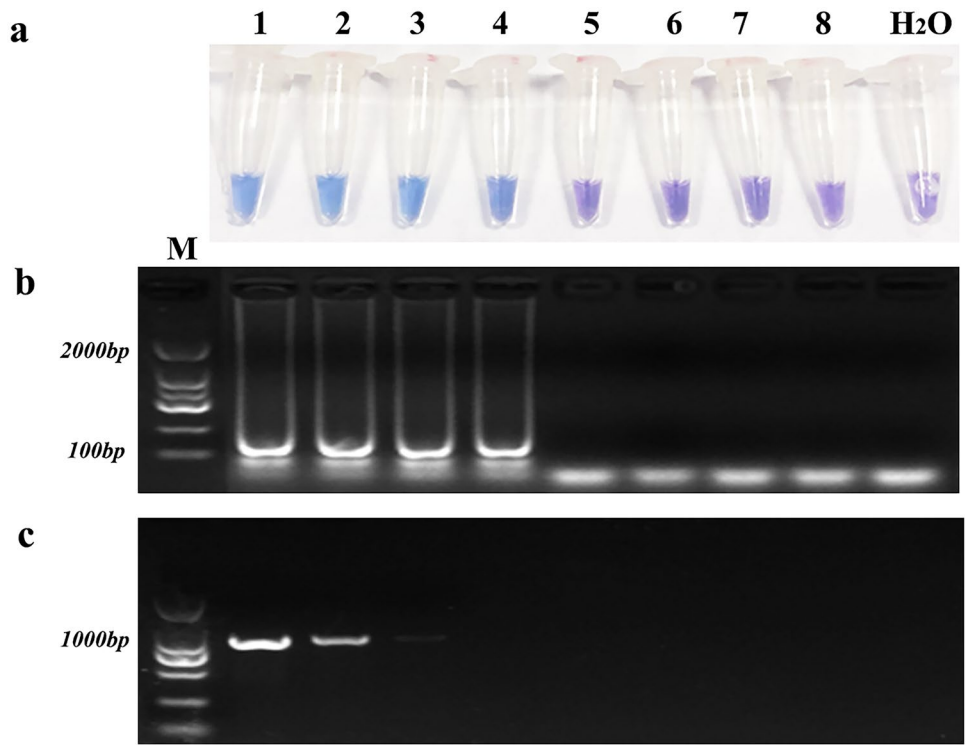

Fig. 3 Sensitivity of LAMP assay specific to *Colletotrichum* spp. pathogenic to strawberry. **a** Color changes evidencing the LAMP products using HNB. **b** Agarose gel electrophoresis of LAMP products. **c** Electrophoresis results of conventional PCR using TUB2-T1 and TUB2-R2 primers (Supplementary Table S3). A gradient dilution of the genomic DNA from *C. aenigma*, one species causing strawberry anthracnose in Shanghai was used for LAMP and a comparative PCR analysis. Lanes 1-8: 100 ng/μL, 10 ng/μL; 1 ng/μL; 100 pg/μL; 10 pg/μL; 1 pg/μL, 100 fg / μL, 10 fg /μL DNA. M:DL2000 DNA ladder

Direct LAMP detection of *Colletotrichum* spp. in symptomatic strawberry plants

Different parts from strawberry plants of cv. Shanghai Angel inoculated with *C. fructicola* were collected at 7-11 days post inoculation together with a control healthy leaf sample (Fig. 4a). The coarse DNA solutions were obtained from the leaf blade, petiole, and crown tissues after a simple pretreatment at 95 ℃ for 10 min in a metal dry bath (Fig. 4b). Then, a LAMP assay was performed using the optimized reaction system as described above. The visual color changes showed that this direct LAMP detection of *Colletotrichum* infection successfully worked with simply pretreated strawberry samples (Fig. 4c). The LAMP amplification indicated by coloring change was confirmed by gel electrophoresis (data not shown). Color changes in reaction tubes appeared since 35 to 40 min bathing at 64 ℃ in three independent repeats of LAMP assay.

Comparatively, a direct PCR was performed using *TUB2* specific primers to detect *Colletotrichum* spp. in diseased strawberry. A consistent result was obtained (Fig. 4d), although about 2.5 hr were used for PCR diagnosis. Apparently, no need for a thermal cycler and an electrophoresis apparatus, a DNA diagnosis of *Colletotrichum* spp. in strawberry samples could be accomplished in one hour via LAMP in a portable dry bath.

LAMP-PCR facilitated a diagnosis of *Colletotrichum* spp. in asymptomatic strawberry

High quality and timely information on latent infection is crucial for controlling the epidemic of strawberry anthracnose. In this study, we validated the ability of LAMP assay followed by a PCR analysis in detecting and differentiating *Colletotrichum* spp. in asymptomatic strawberry plants collected from a production field in a SAAS trail station at Zhuanghang Town, Fengxian District, Shanghai.

Genomic DNA from the outer leaves at a lower position of asymptomatic plants in field was released at 95 ℃ for 10 min and used in a direct LAMP and PCR detection. Seven positive samples were detected from 20 strawberry plants in LAMP assay, while only four positive samples

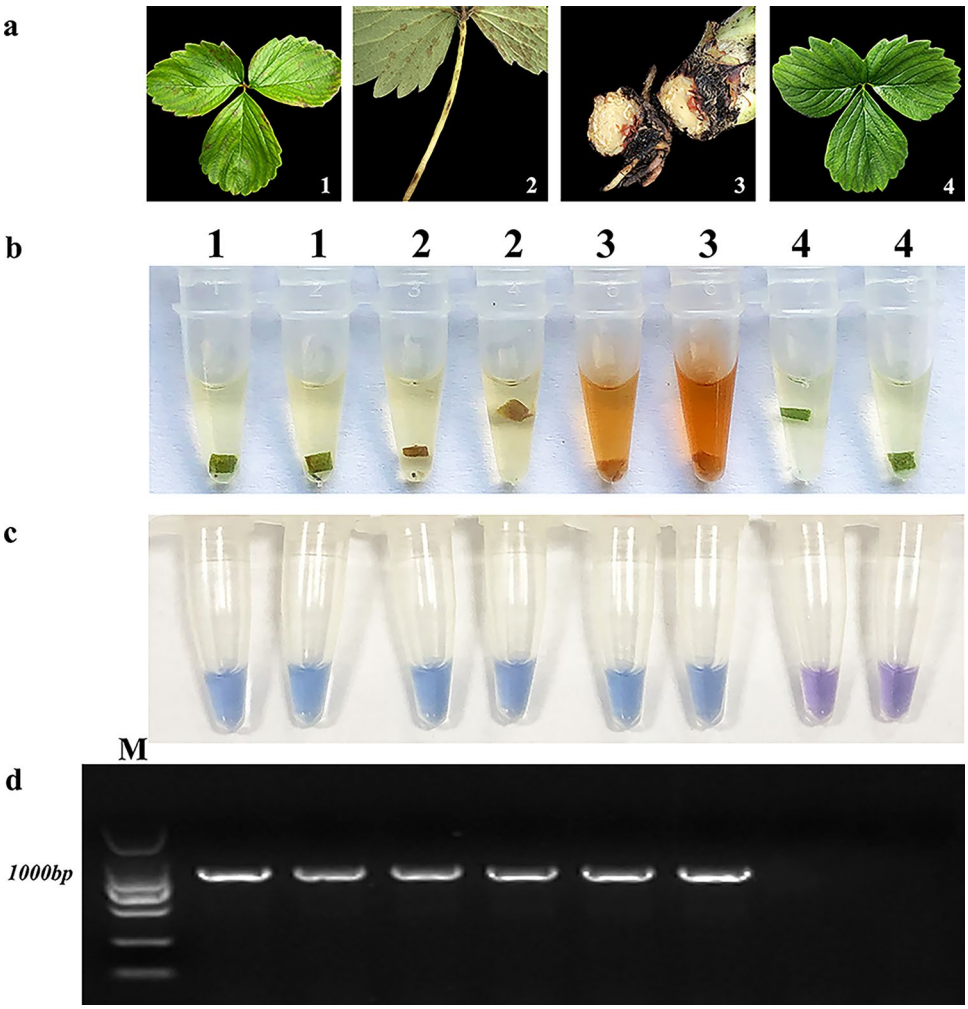

Fig. 4 Direct LAMP and PCR detection of *Colletotrichum* spp. in diseased strawberry. The symptomatic leaf blade (1), petiole (2), and crown (3) tissues were sampled from strawberry plants cv. Shanghai Angel infected with *C. fructicola* at 7-11 days post inoculation (dpi). The healthy leaf blade tissue (4) was used as a negative control. **a** The morphology of diseased and healthy strawberry tissues. **b** Pretreatment of fresh strawberry tissues using lysis buffer A after at 95 ℃ for 10 min. **c** Direct LAMP assay for the detection of *Colletotrichum* spp. in strawberry. **d** Direct PCR detection of *Colletotrichum* spp. in symptomatic strawberry samples

were detected in conventional PCR (Fig. 5a, b). Apparently, the detection ratio of *Colletotrichum* spp. in asymptomatic plants was higher for LAMP assay than PCR. In addition, a two days culture at 28 ℃ on PDA for surface-sterilized strawberry samples revealed the fungal mycelium growth on some samples (Fig. 5c). PCR analysis against the mycelium DNA using *Colletotrichum TUB2* specific primers suggested that LAMP assay result was consistent with tissue separation and mycelium diagnosis (Fig. 5d). Furthermore, PCR analysis using three additional barcodes including ApMat, Marker1, and Marker 2 distinguished among *Colletotrichum* species. Six of seven positive samples were infected by *C. siamense*, and the rest one (No.5) was infected by *C. fructicola*. Therefore, strawberry plant direct LAMP integrated with PCR developed in this study, not only provided a sensitive and practical diagnosis of latent *Colletotrichum* infection on strawberry, but also differentiated anthracnose fungi at a species level, supporting a promising chance to monitor *Colletotrichum* spp. population in field. Finally, the schematic flow of the combined LAMP-PCR assay for latent infection of anthracnose fungi on strawberry developed in this study was summarized in Fig. 6.

Discussion

The current work found that PCR with the Virulent-strain specific primers (Suzuki et al. 2008) amplified a band unique to *C. fructicola* (Fig. 1, Supplementary Fig. S3). This pair of primers had been used in classifying *Colletotrichum* isolates as virulent or less virulent (Gan et al. 2017). Clearly, this barcode might be not relevant to pathogenicity differentiation in *Colletotrichum* genus. In our previous work, both virulent and less virulent strains were identified in *C. fructicola* and *C. siamense*, indicating that a specific species is not closely correlated with its virulence to a certain host (Zhang et al. 2020).

Marker 1 and Marker 2 were developed to distinguish *Colletotrichum* species pathogenic to strawberry in Japan (Gan et al. 2017). In that work, PCR with Marker 1 specific primers obtained one band of polymorphic sizes in different isolates of certain species, either in *C. fructicola*, *C. siamense*, or *C. aenigma*. However, in this work, a uniform band in each species was observed for Marker 1 specific PCR (Supplementary Fig. S3), at least against 40 *Colletotrichum* strains identified by a multi-locus phylogeny analysis (Zhang et al. 2020). This barcode indeed holds an ideal discernibility in identifying *Colletotrichum* at a species level, although the applicability should be tested in more fungal strains reliably identified using multi-gene phylogeny in the future. In addition, current PCR using Marker 1 produced a uniform band in all 19 strains of *C. siamense* whereas a distinct band in *C. murrayae* (Supplementary Fig. S3, Fig. 1). Much work is needed to ascertain whether *C. murrayae* and *C. siamense* were conspecific as previously suggested (Liu et al. 2016).

It was reported that Marker 2 could distinguish among *C. fructicola*, *C. siamense* and *C. aenigma*, since a common specific band was obtained in each species, and species could be differentiated based on amplicon size (Gan et al. 2017). However, the current work suggested that this barcode from PCR was of same size in *C. siamense*, *C. gloeosporioides sensu stricto*, and *C. murrayae*. Marker 2 also displayed polymorphic weak amplification in 20 *C. fructicola* strains (Supplementary Fig. S3). These inconsistences might result from the confusion in identifying causal agents for strawberry anthracnose at a species level for a long period, which is common in characterizing many plant pathogens. For example, a primer CgInt related to ITS rDNA was supposed to be specific to *C. gloeosporioides* (Mills et al. 1992), which has been used for a long time in strawberry anthracnose diagnosis even till 2015 (Rahman et al. 2015). Indeed, the Cg Int primer was found to not match many taxa within *C. gloeosporioides* complex except for *C. fructicola* and *C. siamense* (Weir et al. 2012).

LAMP assay is applicable for a fast, sensitive and on-site diagnosis of plant pathogens. In this study, a LAMP assay was successfully established beginning with a 10 min lysis of strawberry tissues in a parameter of 2-3 mm. This method allowed a direct detection of most reported *Colletotrichum* species of *C. gloeosporioides* and *C. acutatum* complexes within one hour. The current LAMP assay could be further improved for the convenience of strawberry farmers and used in practical fields, although we should be more cautious in providing an accurate diagnosis based on LAMP assay, considering the site of sampling tissues, the potential existence of dead spores of *Colletotrichum* spp., and no differentiation between fungi species. Since the fungal pathogen may localize in an unsampled part of a plant at the initial stage of infection, the possibility of incorrect judgment resulting from sampling limits could not be eliminated in a direct on-site DNA diagnosis. Without an initial culturing of fungi, all DNA diagnosis methods have a risk of confusing between dead and living pathogens. Since different *Colletotrichum* species might use different infection strategies and require distinct management, it is important to distinguish between pathogen species. For example, the aggressive *C. siamense* and *C. fructicola* caused larger lesions on wounded and non-wounded strawberry leaves, while the opportunistic *C. boninense*, *C. karstii*, and *C. miaoliense* only caused lesions on wounded leaves (Chung et al. 2020). Simply, when no aggressive *Colletotrichum* spp. has been detected, fungicide is not needed in a strawberry field, so long as avoiding wounding caused by agricultural practice. These facts re-enforced the need of establish an on-site LAMP

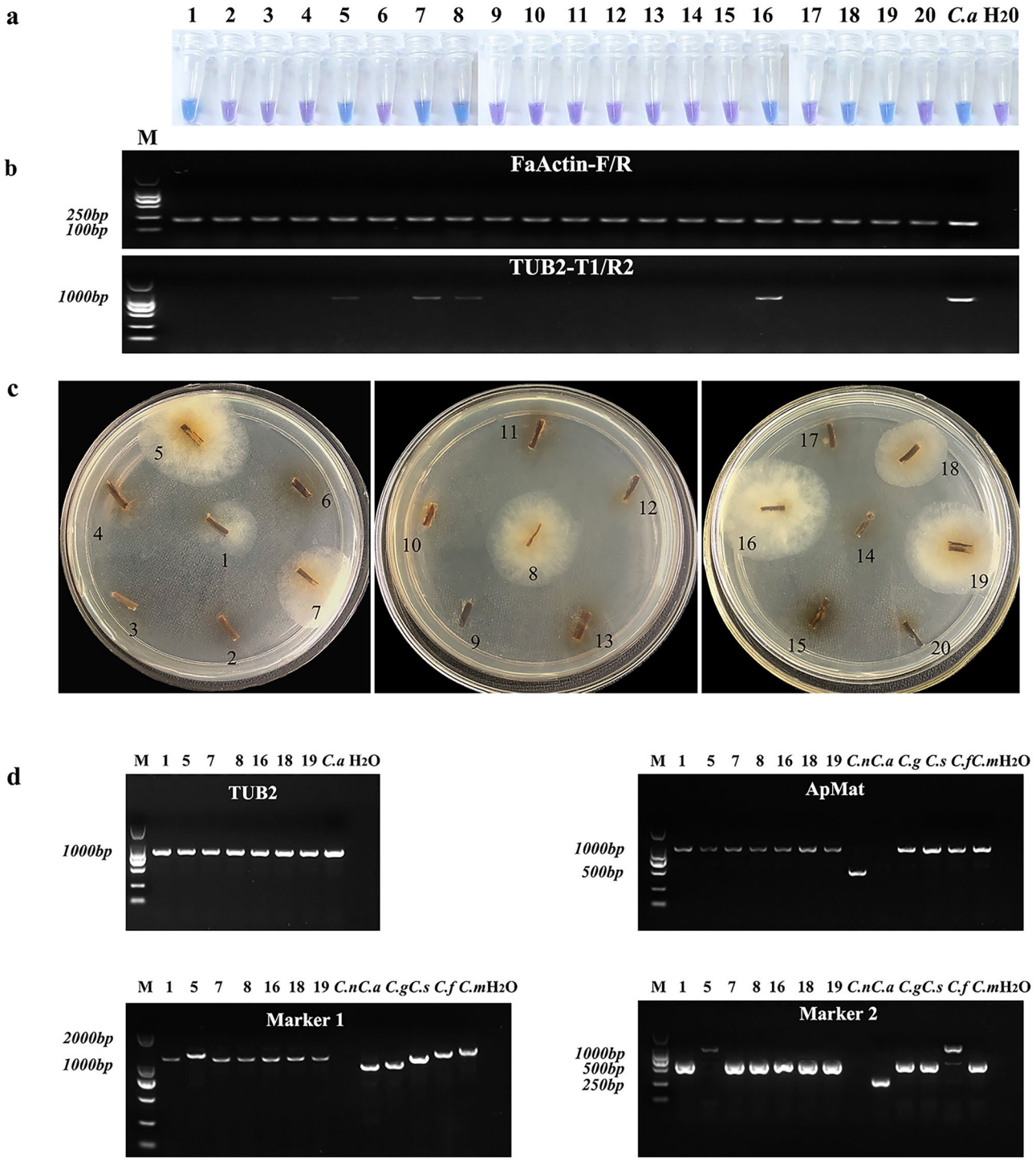

Fig. 5 LAMP and PCR combined assay resolved the detection and differentiation of latent *Colletotrichum* infection on strawberry collected from a production field. **a** Direct LAMP detection of latent *Colletotrichum* spp. infection on strawberry (an outer petiole at a lower position sampled). **b** Direct PCR (35 cycles) using primers specific to *TUB2* detecting latent infection of *Colletotrichum* spp. **c** The fungal colonies associated with the detached leaf petiole samples at 28 ℃ for 2 d. **d** PCR assay (35 cycles) against additional barcodes (Marker1, Marker 2, ApMat) distinguished latent infection on strawberry at a species level

and in lab PCR combined method to ensure a sensitive and comprehensively accurate diagnosis of *Colletotrichum* spp. on strawberry.

The LAMP-PCR combined method developed in this study combined the advantages of LAMP as a sensitive, affordable, and on-site diagnosis of latent *Colletotrichum*

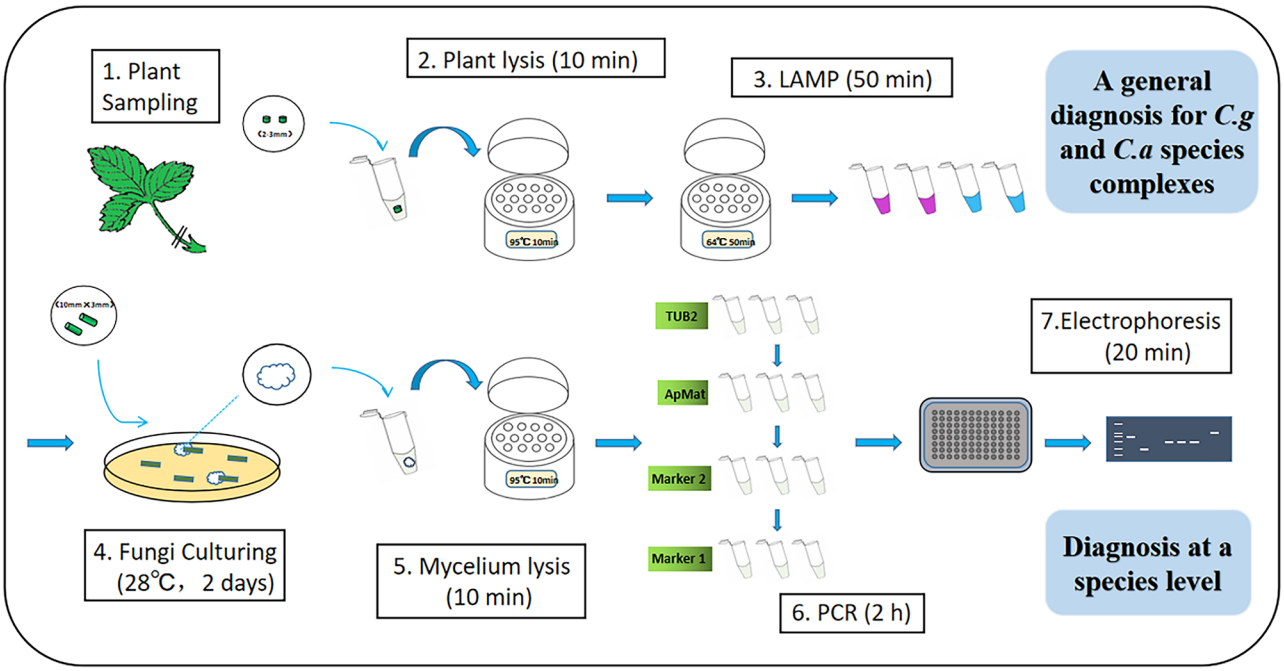

Fig. 6 Schematic flow of the combined LAMP-PCR assay for detecting latent infection of anthracnose fungi on strawberry. The DNA diagnosis consists of seven steps: sampling in the field (Step 1); lysis of fresh strawberry samples at 95 ℃ for 10 min in a digital dry bath (Step 2); a direct LAMP assay for a general detection of *Colletotrichum* at 64 ℃ for 35-50 min (Step 3); culturing and fungi separation at 28 ℃ for 2 d (Step 4); lysis of fresh fungal mycelium at 95 ℃ for 10 min (Step 5); PCR targeting four barcodes successively (*TUB2*, *ApMat*, Marker 2, Marker 1) (Step 6) followed by an electrophoresis analysis on 1-1.5% agarose (Step 7) were performed for a species level diagnosis accomplished in two hours. The experimental details are described in the text

infection on strawberry, as well as the high discernibility of PCR in lab in distinguishing *Colletotrichum* species, which might support a definitive pathogen identification for strawberry anthracnose management. This method could detect *Colletotrichum* spp. of both *C. gloeosporioides* and *C. acutatum* species complexes. Using this method we successfully differentiated two dominant species *C. fructicola* and *C. siamense* in asymptomatic strawberry. Due to the limited inclusivity of fungal isolates tested in current study, the ability of current method to differentiate other species (*C. gloeosporioides*, *C. murrayae*, *C. aenigma*, *C. nymphaeae*) seems promising but validation work is needed in the near future.

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Author contributions K. D. and Q-H. G conceived this work. Y. L. carried out most experiments and data analysis. Y. J. performed pathogen culture work. L-L. S., L-Q. Z., Y-C. H., and Z-Y. N. contributed to purification of all fungal isolates used in this work. W-Z. Y sampled diseased strawberry. Y. L. and K. D. prepared the manuscript. All authors contributed to revising and approved the manuscript.

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Declarations

Research involving human and animal participants This research did not involve any human participants or animals.

Conflict of interest The authors have no conflicts of interest to declare.

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