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Development of a Specific Nested PCR for the Detection of 16SrI and 16SrII Group Phytoplasmas Associated with Yellow Leaf Disease of Areca Palm in Hainan, China

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Abstract: Yellow leaf disease (YLD), caused by areca palm yellow leaf phytoplasma (AYLP), is a devastating disease that severely impacts the sustainable development of the areca palm industry. Efficient and accurate detection methods are considered crucial for the diagnosis and management of YLD. To address issues like false positive results using universal nested PCR primers, this study designed specific outer and internal primers based on the conserved regions of the 16S rDNA sequence of phytoplasmas, resulting in a nested PCR primer set, HNP-1F/HNP-1R and HNP-2F/HNP-2R. This set consistently amplified a single specific target band of 429 bp from 16SrI and 16SrII groups of AYLP. The sensitivity threshold for the 16SrI group of AYLP was 7.5×10^{-7} ng/μL, while that for the 16SrII group of AYLP was 4×10^{-3} ng/μL. To verify and evaluate the efficiency, the developed nested PCR system was used to detect leaf samples collected from trees showing leaf yellowing symptoms in areca palm plantations, and the results showed that the primer set could specifically detect AYLP. This primer set is characterized by rapid and accurate detection, high specificity, and high sensitivity compared to the traditional universal primer set of P1/P7 and R16mF2/R16mR1 or R16mF2/R16mR1 and R16mF2n/R2, providing a scientific basis for the specific diagnosis and early control of yellow leaf disease of areca palm.

Keywords: areca palm (*Areca catechu* L.); yellow leaf disease of areca palm; areca palm yellow leaf phytoplasma; nested PCR; molecular detection

1. Introduction

Areca palm (*Areca catechu* L.) is a perennial evergreen tree belonging to the genus *Areca* in the family Palmaceae. It is native to Malaysia and now widely distributed across South and Southeast Asia including 16 countries and regions such as India and Malaysia [1]. In China, areca palm is mainly cultivated in Hainan and Taiwan, with smaller amounts grown in Yunnan, Guangdong, and Guangxi [2]. The Areca palm holds significant dietary and medicinal value, ranking the first among the "Four Main Southern Medical Plants" (areca palm *Areca catechu* L., *Alpinia oxyphylla* Miq., *Amomum villosum* Lour., and *Morinda officinalis* How.). Its flowers and fruits are rich in various essential nutrients and its fruits, seeds, peels, and flowers can be used medicinally to treat conditions such as beriberi, swelling, and digestive issues [3]. The fruit of the areca palm, arecanut, is economically valuable and, when chewed by customers, promotes salivation, generates heat, and relieves fatigue, making it a popular chewing product. Areca palm is one of Hainan's economically important "Six Trees" crops (coconut *Cocos nucifera* L., areca palm, oil palm *Elaeis guineensis* Jacq., rubber tree *Hevea brasiliensis*

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Muell. Arg., *Dalbergia odorifera* T. Chen, and agaru *Aquilaria agallocha* (Lour.) Roxb) in agriculture, accounting for over 95% of the national planting area [4]. With rising consumer demand and prices in China, the planting area and output value of areca palm in Hainan have increased annually. Currently, areca palm has become one of the key economic sources for more than 2.3 million farmers in Hainan [5], playing a crucial role in the provincial agricultural and rural development and in increasing farmers' income and wealth.

Yellow leaf disease of areca palm (YLD) caused by areca palm yellow leaf phytoplasma (AYLP) is a lethal disease first reported in the central region of Kerala, India in 1914 [6]. This disease was also observed in China in 1981 [7] and Sri Lanka in 2015 [8]. Since its initial discovery in Tunchang County, Hainan Province, China, in 1981, YLD has spread to nearly all areca palm growing areas in Hainan, causing severe damage to the local areca palm industry [5,9]. Due to the lack of effective control measures in production, pathogen detection remains the primary method for managing this disease.

Three phytoplasmas groups or subgroups responsible for YLD have been identified in India: 16SrI-B, 16SrXI-B, and 16SrXVI [10,12]. In China, earlier researches had indicated that the phytoplasmas causing YLD belong to the 16SrI-B and 16SrI-G subgroups [13,14]. Recently, our research team discovered new phytoplasmas, specifically from the 16SrII and 16SrXXXII groups, in YLD samples collected from Hainan [15,16].

To date, various detection methods have been developed for the YLD-causing phytoplasmas, including electron microscopy [18], enzyme-linked immunosorbent assays (ELISA) [19], nested PCR [14,20], loop-mediated isothermal amplification (LAMP) [21], droplet digital PCR (ddPCR) [22], and quantitative real-time PCR (qPCR) [10,23]. However, each of these methods has its advantages and limitations. For instance, electron microscopy assays require complex sample preparation, are time-consuming, and costly. Serological assays may face challenges in preparing antisera and risk of cross-reactivity with host antigens. Those molecular methods developed recently were used to detect AYLP in 16SrI group. Meanwhile, the LAMP technique, while capable of detection at a constant temperature, has a high primer design complexity [24] and is prone to contamination, leading to false positives. Compared to conventional PCR, qPCR and ddPCR involve higher reagent costs, more stringent laboratory conditions, and expensive equipment. Additionally, products from these methods (ELISA, LAMP, and qPCR) cannot be sequenced. In contrast, amplified products of nested PCR using universal phytoplasma primers can be sequenced, making it widely used for phytoplasma detection and classification research. However, nested PCR using universal phytoplasma primers may sometimes result in false-positive outcomes in the amplification of 16S rDNA sequences, significantly affecting detection efficiency [25]. In recent research, our team developed nested PCR primer sets F4/R1 and F2/R2 [20] to address detection challenges posed by the low titer and uneven distribution of AYLP in areca palm plants [4,26]. Though this primer set significantly improved the detection efficiency than universal primer sets like P1/P7 and R16mF2/R16mR1 or P1/P7 and R16mF2n/R2; subsequent studies showed that it, just like the universal primers, sometimes amplified non-specific fragments too (our lab's unpublished data). In addition, research indicate that the 16SrI group is widely distributed in Hainan Province [17] and that there are many of cases where the 16SrII group has been identified in Wenchang City and Danzhou City (our unpublished data) , while only once case of the 16SrXXXII groups was observed [16]. Currently, there is no diagnostic technique available that can simultaneously detect both 16SrI and 16SrII groups of AYLP. To resolve this issue of non-specific amplification and to meet the needs for the detection of 16SrI group AYLP and novel identified 16SrII group AYLP, this study developed a nested PCR detection system comprising two specific primer pairs of four candidates, based on the 16S rDNA sequences of 16SrI and 16SrII group AYLPs and other areca palm-related pathogens. The goal is to establish a new specific nested PCR primers combination for accurate detection of AYLPs (16SrI and 16SrII groups) in Hainan, providing technical support for AYLP detection, identification, and early prevention of YLD in areca palm seedlings and infected plants in the field.

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2. Results

2.1. Application of Universal Nested PCR for Phytoplasma Detection

A total of 335 areca palm genomic DNA samples were collectively tested using the universal nested PCR primer set targeting phytoplasmas. Among these samples, 50 exhibited amplification bands of approximately 1400 bp (Figure 1). The resulting PCR products from these 50 positive samples were subsequently sequenced by Sangon Biotech (Shanghai, China). The sequencing data revealed the following distribution: among the 50 samples, 16 samples showed amplification specific to areca chloroplast, 20 exhibited amplification bands corresponding to bacteria, only 10 displayed specific amplification related to phytoplasmas, and the remaining four samples yielded no detectable sequencing results (Figure 2).

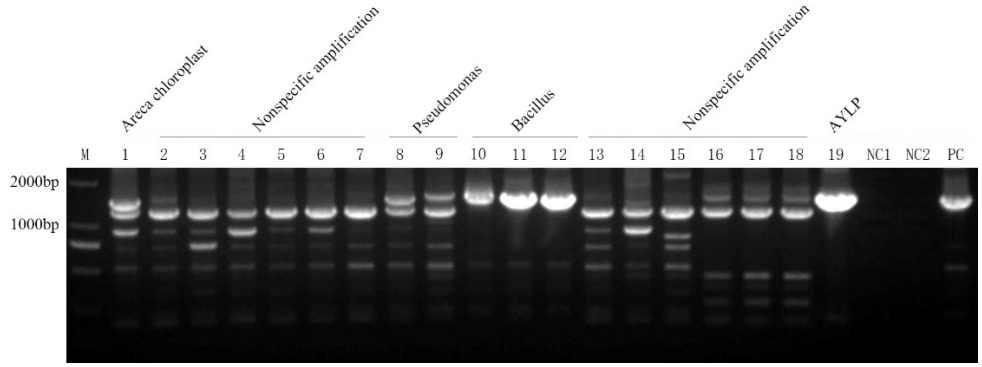

Figure 1. Profile of 16S rDNA sequences amplified by nested PCR with universal primer P1/P7 followed by R16mF2/R16mR1, from areca samples. M: DL2000 DNA Marker; 1-19: areca samples; NC1: blank control of the first round of PCR; NC2: blank control of the second round of PCR; PC: positive control, periwinkle DNA, the same for the following figures.

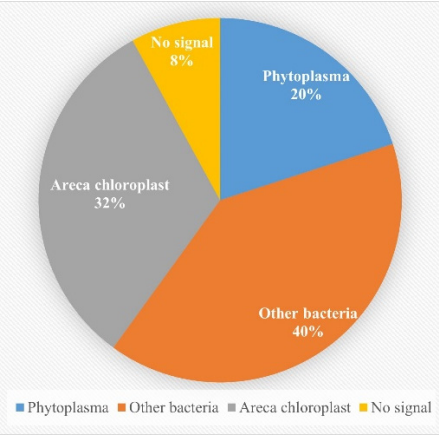

Figure 2. Ratios of different sequences in 50 samples.

2.2. Nested PCR Primer Test and Combination

By performing multiple sequence alignments of 16S rDNA from areca palm yellow leaf phytoplasma, areca chloroplasts, and endophytic bacteria, and following the principles of PCR primer design, we ultimately designed one pair of outer primer HNP-1F/R and three pairs of internal primers HNP-2F/2R, HNP-3F/3R, and HNP-4F/4R (Table 2).

These nested PCR primers were tested using genomic DNA samples from areca palm leaves infected with phytoplasma groups 16SrI and 16SrII, as well as genomic DNA samples from areca palm bacterial leaf blight pathogen and pineapple phytoplasma as references. Both HNP-2F/2R and HNP-3F/3R amplified target bands only in positive control and areca palm samples infected with

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phytoplasma groups 16SrI and 16SrII (Figure 3A and Figure 3B), indicating good performance. However, HNP-4F/4R also amplified a band of approximately 1500 bp in areca palm bacterial leaf blight pathogen DNA (Figure 3C). The combinations of HNP-1F/1R with either HNP-2F/2R or HNP-3F/3R were used to form a nested PCR in subsequent experiments.

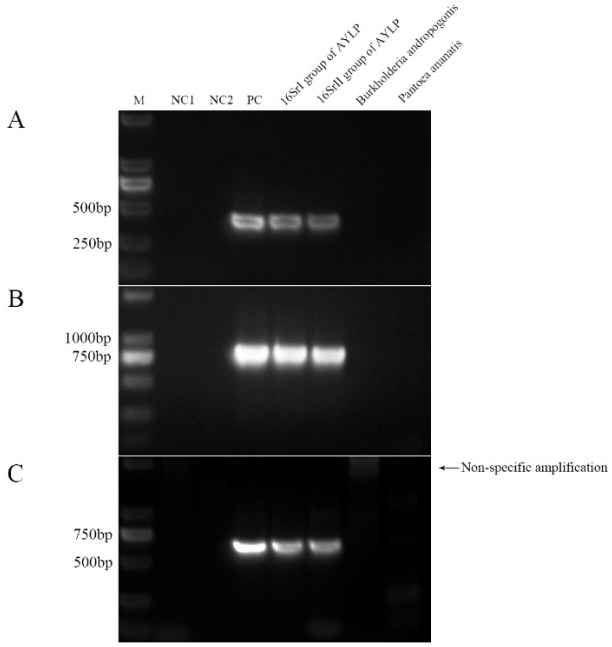

Figure 3. The preliminary screening of three pairs of internal primers designed in this study. A: Profile of 16S rDNA sequences amplified by nested PCR with primer HNP-1F/1R followed by HNP-2F/2R; B: Profile of 16S rDNA sequences amplified by nested PCR with primer HNP-1F/1R followed by HNP-3F/3R; C: Profile of 16S rDNA sequences amplified by nested PCR with primer HNP-1F/1R followed by HNP-4F/4R. M: marker DL2000; NC1: blank control for the first round of PCR; NC2: blank control for the second round of PCR; PC: positive control.

2.3. Specificity Validation of Nested PCR

Using the outer primers HNP-1F/1R and internal primers HNP-2F/2R and HNP-3F/3R, the specificity of detection of areca palm DNA infected with phytoplasmas from the 16SrI and 16SrII groups, as well as DNA from five other pathogens was conducted. The results indicated that HNP-2F/2R only amplified a target band of approximately 429 bp in positive control and in areca palm samples infected with phytoplasmas from the 16SrI and 16SrII groups (Figure 4A). However, HNP-3F/3R, in addition to the three mentioned samples, also amplified a 652bp band in healthy areca palm samples and samples infected with phytoplasmas from the 16SrXXXII group (Figure 4B). Consequently, HNP-2F/2R was selected as the internal primer set for subsequent experiments.

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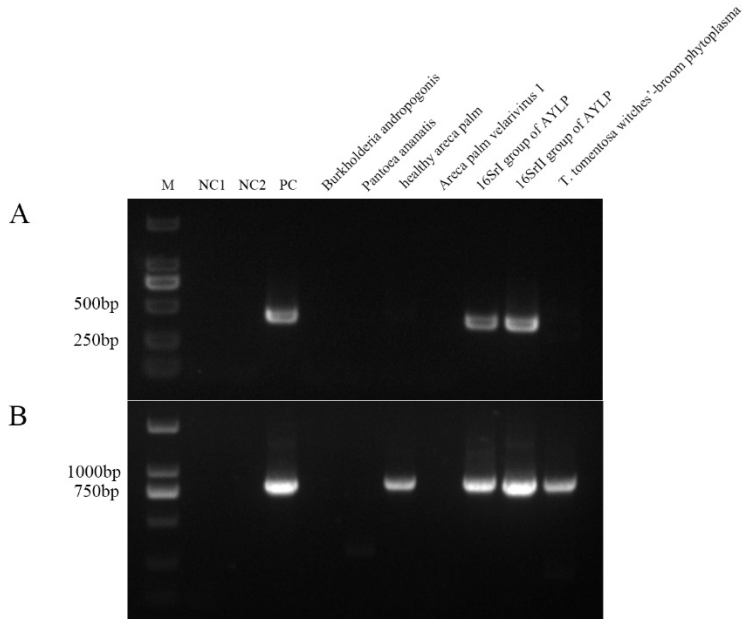

Figure 4. Profile of 16S rDNA sequences amplified by nested PCR with primer HNP-1F/1R followed by HNP-2F/2R and HNP-3F/3R respective for the specificity validation. A: HNP-2F/2R, B: HNP-3F/3R. M: marker DL2000; NC1: blank control for the first round of PCR; NC2: blank control for the second round of PCR; PC: positive control; 1: healthy areca leaf sample; 2: *Burkholderia andropogonis* (*Robbsia andropogonis*); 3: *Pantoea ananatis*; 4: Jujube witches-broom phytoplasma; 5: AYLP of 16SrI group; 6: AYLP of 16SrII group; 7: *Trema tomentosa* witches'-broom phytoplasma; 8: leaf sample infected by areca palm velarivirus 1.

2.4. Optimization of PCR Annealing Temperatures

In the nested PCR reaction, the annealing temperatures for both the outer and internal primers were systematically optimized within the range of 40°C to 60°C. For the outer primers targeting HNP-1F/1R, there was no significant difference in band intensity between annealing temperatures of 40.0°C to 53.6°C. However, as the annealing temperature increased from 56.0°C to 60.0°C, the band intensity decreased (Figure 5A). Therefore, we selected 53.6°C as the optimal annealing temperature.

Regarding the internal primers targeting HNP-2F/2R, a gradient annealing temperature approach was employed. Bands corresponding to the target were consistently visible within the temperature range of 46.4°C to 51.3°C, while no amplification occurred at 60.0°C (Figure 5B). Consequently, we determined the annealing temperature for HNP-2F/2R to be 51.3°C.

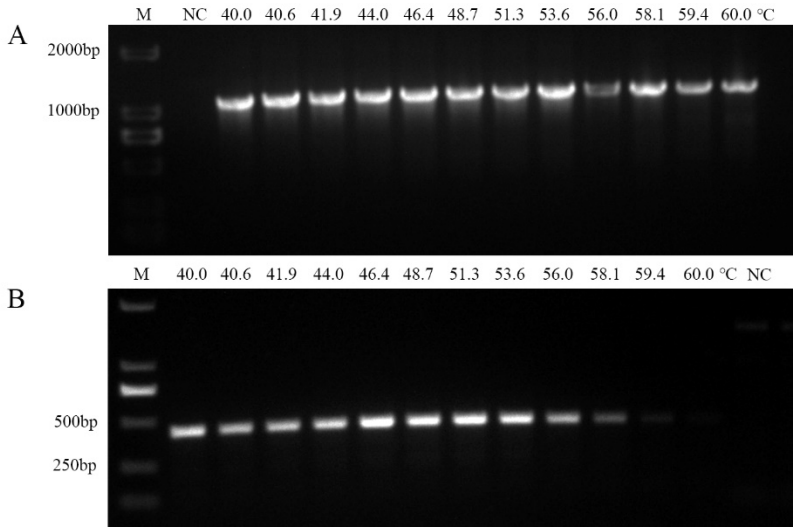

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Figure 5. A: Annealing temperature optimized for the outer primer HNP-1F/1R. M: marker DL2000; NC: blank control. M: marker DL2000; NC: blank control. B: Annealing temperature optimized for the inner primer HNP-2F/2R. M: marker DL2000; NC: blank control.

2.5. Sensitivity Determination of Nested PCR

To assess the sensitivity of this method for detecting the two phytoplasma groups, nested PCR using the outer primers HNP-1F/1R and internal primers HNP-2F/2R was conducted on recombinant plasmids containing 16S rDNA segments from the 16SrI and 16SrII phytoplasma groups.

Stable amplification of a single specific target band was achieved for the 16SrI group across plasmid concentrations ranging from 7.5 ng/μL to 7.5 × 10^{-7} ng/μL. However, as the concentration decreased, the amplification band gradually became fainter and disappeared at 7.5 × 10^{-7} ng/μL (Figure 6A). Similarly, for the 16SrII group, a single specific target band (429 bp) was consistently yielded across plasmid concentrations ranging from 4 ng/μL to 4 × 10^{-3} ng/μL. The amplification band intensity decreased with decreasing plasmid concentration and disappeared at 4 × 10^{-3} ng/μL (Figure 6B).

These results indicate that the nested PCR method has a minimum detection limit of 7.5 × 10^{-7} ng/μL for the 16SrI phytoplasma group and 4 × 10^{-3} ng/μL for the 16SrII phytoplasma group.

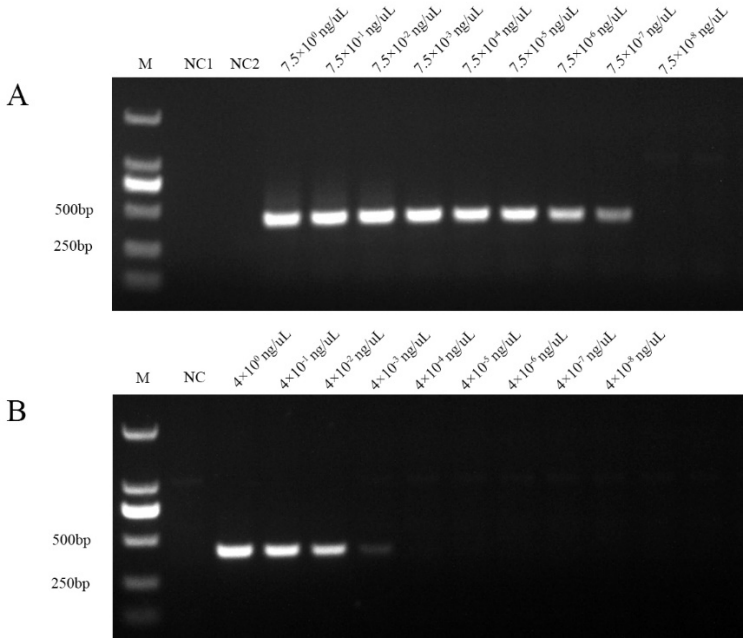

Figure 6. Sensitivity test for nested PCR primer set of HNP-1F/1R and HNP-2F/2R. A: Gel electrophoresis for sensitivity of 16SrI phytoplasma using novel nested PCR. M: marker DL2000; NC1: blank control for the first round of PCR; NC2: blank control for the second round of PCR. B: Gel electrophoresis results for the sensitivity of 16SrII phytoplasma using novel nested PCR M: marker DL2000; NC: blank control.

2.6. Comparison of The Newly Developed Nested PCR with Universal Nested PCR

To compare the detection efficiency of the nested PCR method developed in this study with that of the universal nested PCR, 30 field-collected areca samples were tested.

The universal nested PCR method failed to detect phytoplasma, except in the positive control (Figure 7A). In contrast, our newly developed nested PCR method successfully detected phytoplasma in 10 of the tested areca samples (Figure 7B). Sequencing confirmed that all 10 PCR products corresponded to the 16SrI group phytoplasma. This study highlights the effectiveness of our specific nested PCR approach for detecting AYLP and provides valuable insights for routine diagnosis of yellow leaf disease of areca palm research.

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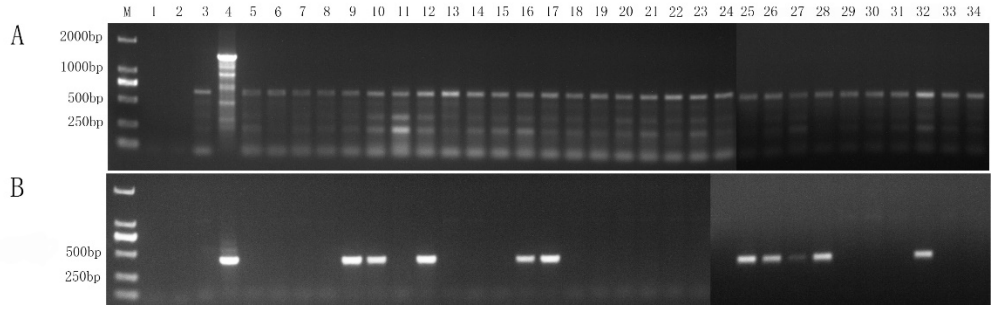

Figure 7. Comparison of Profile of 16S rDNA sequences amplified using universal primers with the novel primers developed in this study. M: marker DL2000; 1: blank control for the first round of PCR; 2: blank control for the second round of PCR; 3: healthy areca leaf sample; 4: positive control; 5-34: areca leaf samples collected in the fields.

2.7. Construction and Analysis of Phylogenetic Trees

A phylogenetic tree was constructed using sequences of representative strains from the phytoplasma groups/subgroups, based on the respective amplified fragments of R16mF2/R1 and HNP-2F/2R, using MEGA 11 software. In both phylogenetic trees, phytoplasma of the 16SrI and 16SrII groups were each clustered into a separate branch. Additionally, the phylogenetic tree constructed from the sequence fragments from HNP-2F/2R showed differences in the subdivision of 16SrI subgroups compared to the tree from the R16mF2/R1 sequence fragments. This indicates that the nested PCR method established in this study can identify and group the phytoplasmas associated with YLD in the 16SrI and 16SrII groups but cannot differentiate at the subgroup level within the 16SrI group (Figures 8A and 8B). The multiple sequence alignment also indicates that the fragments amplified by HNP-2F/2R exhibit high similarity to the 16SrI group AYLP (Figure 8C).

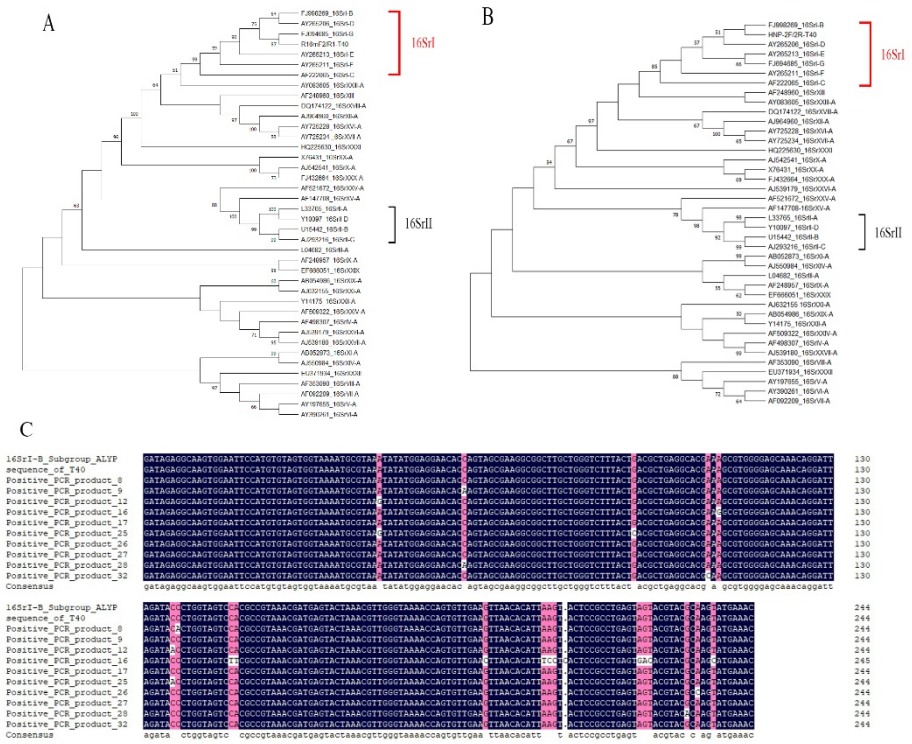

Figure 8. Phylogenetic tree constructed based on 16S rDNA gene sequences of phytoplasmas. A: Phylogenetic tree constructed from the amplified fragments of universal primers targeting the 16S rDNA gene in phytoplasmas. B: Phylogenetic tree constructed from the amplified fragments of nested primers established in this study for the 16S rDNA gene in phytoplasmas. C: Multiple sequence alignment of 10 positive PCR product sequences and the T40 sequence used for constructing the phylogenetic tree with 16SrI-B subgroup AYLP.

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3. Discussion

Phytoplasmas, originally known as mycoplasma-like organisms (MLOs) [27], belong to the prokaryote group without cell walls [28,29]. Phytoplasmas are prokaryotic microorganisms that specifically parasitize the sieve tube cells in the phloem of plants. Researches have shown that their titer within plants is uneven and varies with temperature [30,31]. Unfortunately, phytoplasmas are difficult to cultivate artificially [32], which hinders their systematic identification and classification using traditional isolation and culture methods commonly employed for other prokaryotic and eukaryotic organisms (such as plant pathogenic bacteria and fungi) [33,34]. Therefore, the establishment of specific and sensitive detection methods is crucial for the study of aetiology, epidemiology, and control of phytoplasma diseases, including areca palm yellow leaf disease. In this study, internal and outer primers based on the 16S rDNA specific regions of AYLP were designed. A primer set, HNP-1F/HNP-1R and HNP-2F/HNP-2R, was ultimately screened for specific amplification of AYLP from the novel designed primers. The annealing temperature was optimized, and then the optimal nested PCR system for detecting areca palm yellow leaf phytoplasma was established.

The universal primers P1/P7 and R16mF2/R1 are commonly used for detecting phytoplasmas from different hosts. However, their non-specific amplification is quite notable. In this study, these universal primers were used for the amplification of 16S rDNA of 335 areca palm leaf samples. The result showed that among the 50 samples that amplified with the target size bands (about 1400 bp), only 10 (20%) were ultimately confirmed as areca palm yellow leaf phytoplasma (AYLP) according to the results of sequences alignment; 16 (32%) were identified as areca palm chloroplast, and 20 (40%) were other bacteria, with the remaining samples showing no signal. This result indicated that the specificity of the universal primers P1/P7 and R16mF2/R1 for the amplification of AYLP is relatively poor, resulting in a high false-positive rate (72% to 80%). A similar result was found in the detection of the pathogenic phytoplasma in sisal purple leafroll disease [35]. In our preliminary studies during 2018 and 2019, 100 leaf samples were detected using universal primer sets P1/P7 and R16mF2/R16mR1, only one sample was successfully amplified, its sequence was verified as AYLP. To solve the problem of the low detection efficiency of AYLP, a nested PCR method had been developed with novel primer sets F4/R1 and F2/R2 [20] with a target band of 525 bp had been designed based on 16S rDNA gene sequences of AYLP. Though this primer set significantly improved detection efficiency (80 out of 482, 17%) than universal primer set P1/P7 and R16mF2/R16mR1 (1 out of 100, 1%), subsequent studies showed that this primer set also led to non-specific amplification of sequences (for example, *Bacillus* sp.) in the areca leaf and suspected vector insects (our lab unpublished data). The newly nested PCR detection method was developed by performing multiple sequence alignments of the 16S rDNA sequences of the reported areca palm yellow leaf phytoplasma and other bacteria. This approach was used to select specific regions of the phytoplasma for primer design and screening, ensuring high specificity. Specificity tests demonstrated that this method could detect the 16SrI and 16SrII groups of phytoplasma currently found in areca palms, while other groups of phytoplasma and other bacteria were not detected as positive.

In terms of detection sensitivity, the newly developed nested PCR detection method has a minimum detection limit of 16 copies/μL (7.5×10^{-7} ng/μL) for the 16SrI group of AYLP. In comparison, the existing TaqMan qPCR detection method has a limit of 1.16 copies/μL [23], and ddPCR has a limit of 0.07 copies/μL [22]. However, the nested PCR detection method developed in this study can also simultaneously detect the 16SrII group of phytoplasmas, with a minimum detection limit of 1.3×10^{3} copies/μL (4×10^{-3} ng/μL).

Regarding cost and detection efficacy, this newly established method is more cost-effective and less prone to contamination compared to qPCR and ddPCR. Additionally, all amplified fragments from the samples sequenced thus far have been identified as phytoplasmas. This method also allows for the construction of phylogenetic trees from the sequencing data to identify positive samples, a capability that current qPCR and ddPCR methods lack.

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The nested PCR detection method developed in this study can be used for the detection and identification of areca palm yellow leaf phytoplasma (16SrI and 16SrII groups). It provides technical support for subsequent AYLP detection, identification, and early prevention and control of areca palm yellow leaf disease.

4. Materials and Methods

4.1. Materials

Areca leaf samples showing yellow symptoms collected from the field and stored in our laboratory were used in this study (Table 1).

Table 1. Areca leaf samples used in this study.

Samples	Taxonomy	Host plant	Location
J069	16SrI Group	Areca palm *Areca catechu*	Wenchang, Hainan
S97	16SrI Group	Areca palm *A. catechu*	Wenchang, Hainan
T40	16SrI Group	Areca palm *A. catechu*	Wenchang, Hainan
H-9	16SrI Group	Periwinkle *Catharanthus roseus*	Wenchang, Hainan
A056	16SrII Group	Areca palm *A. catechu*	Wenchang, Hainan
W4	16SrII Group	Areca palm *A. catechu*	Wenchang, Hainan
MW1-1	*Burkholderia andropogonis*	Areca palm *A. catechu*	Wenchang, Hainan
I027	Areca palm velarivirus 1	Areca palm *A. catechu*	Wenchang, Hainan
TC-1	*Pantoea ananatis*	Areca palm *A. catechu*	Tunchang, Hainan
SHM-1	16SrXXXII Group	*Trema tomentosa*	Qionghai, Hainan
PT-1	16SrI Group	*Paulownia* sp.	Taian, Shandong
ZF-1	16SrIV Group	Jujube *Ziziphus jujuba*	Taian, Shandong

4.2. Extraction of Total DNA from Areca Plam Leaf Samples Showing Yellow

Leaf samples of areca palm weighing approxima was 0.1 g were taken and cut into 1 mm pieces. DNA was extracted following the instructions provided by TianGen Biotech Co., Ltd. (Beijing, China), using the plant genomic DNA extraction kit, quantified using Nanodrop 2000, and stored at -20 °C for subsequent use.

4.3. Construction of Recombinant Plasmids for 16S rDNA of Areca Palm Yellow Leaf Phytoplasma

Yellow leaf disease (YLD) is a major disease affecting areca palm plants. Rapid detection of the pathogen responsible for YLD is crucial for effective management. In this study, universal nested PCR primers P1/P7 and R16mF2/R16mR1 (Table 2) targeting the 16S rDNA of phytoplasmas were employed to amplify the total DNA extracted from Areca leaf showing YLD. The reaction mixture consisted of 2 μL of DNA template, 12.5 μL of 2 × Taq PCR Master Mix (Aidlab Biotechnologies Co., Ltd, Beijing, China, PC0902), and 1 μL of each primer (10 μM), with ddH_2O added to a final volume of 25 μL. The PCR procedure included an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes.

After the first round of PCR using external primers P1/P7, the product was diluted 20-fold, and a second round of amplification was performed using internal primers R16mF2/R16mR1. The procedure was like the first round, except for an annealing temperature of 50 °C. The amplified

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products were analyzed by electrophoresis on a 1% agarose gel (120 V, 35 minutes) and visualized using a gel imaging system (Synoptics Ltd, USA, GBOX-F3-LFB).

To construct recombinant plasmids, the PCR products were purified using a gel extraction kit (Tiangen Biotech, Beijing, China, DP209-02). Subsequently, a rapid ligation kit (Sangon Biotech, Shanghai, China, B522214-0020) was used to ligate the purified products into the T-vector. The resulting plasmids were transformed into DH5α competent cells (Sangon Biotech, Shanghai, China, B528413-0010). Positive clones were selected in LB sterile liquid medium supplemented with 50 ng/μL ampicillin, and plasmid DNA was extracted using a plasmid mini-prep kit (Tiangen Biotech, Beijing, China, DP103-02) and sequenced by Sangon Biotech (Shanghai, China).

Table 2. Primers for amplification of 16S rDNA gene using nested-PCR.

Primer	Primer sequence (5′- 3′)	Annealing temperature (℃)	Target fragment length (bp)	References
P1	AAGAATTTGATCCTGGCTCAGGATT	55	1800	[36]
P7	CGTCCTTCATCGGCTCTT			
R16mF2	CATGCAAGTCGAACGGA	50	1500	[37]
R16mR1	CTTAACCCCAATCATCGA			
HNP-1F	TTCTTGTTTTTAAAAGACCT	44	1072	This study
HNP-1R	AAACTTGCGCTTCAGCT			
HNP-2F	TGTGGTCTAAGTGCAAT	48	429	This study
HNP-2R	CTGATAACCTCCACTGTGTT			
HNP-3F	TTCTTGTTTTTAAAAGACCT	50	837	This study
HNP-3R	ATAACCTCCACTGTGTTTCT			
HNP-4F	AATGCTCAACATTGTGATGCT	48	652	This study
HNP-4R	AAACTTGCGCTTCAGCT			

4.4. Design and Primary Screening of Specific Primers

In this study, outer and internal primers were designed and screened for detecting the 16S rDNA of phytoplasmas associated with yellow leaf disease of areca palm (YLD). For this, previously reported 16S rDNA sequences from the 16SrI-B subgroup and the 16SrI-G subgroup of YLD phytoplasmas were utilized, along with the 16S rDNA sequence of phytoplasmas from the 16SrII subgroup, which was previously characterized in our laboratory. To enhance specificity, these 16S rDNA sequences were also aligned and compared with those from Areca chloroplasts, areca bacterial leaf spot pathogens, and other bacterial species (Table 3). Based on sequence variations, several nested PCR primers were designed and synthesized in Sangon Biotech (Shanghai, China).

In the first round of amplification, genomic DNA samples from areca palm leaves infected with phytoplasma groups 16SrI and 16SrII, as well as DNA of areca bacterial leaf blight pathogen (*Burkholderia andropogonis*) and *Pantoea ananatis*, were amplified using the primers HNP-1F/1R. Chrysanthemum genomic DNA infected with phytoplasma group 16SrI was used as a positive control, and H_2O served as the negative control. The first-round PCR products were diluted 20 times,

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and three pairs of nested primers (HNP-2F/2R, HNP-3F/3R, and HNP-4F/4R) were used to amplify the first-round PCR products, aiming to select the better nested PCR primer combination.

Table 3. Reference sequences used in this study.

NO.	Samples	GenBank accession number	Application
1	16Sr I-B SubGroup AYLP	FJ998269	Primer Design/Phylogenetic analysis
2	16Sr I-G SubGroup AYLP	FJ694685	Primer Design/Phylogenetic analysis
3	16Sr II-A SubGroup AYLP	OQ586085	Primer Design
4	Areca Catechu Chloroplast	NC_050163	Primer Design
5	*Burkholderia andropogonis*	NR_104960	Primer Design
6	*Pantoea ananatis*	MW174802	Primer Design
7	*Chrysophyllum albidum*	LC110196	Primer Design
8	*Curtobacterium citreum*	MF319766	Primer Design
9	*Curtobacterium luteum*	JX437941	Primer Design
10	*Sphingomonas yantingensis*	MF101149	Primer Design
11	*Bacillus cereus* (*Robbsia andropogonis*)	HQ833025	Primer Design
12	*Staphylococcus epidermidis*	CP040883	Primer Design
13	*Xanthomonas sacchari*	MN889285	Primer Design
14	*Xanthomonas campestris*	JX415480	Primer Design
15			
16	16SrI-C SubGroup	AF222065	Phylogenetic analysis
17	16SrI-D SubGroup	AY265206	Phylogenetic analysis
18	16SrI-E SubGroup	AY265213	Phylogenetic analysis
19	16SrI-F SubGroup	AY265211	Phylogenetic analysis
20			
21	16SrII-A SubGroup	L33765	Phylogenetic analysis
22	16SrII-B SubGroup	U15442	Phylogenetic analysis
23	16SrII-C SubGroup	AJ293216	Phylogenetic analysis
24	16SrII-D SubGroup	Y10097	Phylogenetic analysis
25	16SrIII-A SubGroup	L04682	Phylogenetic analysis
26	16SrIV-A SubGroup	AF498307	Phylogenetic analysis
27	16SrV-A SubGroup	AY197655	Phylogenetic analysis
28	16SrVI-A SubGroup	AY390261	Phylogenetic analysis
29	16SrVII-A SubGroup	AF092209	Phylogenetic analysis
30	16SrVIII-A SubGroup	AF353090	Phylogenetic analysis
21	16SrIX-A SubGroup	AF248957	Phylogenetic analysis
32	16SrX-A SubGroup	AJ542541	Phylogenetic analysis
33	16SrXI-A SubGroup	AB052873	Phylogenetic analysis
34	16SrXII-A SubGroup	AJ964960	Phylogenetic analysis
35	16SrXIII Group	AF248960	Phylogenetic analysis
36	16SrXIV-A SubGroup	AJ550984	Phylogenetic analysis
37	16SrXV-A SubGroup	AF147708	Phylogenetic analysis
38	16SrXVI-A SubGroup	AY725228	Phylogenetic analysis
39	16SrXVII-A SubGroup	AY725234	Phylogenetic analysis
40	16SrXVIII-A SubGroup	DQ174122	Phylogenetic analysis
41	16SrXIX-A SubGroup	AB054986	Phylogenetic analysis
42	16SrXX-A SubGroup	X76431	Phylogenetic analysis
43	16SrXXI-A SubGroup	AJ632155	Phylogenetic analysis

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44	16SrXXII-A SubGroup	Y14175	Phylogenetic analysis
45	16SrXXIII-A SubGroup	AY083605	Phylogenetic analysis
46	16SrXXIV-A SubGroup	AF509322	Phylogenetic analysis
47	16SrXXV-A SubGroup	AF521672	Phylogenetic analysis
48	16SrXXVI-A SubGroup	AJ539179	Phylogenetic analysis
49	16SrXXVII-A SubGroup	AJ539180	Phylogenetic analysis
50	16SrXXIX Group	EF666051	Phylogenetic analysis
51	16SrXXX-A SubGroup	FJ432664	Phylogenetic analysis
52	16SrXXXI Group	HQ225630	Phylogenetic analysis
53	16SrXXXII Group	EU371934	Phylogenetic analysis

4.5. Specificity Verification of Nested PCR

The specificity of the method developed in the current study was validated by using external primers HNP-1F/1R to amplify genomic DNA from various sources, including healthy areca palm leaves, areca palm bacterial leaf spot pathogens, pineapple pan-genus bacteria, YLD phytoplasmas (16SrI and 16SrII), and kenaf plants infected with 16SrXXXII phytoplasmas. Additionally, the complementary DNA was included from areca yellow leaf virus 1 (Table 1). After diluting the first round PCR product 20-fold and using it as a template, a second-round amplification was performed with the internal primers pairs mentioned above. The nested PCR reaction system and cycling conditions were identical to those described in section 1.3. The amplified products were analyzed by electrophoresis on a 1% agarose gel (120 V, 35 minutes) and visualized using a gel imaging system (Synoptics Ltd, USA, GBOX-F3-LFB).

4.6. Optimization of Annealing Temperatures for Nested PCR with Internal Primers

To optimize the annealing temperatures for the nested PCR using internal primers in the detection of phytoplasmas, the HNP-1F/1R + HNP-2F/2R primer set was utilized, targeting the positive Chrysanthemum phytoplasma genomic DNA samples (Table 1).

The first round of amplification using primers HNP-1F/1R, the reaction mixture was same as in section 1.3. The PCR cycling conditions included an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing with a temperature gradient ranging from 40 °C to 60 °C (in 12 steps), annealing for 30 seconds, extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes.

After the completion of the first PCR round, the product was diluted 20-fold, and the second round of amplification using primers HNP-2F/2R was performed. The reaction mixture for the second round was the same as the first, with a temperature gradient ranging from 40 °C to 60 °C (also in 12 steps). The cycling conditions remained consistent, and the amplified products were analyzed by electrophoresis on a 1% agarose gel (120 V, 35 minutes) and visualized using a gel imaging system.

4.7. Establishment of Nested PCR Reaction System

In this study, a nested PCR reaction system using external primers HNP-1F/1R was established for the first round of amplification. After completing the first PCR round, the resulting product was diluted 20-fold, and the second round of amplification was performed using internal primers HNP-2F/2R. The mixture and PCR cycling condition for the two rounds of reactions were the same as in section 1.6, but the annealing temperature was determined to be 42.5 °C (while for the second PCR round, the annealing temperature was 46 °C).

4.8. Sensitivity Testing of Newly Developed Nested PCR

Sensitivity testing was conducted for the nested PCR developed in this study, using recombinant plasmids S97-1 and W4-1, each containing 16S rDNA segments from 16SrI and 16SrII of AYLP, respectively. These plasmids were subjected to a 10-fold serial dilution. S97-1 was diluted from 7.5 ng/μL to 7.5×10^{-8} ng/μL. W4-1 was diluted from 4 ng/μL to 400 zg/μL. The HNP-1F/1R + HNP-2F/2R

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nested PCR primer set was employed to amplify the diluted S97-1 and W4-1 recombinant plasmids. The nested PCR reaction system and cycling conditions were consistent with those described in section 1.6.

4.9. Comparison of Newly Developed Nested PCR Method with Universal Nested PCR Method

In this study, 30 samples of areca palm showing yellow symptoms were simultaneously tested to evaluate the performance of two detection methods using universal primers and the newly developed nested PCR primer set.

The universal nested PCR method followed the reaction steps described in section 1.3. The newly established nested PCR method followed the reaction steps outlined in section 1.6.

4.10. Phylogenetic Analysis of the Fragment Amplified from Primers HNP1F/1R and HNP-2F/2R

The sequence fragments obtained from the aforementioned sequencing and assembly process, along with 16S rDNA sequences of different groups of plant pathogens from GenBank, were used to construct a phylogenetic tree. The Neighbor-Joining (NJ) method in MEGA 11 was employed with a bootstrap value of 1000 [35].

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Data Availability Statement: DNA sequences are available in the GenBank database, with the accession numbers listed in the Results. All other relevant data are within the paper and Supplementary Materials.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

YLD	Yellow leaf disease of areca palm
AYLP	areca palm yellow leaf phytoplasma
LAMP	loop-mediated isothermal amplification
qPCR	quantitative real-time PCR
ddPCR	droplet digital PCR

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Appendix

Table A1. Areca palm leaf samples used in the detection assays using novel nested PCR in this study.

No	Sample	Result	City/County	Time	No	Sample	Result	City/County	Time
1	J069	Positive	Wenchang	2022	169	ZB1-2	Negative	Wenchang	2022
2	A056	Positive	Wenchang	2022	170	MQR4	Negative	Ledong	2021
3	T40	Positive	Wenchang	2022	171	HL3-4	Negative	Ledong	2021
4	W4	Positive	Wenchang	2022	172	HL3-1	Negative	Ledong	2021
5	S97	Positive	Wenchang	2022	173	HL1-2	Negative	Ledong	2021
6	M25	Positive	Wenchang	2023	174	HZ3-2	Negative	Ledong	2021
7	H61	Positive	Wenchang	2023	175	HZ5-2	Negative	Ledong	2021
8	H64	Positive	Wenchang	2023	176	T52	Negative	Ledong	2021
9	R71	Positive	Waning	2022	177	NY1-2	Negative	Wenchang	2022
10	R72	Positive	Waning	2022	178	ZB2-2	Negative	Wenchang	2022
11	R61	Negative	Waning	2022	179	M22	Negative	Wenchang	2022
12	R62	Negative	Waning	2022	180	M23	Negative	Wenchang	2022
13	R63	Negative	Waning	2022	181	M25	Negative	Wenchang	2022
14	R64	Negative	Waning	2022	182	M26	Negative	Wenchang	2022
15	R65	Negative	Waning	2022	183	M27	Negative	Wenchang	2022
16	R66	Negative	Waning	2022	184	M30	Negative	Wenchang	2022
17	R67	Negative	Waning	2022	185	M31	Negative	Wenchang	2022
18	R68	Negative	Waning	2022	186	L30	Negative	Wenchang	2021
19	R69	Negative	Waning	2022	187	L32	Negative	Wenchang	2021
20	R70	Negative	Waning	2022	188	L33	Negative	Wenchang	2021
21	R73	Negative	Waning	2022	189	S40	Negative	Wenchang	2023
22	R74	Negative	Waning	2022	190	S41	Negative	Wenchang	2023
23	R75	Negative	Waning	2022	191	S42	Negative	Wenchang	2023
24	R76	Negative	Waning	2022	192	S43	Negative	Wenchang	2023
25	R77	Negative	Waning	2022	193	S44	Negative	Wenchang	2023
26	R78	Negative	Waning	2022	194	S45	Negative	Wenchang	2023
27	R79	Negative	Waning	2022	195	S46	Negative	Wenchang	2023
28	R80	Negative	Waning	2022	196	S47	Negative	Wenchang	2023
29	R81	Negative	Waning	2022	197	S50	Negative	Wenchang	2023
30	R82	Negative	Waning	2022	198	W11	Negative	Wenchang	2023
31	R83	Negative	Waning	2022	199	W12	Negative	Wenchang	2023
32	R84	Negative	Waning	2022	200	W13	Negative	Wenchang	2023
33	R85	Negative	Waning	2022	201	W14	Negative	Wenchang	2023
34	R86	Negative	Waning	2022	202	W15	Negative	Wenchang	2023
35	R87	Negative	Waning	2022	203	W16	Negative	Wenchang	2023
36	R88	Negative	Waning	2022	204	W17	Negative	Wenchang	2023
37	R89	Negative	Waning	2022	205	W18	Negative	Wenchang	2023
38	R90	Negative	Waning	2022	206	W19	Negative	Wenchang	2023
39	W1	Negative	Waning	2022	207	W20	Negative	Wenchang	2023
40	W2	Negative	Wenchang	2022	208	W21	Negative	Wenchang	2023
41	W3	Negative	Wenchang	2022	209	W22	Negative	Wenchang	2023
42	W3-4	Negative	Wenchang	2022	210	W23	Negative	Wenchang	2023
43	A09	Negative	Wenchang	2022	211	W24	Negative	Wenchang	2023
44	A12	Negative	Wenchang	2022	212	W25	Negative	Wenchang	2023
45	A13	Negative	Wenchang	2022	213	W26	Negative	Wenchang	2023
46	A14	Negative	Wenchang	2022	214	W27	Negative	Wenchang	2023
47	A16	Negative	Wenchang	2022	215	W28	Negative	Wenchang	2023
48	A19	Negative	Wenchang	2022	216	W29	Negative	Wenchang	2023
49	A22	Negative	Wenchang	2022	217	W30	Negative	Wenchang	2023
50	A24	Negative	Wenchang	2022	218	W31	Negative	Wenchang	2023
51	A27	Negative	Wenchang	2022	219	W32	Negative	Wenchang	2023
52	A30	Negative	Wenchang	2022	220	W33	Negative	Wenchang	2023
53	A31	Negative	Wenchang	2022	221	W34	Negative	Wenchang	2023
54	A32	Negative	Wenchang	2022	222	W35	Negative	Wenchang	2023

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55	A34	Negative	Wenchang	2022	223	W36	Negative	Wenchang	2023
56	A36	Negative	Wenchang	2022	224	W37	Negative	Wenchang	2023
57	A37	Negative	Wenchang	2022	225	W38	Negative	Wenchang	2023
58	A39	Negative	Wenchang	2022	226	W39	Negative	Wenchang	2023
59	A49	Negative	Wenchang	2022	227	B2-1	Negative	Baoting	2021
60	A50	Negative	Wenchang	2022	228	B2-2	Negative	Baoting	2021
61	A51	Negative	Wenchang	2022	229	B2-4	Negative	Baoting	2021
62	A55	Negative	Wenchang	2022	230	B2-5	Negative	Baoting	2021
63	A57	Negative	Wenchang	2022	231	B2-6	Negative	Baoting	2021
64	A59	Negative	Wenchang	2022	232	B2-7	Negative	Baoting	2021
65	A60	Negative	Wenchang	2022	233	B2-9	Negative	Baoting	2021
66	B13	Negative	Baoting	2020	234	B4-1	Negative	Baoting	2021
67	B4	Negative	Baoting	2020	235	B4-2	Negative	Baoting	2021
68	B17	Negative	Baoting	2020	236	B4-3	Negative	Baoting	2021
69	B20	Negative	Baoting	2020	237	B4-4	Negative	Baoting	2021
70	B21	Negative	Baoting	2020	238	B4-5	Negative	Baoting	2021
71	B23	Negative	Baoting	2020	239	B4-6	Negative	Baoting	2021
72	B24	Negative	Baoting	2020	240	B4-7	Negative	Baoting	2021
73	B25	Negative	Baoting	2020	241	B4-8	Negative	Baoting	2021
74	B27	Negative	Baoting	2020	242	B4-9	Negative	Baoting	2021
75	B30	Negative	Baoting	2020	243	B4-10	Negative	Baoting	2021
76	B31	Negative	Baoting	2020	244	B4-11	Negative	Baoting	2021
77	B32	Negative	Baoting	2020	245	B4-12	Negative	Baoting	2021
78	B33	Negative	Baoting	2020	246	B4-13	Negative	Baoting	2021
79	B37	Negative	Baoting	2020	247	B4-14	Negative	Baoting	2021
80	B41	Negative	Baoting	2020	248	B4-15	Negative	Baoting	2021
81	B44	Negative	Baoting	2020	249	B4-16	Negative	Baoting	2021
82	B46	Negative	Baoting	2020	250	B4-17	Negative	Baoting	2021
83	B47	Negative	Baoting	2020	251	B4-18	Negative	Baoting	2021
84	B49	Negative	Baoting	2020	252	B4-19	Negative	Baoting	2021
85	B53	Negative	Baoting	2020	253	B5-4	Negative	Baoting	2021
86	B54	Negative	Baoting	2020	254	B5-5	Negative	Baoting	2021
87	B56	Negative	Baoting	2020	255	B5-6	Negative	Baoting	2021
88	B57	Negative	Baoting	2020	256	B5-10	Negative	Baoting	2021
89	B61	Negative	Baoting	2020	257	B6-1	Negative	Baoting	2021
90	B63	Negative	Baoting	2020	258	B6-2	Negative	Baoting	2021
91	B76	Negative	Baoting	2020	259	B6-4	Negative	Baoting	2021
92	C21	Negative	Qionghai	2020	260	B6-5	Negative	Baoting	2021
93	C26	Negative	Qionghai	2020	261	B6-6	Negative	Baoting	2021
94	C27	Negative	Qionghai	2020	262	B6-8	Negative	Baoting	2021
95	C41	Negative	Qionghai	2020	263	B6-10	Negative	Baoting	2021
96	C47	Negative	Qionghai	2020	264	B6-11	Negative	Baoting	2021
97	C61	Negative	Qionghai	2020	265	F04-2	Negative	Tunchang	2020
98	C68	Negative	Qionghai	2020	266	F05-2	Negative	Tunchang	2020
99	C69	Negative	Qionghai	2020	267	F05-1	Negative	Tunchang	2020
100	C70	Negative	Qionghai	2020	268	F06-1	Negative	Tunchang	2020
101	C73	Negative	Qionghai	2020	269	F07-1	Negative	Tunchang	2020
102	C85	Negative	Qionghai	2020	270	F022-1	Negative	Tunchang	2020
103	C87	Negative	Qionghai	2020	271	F028-2	Negative	Tunchang	2020
104	C89	Negative	Qionghai	2020	272	F023-2	Negative	Tunchang	2020
105	C90	Negative	Qionghai	2020	273	F035-1	Negative	Tunchang	2020
106	C91	Negative	Qionghai	2020	274	F036-1	Negative	Tunchang	2020
107	C95	Negative	Qionghai	2020	275	F036-2	Negative	Tunchang	2020
108	C99	Negative	Qionghai	2020	276	F037-1	Negative	Tunchang	2020
109	C100	Negative	Qionghai	2020	277	F037-2	Negative	Tunchang	2020
110	BSL-1	Negative	Ding'an	2023	278	F038-1	Negative	Tunchang	2020
111	BSL-2	Negative	Ding'an	2023	279	F038-2	Negative	Tunchang	2020
112	BSL-3	Negative	Ding'an	2023	280	F039-1	Negative	Tunchang	2020
113	BSL-4	Negative	Ding'an	2023	281	F040-1	Negative	Tunchang	2020

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114	BSL-5	Negative	Ding'an	2023	282	F040-2	Negative	Tunchang	2020
115	BSL-6	Negative	Ding'an	2023	283	F041-2	Negative	Tunchang	2020
116	BSL-7	Negative	Ding'an	2023	284	F044-1	Negative	Tunchang	2020
117	BSL-8	Negative	Ding'an	2023	285	F044-2	Negative	Tunchang	2020
118	BSL-9	Negative	Ding'an	2023	286	F045-1	Negative	Tunchang	2020
119	BSL-10	Negative	Ding'an	2023	287	F045-2	Negative	Tunchang	2020
120	BSL-11	Negative	Ding'an	2023	288	F049-2	Negative	Tunchang	2020
121	BSL-12	Negative	Ding'an	2023	289	F055-2	Negative	Tunchang	2020
122	BSL-13	Negative	Ding'an	2023	290	F099-1	Negative	Tunchang	2020
123	BSL-14	Negative	Ding'an	2023	291	C002-1	Negative	Qionghai	2020
124	BSL-15	Negative	Ding'an	2023	292	C007-2	Negative	Qionghai	2020
125	BSL-16	Negative	Ding'an	2023	293	C013-2	Negative	Qionghai	2020
126	BSL-17	Negative	Ding'an	2023	294	C018Y-1	Negative	Qionghai	2020
127	BSL-18	Negative	Ding'an	2023	295	C018Y-2	Negative	Qionghai	2020
128	BSL-19	Negative	Ding'an	2023	296	C021-1	Negative	Qionghai	2020
129	BSL-20	Negative	Ding'an	2023	297	C023-1	Negative	Qionghai	2020
130	XS-1	Negative	Ding'an	2023	298	C026-1	Negative	Qionghai	2020
131	XS-2	Negative	Ding'an	2023	299	C026-2	Negative	Qionghai	2020
132	XS-3	Negative	Ding'an	2023	300	C031-2	Negative	Qionghai	2020
133	XS-4	Negative	Ding'an	2023	301	C048-1	Negative	Qionghai	2020
134	XS-5	Negative	Ding'an	2023	302	C064-1	Negative	Qionghai	2020
135	XS-6	Negative	Ding'an	2023	303	C065-2	Negative	Qionghai	2020
136	XS-7	Negative	Ding'an	2023	304	C071-2	Negative	Qionghai	2020
137	XS-8	Negative	Ding'an	2023	305	C072-2	Negative	Qionghai	2020
138	XS-9	Negative	Ding'an	2023	306	C076-2	Negative	Qionghai	2020
139	XS-10	Negative	Ding'an	2023	307	C077-2	Negative	Qionghai	2020
140	XS-11	Negative	Ding'an	2023	308	C078-1	Negative	Qionghai	2020
141	LK-1	Negative	Ding'an	2023	309	C080-2	Negative	Qionghai	2020
142	LK-2	Negative	Ding'an	2023	310	C056-2	Negative	Qionghai	2020
143	LK-3	Negative	Ding'an	2023	311	C065-2	Negative	Qionghai	2020
144	LK-4	Negative	Ding'an	2023	312	D008-2	Negative	Ding’an	2020
145	LK-5	Negative	Ding'an	2023	313	D039-1	Negative	Ding’an	2020
146	LK-6	Negative	Ding'an	2023	314	D062-2	Negative	Ding’an	2020
147	LK-7	Negative	Ding'an	2023	315	D093-2	Negative	Ding’an	2020
148	LK-8	Negative	Ding'an	2023	316	ZY2-1	Negative	Wenchang	2020
149	LK-9	Negative	Ding'an	2023	317	7-4-2	Negative	Wenchang	2020
150	LK-10	Negative	Ding'an	2023	318	003-2	Negative	Wenchang	2020
151	LK-11	Negative	Ding'an	2023	319	B021-2	Negative	Baoting	2020
152	LK-12	Negative	Ding'an	2023	320	B027-2	Negative	Baoting	2020
153	LK-13	Negative	Ding'an	2023	321	B029-2	Negative	Baoting	2020
154	LK-14	Negative	Ding'an	2023	322	B030-1	Negative	Baoting	2020
155	LK-15	Negative	Ding'an	2023	323	B032-1	Negative	Baoting	2020
156	LK-16	Negative	Ding'an	2023	324	B037-2	Negative	Baoting	2020
157	LK-17	Negative	Ding'an	2023	325	B038-2	Negative	Baoting	2020
158	LK-18	Negative	Ding'an	2023	326	B042-1	Negative	Baoting	2020
159	LK-19	Negative	Ding'an	2023	327	B042-2	Negative	Baoting	2020
160	LK-20	Negative	Ding'an	2023	328	B055-2	Negative	Baoting	2020
161	I027	Negative	Qionghai	2022	329	B059-1	Negative	Baoting	2020
162	N01	Negative	Lingshui	2022	330	B059-2	Negative	Baoting	2020
163	N06	Negative	Lingshui	2022	331	B056-1	Negative	Baoting	2020
164	N07	Negative	Lingshui	2022	332	B056-2	Negative	Baoting	2020
165	N012	Negative	Lingshui	2022	333	B061-1	Negative	Baoting	2020
166	N013	Negative	Lingshui	2022	334	B069-1	Negative	Baoting	2020
167	ZH-40	Negative	Lingshui	2022	335	B069-2	Negative	Baoting	2020
168	NLT	Negative	Lingshui	2022					

Note: a total of 10 leaf samples were positive in the detection using our novel nest PCR.

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