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Rapid Identification of Tropical Important Mealybugs Based on a Multiplex PCR Assay

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Abstract: The mealybug can severely threaten agricultural and horticultural crops and has a widespread distribution in tropical regions, particularly in high-risk invasion areas such as Hainan, which is an important trade port with superior geographical conditions. Traditional morphological methods can no longer meet the requirements for the rapid and precise identification of different insect stages or debris. DNA barcoding has been used to establish efficient molecular identification tools. In this study, a multiplex polymerase chain reaction (mPCR) assay based on the cytochrome c oxidase subunit I (COI) gene was successfully constructed for the rapid identification of mealybugs. The 5′ end COI gene fragments of 12 mealybug species were amplified and sequenced. Furthermore, an mPCR assay was established to identify three common mealybug species in Hainan, namely *Dysmicoccus neobrevipes*, *Maconellicoccus hirsutus*, and *Paracoccus marginatus*. Condition optimization, sensitivity detection, and field sample testing results prove that the assay can identify the three target species through a single PCR amplification. A sample DNA concentration of as low as 0.1–1 ng/μL can be detected. Additionally, the assay in conjunction with barcode sequencing can identify mealybugs collected in the field, clarifying the distribution and host plants of 12 mealybug species commonly found in Hainan. Thus, the rapid identification of important mealybug species is realized. The establishment of this technology provides an economical and efficient molecular tool for the quarantine and monitoring of mealybugs in Hainan and other regions, which are essential for the detection, monitoring, and early warning of invasive organisms.

Keywords: DNA barcoding; rapid identification; plant quarantine; invasive alien species

Citation: Xi, Y.; Yan, W.; Liu, K.; Cai, B.; Wu, S. Rapid Identification of Tropical Important Mealybugs Based on a Multiplex PCR Assay. *Agronomy* **2024**, *14*, 2786. https://doi.org/10.3390/agronomy14122786

Academic Editor: Christos G. Athanassiou

Received: 13 October 2024
Revised: 18 November 2024
Accepted: 20 November 2024
Published: 23 November 2024

1. Introduction

Mealybugs (Hemiptera: Coccoidea) are a class of small insects that pose a potential economic threat to agricultural, forestry, and horticultural crops [1]. They are highly specialized and widely distributed, mainly in tropical and subtropical regions. They inflict damage on various crops, fruits, trees, and ornamental plants by sucking the sap from tender plant parts, leading to stunted plant growth, yellowing of leaves, deformities, and even death. In addition, mealybugs can transmit plant viruses, such as areca palm velarivirus 1 (APV1) [2] and pineapple mealybug wilt-associated viruses (PMWaVs) [3]. They also secrete honeydew, which induces sooty mold, further impacting plant health [4]. Mealybugs pose a serious threat to agricultural and forestry production because of their diverse populations, minute size, high concealment, and ease of spread through international trade.

As the southernmost province of China, Hainan is renowned not only for its unique geographical location and rich biodiversity but also for being an important international trade port. The unique geographic location and the frequent trade exchanges have made

Hainan a high-risk area for invasive pests, such as mealybugs. *Dysmicoccus neobrevipes* [5], *Phenacoccus solenopsis* [6], and *Planococcus minor* [7], which are on the list of imported quarantine pests in China, have been recorded in Hainan, thereby causing considerable negative impacts on the domestic economy and ecosystem. Additionally, the important invasive mealybug *Paracoccus marginatus* [8] and native species, such as *Maconellicoccus hirsutus*, are widely distributed in Hainan and have a considerable impact on the production of cassava crops and tropical fruits, such as papaya and guava [9]. Therefore, *D. neobrevipes*, *P. marginatus*, and *M. hirsutus* are the three most concerned species in this study due to their agricultural importance, prevalence in the region, and the absence of existing identification methods. However, traditional identification methods that rely on morphological characteristics or host plants face challenges in quarantine inspection and community ecology studies. Sexual dimorphism is general for mealybug species, and their morphological identification is usually based on female adult individuals. Although there are taxonomic keys mainly based on the morphological characteristics of female adults, morphological identification remains problematic for non-specialists. Additionally, the identification of immature mealybugs, such as nymphs and eggs, is even more difficult and often impossible. Especially during customs quarantine interceptions or field collections, samples may be in a damaged state or an unsuitable life stage for identification [10]. Furthermore, most mealybugs exhibit a broad host plant spectrum. With considerable overlap in the host plants of different mealybug species, the information on host plants for their precise identification can be also relatively limited. The difficulties of identification will lead to considerable problems in an underestimation of the species richness and the risk of invasion. Therefore, accurate identification and rapid detection of mealybugs are particularly important for quarantine inspection and field monitoring in Hainan.

The advancement of molecular biology techniques has facilitated DNA barcoding as a rapid and precise approach for species identification [11]. It has the advantages of simple operation, high accuracy, and no restrictions on the morphology and developmental stages of species [12]. This technology can analyze interspecies evolutionary relationships and accurately position individuals into known taxonomic units through multiple alignments and phylogenetic analysis. The maternally inherited mitochondrial cytochrome c oxidase subunit I (COI) gene is widely used as a molecular marker for species identification [13]. To date, there have been two kinds of approaches for molecular identification for field-collected samples, namely PCR-based and DNA sequence-based methods [14]. DNA sequence-based methods are relatively costly and time-consuming when a large number of specimens need to be sequenced, although they do not need prior knowledge of all potential species in the study area [15]. On the other hand, simplicity and speed can be achieved using diagnostic PCR approaches. The multiplex polymerase chain reaction (mPCR) system can overcome these limitations for a defined set of taxa, as it allows for the amplification of the targets in a single reaction without the following sequencing. It uses multiple pairs of specific primers to amplify multiple target sequences in parallel, with each primer pair producing different sizes of PCR products. As a result, it becomes more economical and more efficient than several single PCR reactions. This technology is particularly suitable for identifying the species that are likely to coexist in specific environments yet are challenging to differentiate. Therefore, the mPCR has been widely used in fields such as the rapid identification of insects and algae [16,17], the analysis of host–parasitoid food webs [18], the detection of insect contaminants in food [19], and the diagnosis of pathogenic bacteria [20]. With carefully designed reaction systems, the mPCR technology can provide an efficient and reliable molecular tool for the accurate identification of invasive mealybugs. Thus, it can effectively improve the inspection and monitoring of important invasive mealybugs at ports, which are essential for effectively blocking their spread, ensuring biological safety, and maintaining ecological balance.

2. Materials and Methods

2.1. Source of Mealybugs

The specimens of 12 mealybug species were provided by the Animal and Plant Quarantine Center, Haikou Customs District (Haikou, China). For each species, three to five female adult specimens were obtained and stored in absolute ethyl alcohol. Additionally, to test the newly developed molecular approaches (see below), living mealybug samples were collected from 11 cities and counties in Hainan Province. A detailed field survey was conducted on tropical fruits, economic crops, greening plants, and flowers in various cities and counties of Hainan through ground transect surveys. These surveys provide detailed information, such as the host plants of mealybugs and the collection sites. After collection, the mealybug samples were preserved in absolute ethyl alcohol and stored at −20 °C for further use.

2.2. Non-Destructive DNA Extraction

Before slide specimen preparation, a small hole was punched between the middle and hind legs of the mealybug using a $00^{\#}$ insect pin. The individual was transferred to a 1.5 mL Eppendorf tube, and 20 µL of proteinase K, 20 µL of RNase A, and 100 µL of Buffer A (Solarbio Universal Genomic DNA Extraction Kit, Solarbio Technology Co., Ltd., Beijing, China) were added. The sample was incubated at 56 °C for 8–10 h to undergo lysis until it became transparent. After the sample for slide preparation was removed, the remaining solution was used for DNA extraction, following the instructions of the Solarbio Universal Genomic DNA Extraction Kit (Solarbio Technology Co., Ltd., Beijing, China). The quality and concentration of the extracted DNA samples were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA extractions were stored at −20 °C until use.

2.3. PCR Amplification and Sequencing

The universal primers PcoF1 (5′-CCTTCAACTAATCATAAAAATATYAG-3′) and LepR1 (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) [21] for mealybugs were used to amplify the DNA barcode COI gene fragments of the 12 mealybug species. The PCR amplification was performed in a total volume of 50 µL containing 25 µL of 2× Phanta Max Buffer, 1 µL of dNTP Mix, 1 µL of each 10 µM upstream and downstream primers, 1 µL of Phanta Max Super-Fidelity DNA polymerase (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China), 1 µL of DNA template, and ddH_2O to make up to 50 µL. The PCR was performed at 94 °C for 5 min at five cycles of 94 °C for 20 s, 45 °C for 30 s, and 72 °C for 20 s. Then, there were 35 cycles of 94 °C for 20 s, 52 °C for 30 s, and 72 °C for 20 s, followed by a final extension at 72 °C for 8 min. The PCR products were visualized using 1.5% agarose gel electrophoresis. Within each batch of samples, molecular-grade water was included as negative PCR controls. The PCR products that match the target fragment length were purified and sent to BoShang (Nanshan) Biotech Co., Ltd., (Shanghai, China) for Sanger sequencing. The obtained raw sequences were trimmed, assembled, and manually proofread. Then, they were blasted on the NCBI website (https://www.ncbi.nlm.nih.gov/, accessed on 6 November 2024) to ensure that the target fragments were correctly amplified and to confirm the species information of the samples. The genetic distances of the COI sequences obtained from the specimens of 12 mealybug species were calculated with a K2P (Kimura-2-parameter) model by MEGA 11.0 software [22]. The maximum within-species distance and the minimum between-species distance for each species pair were assessed. Afterward, a neighbor-joining (NJ) phylogenetic tree based on COI sequences of 12 mealybug species and an outgroup (*Icerya seychellarum*) was conducted with 1000 bootstrap replicates [22].

2.4. Construction and Optimization of the mPCR Assay

The COI gene sequence fragments of the 12 mealybug species obtained from sequencing were organized and aligned in the Geneious Prime v.2022.0.1 software (Biomatters, Auckland, New Zealand). At least three sequences of each species were used for

primer design. The mPCR-specific primers were designed for three target mealybug species (*D. neobrevipes*, *P. marginatus*, and *M. hirsutus*). The specific primer pairs were designed by software such as NetPremier (PMCID: 1392910) from PREMIER Biosoft (https://www.premierbiosoft.com/NetPrimer/, accessed on 19 November 2024) and Primer-BLAST from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast, accessed on 9 April 2023) to predict the melting temperature (Tm), free energy (ΔG), and dimers and to ensure the specificity of the primers. The primers were designed to meet the following rules. (1) The lengths of the PCR amplification products between different species differ by at least approximately 100 bp. (2) The Tm values of all primers are as close as possible and greater than 58 °C. (3) The first base at the 3′ end of the specific primers for the target species must be specific, and at least two of the last five bases must be specific bases.

To test the specificity of the developed mPCR assay, it was performed for three target mealybug species and nine other non-target species. For each species, at least three samples were used. Moreover, the DNA extracts of *D. neobrevipes*, *M. hirsutus*, and *P. marginatus* were initially adjusted to a concentration of 100 ng/μL. Then, the template DNA was diluted in a gradient of 10-fold dilutions using Tris-EDTA buffer (TE) to test the sensitivity of the assay for the three target mealybugs.

DNA extracts of field-collected mealybugs from a field survey (see above) were used for testing the newly developed mPCR assay to evaluate the utility for the identification of mealybug communities in nature. The COI barcode region of the field-collected mealybugs, which did not obtain target bands with the mPCR assay, were amplified using universal primers (PcoF1 and LepR1) and subjected to sequencing to identify their species. Within each batch of samples, molecular-grade water was included as negative PCR controls. All PCR products were visualized using 1.5% agarose gel electrophoresis. Additionally, three mPCR products of each target species that were amplified in the specificity test and the field sample test were purified and sent for sequencing to confirm the correct amplification.

3. Results

3.1. Amplification and Analysis of the COI Gene

The universal primers for the mealybug (PcoF1 and LepR1) were used to amplify the DNA barcode COI gene fragment (5′ end). The electrophoresis results showed a single band of approximately 700 bp (Figure S1). After the raw sequences were trimmed, COI gene fragments (approx. 600–650 bp) were obtained for each mealybug sample. Finally, a set of high-quality DNA sequence data for 12 species of mealybugs was successfully obtained (Table 1). Three sequences of each species were acquired, except for *P. marginatus* and *P. minor*, of which five and four sequences were successfully obtained. The difference between the COI sequences of all samples within the 12 studied mealybug species has been assessed. The maximum genetic distances within species were 0–0.01 ($0.0009_{mean} \pm 0.0004_{se}$), whereas the minimum distances between species were 0.02–0.21 ($0.12_{mean} \pm 0.001_{se}$) (Table S1). An NJ tree based on the COI sequences of all species revealed that the sequences of all morphologically assigned species were clustered on their respective branches (Figure S2).

Table 1. COI gene sequences from 12 mealybug species considered in this study.

Genus	Species	Number of COI Sequences	GenBank Accession No.
Dysmicoccus	*Dysmicoccus neobrevipes*	3	PQ558664, PQ568066, PQ568067
Maconellicoccus	*Maconellicoccus hirsutus*	3	PQ567098–PQ567100
Paracoccus	*Paracoccus marginatus*	5	PQ567076–PQ567080
Phenacoccus	*Phenacoccus madeirensis*	3	PQ567106–PQ567108
	Phenacoccus solenopsis	3	PQ568059–PQ568061
	Phenacoccus solani	3	PQ568071–PQ568073

Table 1. *Cont.*

Genus	Species	Number of COI Sequences	GenBank Accession No.
Planococcus	*Planococcus minor*	4	PQ568023–PQ568026
	Planococcus citri	3	PQ569629–PQ569631
Ferrisia	*Ferrisia virgata*	3	PQ568063–PQ568065
Pseudococcus	*Pseudococcus jackbeardsleyi*	3	PQ568007–PQ568009
	Pseudococcus cryptus	3	PQ569954–PQ569956
Nipaecoccus	*Nipaecoccus viridis*	3	PQ567102–PQ567104

3.2. Assay for Identifying Three Mealybug Species

In total, 39 COI sequences of 12 mealybug species were used for primer design (Table 1). For each species, at least three sequences were included. Specific primers for multiplex PCR were carefully designed by targeting the *D. neobrevipes*, *M. hirsutus*, and *P. marginatus* (Table 2). The locations of species-specific primer pairs have been indicated in the alignment for the COI sequences of 12 mealybug species (Figure S3).

Table 2. Specific primer pairs of mPCR for three mealybug species.

Species	Primer Name and Sequence (5′–3′)	Length (bp)	Amplicon (bp)
Dysmicoccus neobrevipes	Dn-F: TTTTTTATAACAATACCTATTATTATTGGTAGT	33	397
	Dn-R: AATAGGAATTGAAATAATTAATAGAAATGTT	31	
Maconellicoccus hirsutus	Mh-F: TATTATATAATAATTACTTTACATGCCTTTTTA	33	260
	Mh-R: GTGTAATATAATTTTGATTAATTAGGGGT	29	
Paracoccus marginatus	Pm-F: GATTTTGATTATTAATTCCATCTTTAATT	29	166
	Pm-R: AAATTGAAGATAAACCATTTAAATGTAAT	29	

Applying the newly designed primers, the final optimized mPCR was performed in a total volume of 50 µL containing 25 µL of 2× Gold Multiplex PCR Mix (Jiangsu Cowin Biotech Co., Ltd., Taizhou, China), 1 µL of each 10 µM upstream and downstream primer (with a final concentration of 0.2 µM for each primer), 2.5 µL of DNA template, and ddH_2O to make up to 50 µL. The PCR was performed at 95 °C for 10 min, with 35 cycles of 95 °C for 30 s, 58 °C for 90 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min.

3.3. Assay Specificity and Sensitivity

The DNA extracts of 12 mealybug species were used as templates for mPCR amplification to verify the effectiveness and specificity of the primers (Figure 1). The mPCR assay could amplify a bright single band for the three target mealybugs, as well as the positive mixed samples, with band sizes matching the primer design expectations (397 bp for *D. neobrevipes*, 260 bp for *M. hirsutus*, and 166 bp for *P. marginatus*; Table 2). The three sequences obtained from the mPCR products of each target species were consistent with the species sequences. Moreover, the amplification of the remaining nine nontarget mealybug species with this assay resulted in no bands, indicating that the three pairs of specific primers have very strong specificity and can be used for the rapid identification of three target mealybugs in the Hainan region.

For the sensitivity test of the mPCR assay, the maximum sensitivity of the DNA samples that can be detected by the mPCR and electrophoresis process is 0.1 ng/µL for *D. neobrevipes* and *M. hirsutus*, respectively, as well as 1 ng/µL for *P. marginatus* (Figure 2).

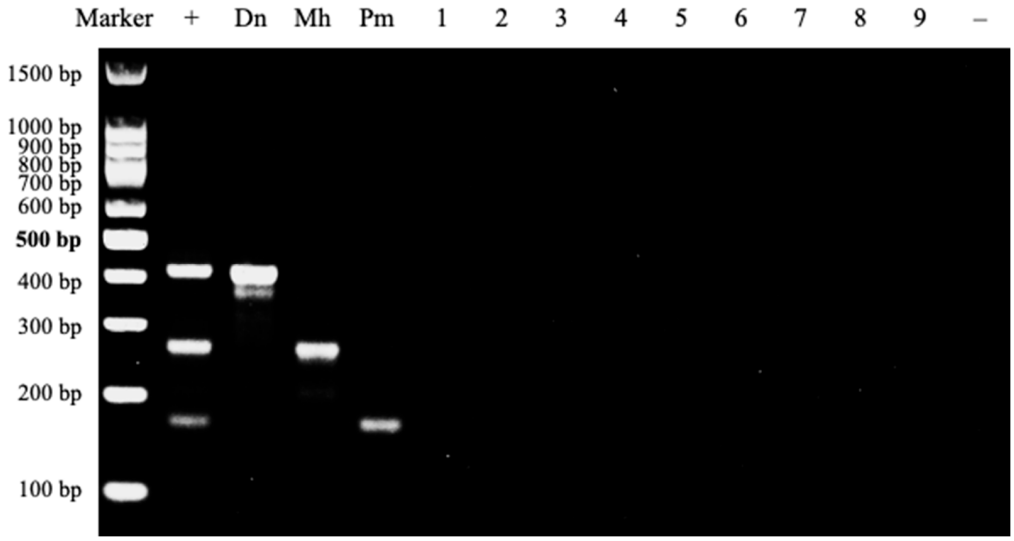

Figure 1. Specificity test of the mPCR assay for three target mealybug species. +: positive control (mixed DNA samples of three target mealybug species); Dn: *Dysmicoccus neobrevipes*; Mh: *Maconellicoccus hirsutus*; Pm: *Paracoccus marginatus*; 1: *Phenacoccus solenopsis*; 2: *Phenacoccus solani*; 3: *Phenacoccus madeirensis*; 4: *Ferrisia virgata*; 5: *Pseudococcus jackbeardsley*; 6: *Planococcus minor*; 7: *Planococcus citri*; 8: *Nipaecoccus viridis*; 9: *Pseudococcus cryptus*; −: negative control (ddH_2O).

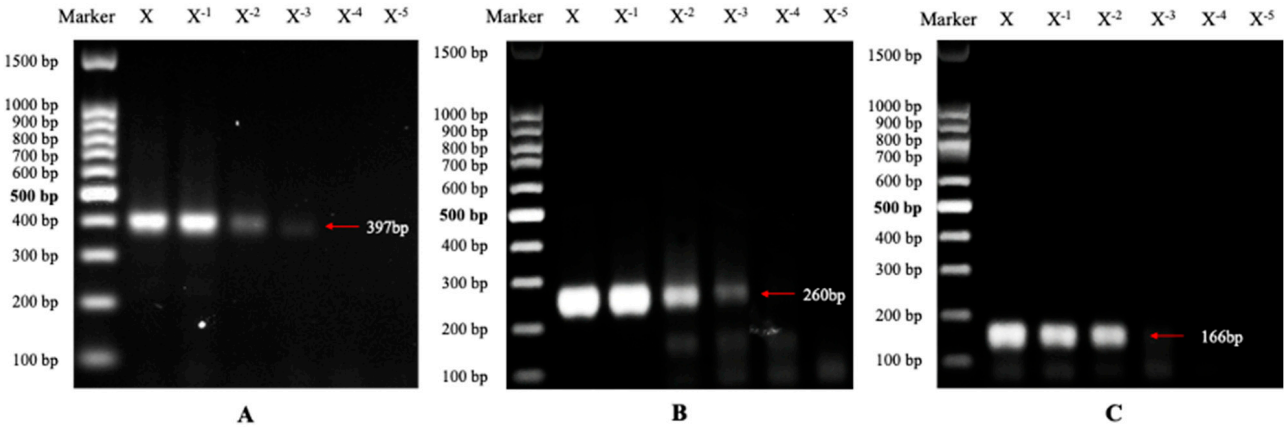

Figure 2. Sensitivity test of the mPCR assay for three target mealybug species. (**A**): *Dysmicoccus neobrevipes*; (**B**): *Maconellicoccus hirsutus*; (**C**): *Paracoccus marginatus*; $X-X^{-5}$ represents the DNA sample concentrations of 10^2, 10, 1, 10^{-1}, $10^{-2,}$ and 10^{-3} ng/μL, respectively.

3.4. Field Sample Tests

A total of 121 mealybug samples were collected from 22 host plants in 11 cities and counties in Hainan Province, China (Table S2). In Hainan, the host plants of mealybugs were mainly tropical fruits, ornamental plants, and weeds. Various mealybugs also damaged important crops, such as cassava, cowpea, sisal, and betel palm. After screening these samples with an mPCR assay, 48 mealybugs (39.67%) were successfully identified as their three target species (*D. neobrevipes*, *M. hirsutus*, and *P. marginatus*). The assay could specifically amplify the DNA of the three target species with single bands (Figure 3). The obtained sequences of the PCR products, which were sent for sequencing verification, were consistent with the COI sequences of the respective species. This finding indicates that the mPCR assay can be used for the rapid identification of the three target species.

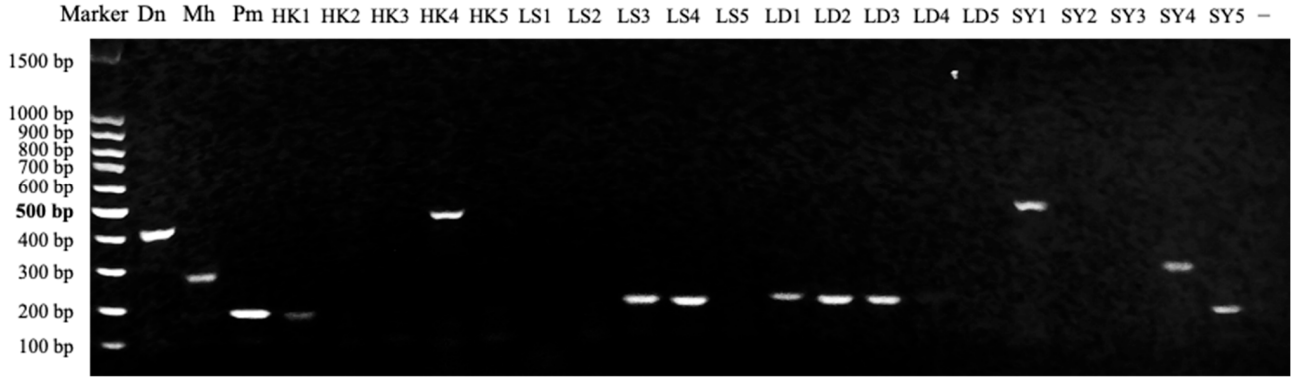

Figure 3. Application of the mPCR assay for field-collected sample identification. Db: *Dysmicoccus neobrevipes*; Mh: *Maconellicoccus hirsutus*; Pm: *Paracoccus marginatus*; HK: Haikou samples; LS: Lingshui samples; LD: Ledong samples; SY: Sanya samples; 1–5 indicates five field-collected samples, respectively; −: negative control (ddH_2O).

To identify the mealybug species that were not of the three target species of the mPCR assay, the COI barcode regions of the remaining 74 samples were amplified and sequenced. Seventy-one samples (57.85%) were identified at the species level through a sequencing approach, whereas three samples (2.48%) were not.

Ultimately, 12 mealybug species, belonging to eight genera, were identified through DNA-based molecular identification. Among them, seven invasive mealybug species were found (*D. neobrevipes*, *P. marginatus*, *P. madeirensis*, *P. solani*, *P. solenopsis*, *P. minor,* and *P. jackbeardsleyi*), including three quarantine mealybugs (*D. neobrevipes*, *P. solenopsis,* and *P. minor*), and the remaining five mealybug species are native mealybugs. Additionally, a sample was identified as *Icerya seychellarum* (Monophlebidae) (Table 3).

Table 3. Identification of field-collected mealybug samples with DNA-based methods.

Methods	Species	Number of Samples (n = 121)	Percentage (%)
Multiplex PCR	*Dysmicoccus neobrevipes*	11	9.09
	Maconellicoccus hirsutus	5	4.13
	Paracoccus marginatus	32	26.45
Sangerse quencing	*Ferrisia virgata*	11	9.09
	Nipaecoccus viridis	3	2.48
	Phenacoccus madeirensis	5	4.13
	Phenacoccus solani	5	4.13
	Phenacoccus solenopsis	7	5.79
	Planococcus citri	6	4.96
	Planococcus minor	14	11.57
	Pseudococcus cryptus	10	8.26
	Pseudococcus jackbeardsleyi	8	6.61
	Icerya seychellarum	1	0.83
	Pseudococcidae sp.	3	2.48

Among the mealybug species identified in this study, invasive *P. marginatus* was the most widely distributed and was found in eight cities or counties in Hainan. Each of the three quarantine mealybugs was also found in three or four sampling sites. The native species of the genera *Maconellicoccus* and *Nipaecoccus* are only found sporadically in Hainan, whereas F. virgata were found at four sampling sites (Table S3).

Also, the invasive mealybug, *P. marginatus,* was found on the most diverse host plants (nine taxa), in which the most frequent was papaya. The three quarantine mealybugs were also found on various host plants (six for *D. neobrevipes*, four for *P. solenopsis*, and seven for *P. minor*). The economically important crops, cassava and papaya, could be the host of the

most mealybugs found in this study. Eight and seven mealybug species or taxa were found on cassava and papaya, respectively (Table S4).

4. Discussion

The COI gene sequence has been used as a widely applicable animal barcode due to its standard PCR amplification region, which is relatively conserved and generally exhibits species-level resolution [14]. Using the COI gene as a DNA barcode can effectively overcome the shortcomings of traditional morphological identification. Thus, DNA barcode technology is widely used in the identification of small and cryptical insects, such as mealybugs [23]. In this study, we successfully obtained the COI barcode sequences of 12 mealybug species collected in Hainan and confirmed their morphological identification with molecular information. In some insects, a single DNA marker may not be sufficient to distinguish species accurately, and multiple DNA markers must be combined to improve the accuracy of identification [11,24]. In this study, the results show that three to five standard 5′ end COI gene sequences of approximately 600–650 bp could be used to distinguish all 12 common mealybug species found in Hainan. Although a relatively low number of sequences should be taken into account when interpreting this result, the present findings and the mPCR assay based on the sequence information are still reliable [18,25]. A good estimation of the distance in COI sequences has been shown to be based on more than 20 individuals per species [26], and the sample number has been found to be positively correlated with the distance between species [27]. However, the barcode gap is usually not affected by sample size, and even the COI sequences obtained from a relatively small number of samples can be used for the identification of specimens collected from a larger geographic scale [27].

Molecular identification using DNA barcoding traditionally involves PCR amplification of the COI gene fragment, followed by sequencing and sequence analysis. It is a relatively cumbersome process and takes a long time for species identification. Additionally, methods such as high-throughput sequencing [28], microsatellite markers [29], and multiplex real-time PCR [30], which have been developed in recent years for molecular identification, have issues such as high cost, long cycle time, complex operation, and instrument dependence. Therefore, they are unsuitable for the rapid detection required in custom port quarantine and other similar situations. As an efficient molecular diagnostic tool, mPCR technology plays an increasingly important role in biological research. It can amplify multiple DNA fragments simultaneously, thereby allowing for the detection of multiple target species at the same time and greatly improving the efficiency and throughput of experiments. In this study, we designed and optimized three pairs of primers based on the size of the obtained COI gene fragments targeting important mealybugs, *D. neobrevipes*, *M. hirsutus*, and *P. marginatus*, which were the most concerned species due to their agricultural importance, prevalence in the region, and the absence of existing identification methods. We added them to the same PCR reaction assay, thereby enabling the identification of three species in one PCR reaction. Given the same principle, multiple mPCR systems can be designed to identify other mealybug species simultaneously. Additionally, this established mPCR assay can successfully distinguish three target mealybug species from all 12 common mealybug species in Hainan. The species-specific primer pairs used in this assay could be also applied together with additional assays to target expanding the species that can be established in future studies. When applied in port quarantine, this technology can greatly reduce the inspection cycle and lower costs, thereby making it more economical and effective than other molecular identification techniques. However, multiplex PCRs have limits for the analysis of an unknown species spectrum, although they provide a high throughput and a highly sensitive method with a high degree of resolution [31]. In this case, morphologic identification and a DNA sequence-based approach are still necessary and reliable for providing an integrated picture of the entire mealybug spectrum, which is especially important when facing the presence of rare or unexpected species. Furthermore, the latest techniques for rapid detection, such as loop-mediated isothermal amplification

(LAMP) [32] and recombinase polymerase amplification (RPA) assays [33], are also practical or even more effective for field study and quarantine inspection. The species-specific primer pairs and sequence regions found in this study would be needed for establishing the LAMP or RPA assays.

Mealybugs are highly concealed in agricultural and forestry production, as well as in the import and export of goods. However, their spread is usually strictly limited by quarantine regulations, which is why their detection and monitoring have high requirements. Many countries have implemented strict procedures, such as hot water immersion [34], to clean and disinfect seedlings and prevent the spread of invasive alien species. Thus, during the quarantine process, directly obtaining a large number of live mealybugs for DNA sample extraction may not be possible. Extracting trace DNA from the environment, such as nursery cleaning wastewater or soil, can be considered. The assay in this study can successfully detect DNA samples at 0.1–1 ng/μL. Thus, the diagnostic PCR approach has the potential to be used in conjunction with environmental DNA (eDNA) technology [35] to identify specific species, thereby achieving the precise detection and early warning of mealybugs.

Around 40% of the field-collected mealybugs could be identified as the three target species of the mPCR assay (*D. neobrevipes*, *M. hirsutus*, and *P. marginatus*). These samples could be distinguished from all field-collected species, even involving a species in Monophlebidae, *I. seychellarum*, which was not considered in the primer design. This result indicates that the established mPCR assay has good specificity and great potential for identifying the samples from field surveys. However, there was still a high proportion of remaining samples, which needed to be identified using the sequencing approach. Therefore, multiple mPCR systems covering more mealybug species need to be designed and tested in further studies, especially for those highly concerning mealybugs, such as *P. minor*, *P. solenopsis*, and *P. jackbeardsleyi*, which were found to be widely distributed in different geographic regions and on diverse host plants. Also, rapid identification tools are needed for the mealybugs that damage economically important crops, such as cassava and papaya, to deal with their complex mealybug communities. Additionally, the field sample test found three unidentified samples, which might be an indication of new mealybug species or a warning of another invasion event. For further study, a thorough investigation of the unidentified field samples is still needed.

5. Conclusions

In this study, the 5′ end COI gene sequences of 12 mealybug species in Hainan were obtained. Based on these COI sequences, an mPCR assay was successfully established. It could achieve rapid and accurate identification of *D. neobrevipes*, *M. hirsutus*, and *P. marginatus*, which have significant importance in tropical regions. This assay can simultaneously amplify multiple target DNA sequences in a single PCR reaction, with each target sequence producing PCR products of different sizes. Moreover, it showed high specificity and sensitivity in the identification of these three mealybugs. Combining the mPCR assay and barcode sequencing, a set of mealybug samples collected from multiple locations in Hainan was individually identified. Twelve mealybug species were found, and their distribution and host plants in Hainan were clarified. Compared with traditional morphological or sequencing-based identification methods, this technology significantly improves the efficiency of mealybug species identification. It also provides an effective molecular tool for mealybug quarantine and monitoring in Hainan and other tropical regions. Thus, it helps to effectively block the spread of mealybugs and protect local agricultural production and ecological safety.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14122786/s1: Figure S1: Electrophoretogram of the DNA barcode COI gene amplification of 12 mealybug species; Figure S2: Neighbor-joining phylogenetic tree based on COI sequences of 12 mealybug species and an outgroup, *Icerya seychellarum*; Figure S3: The locations of species-specific primer pairs in the alignment for the COI sequences of 12 mealybug species; Table S1: Minimum between species and maximum within species genetic distances of 12 mealybug species based on COI sequences; Table S2: Sample collection information of mealybugs in Hainan; Table S3: Identified mealybug samples collected at each sampling site; Table S4: Identified mealybug samples collected from different host plants.

Author Contributions: Conceptualization, Y.X., B.C. and S.W.; methodology, Y.X.; software, Y.X.; validation, W.Y.; formal analysis, Y.X.; investigation, Y.X. and W.Y.; resources, W.Y. and B.C.; data curation, Y.X. and W.Y.; writing—original draft preparation, Y.X. and W.Y.; writing—review and editing, K.L., B.C. and S.W.; visualization, Y.X. and W.Y.; supervision, B.C. and S.W.; project administration, B.C. and S.W.; funding acquisition, B.C. and S.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Project of China (2021YFC2600600) and the Hainan Major Science and Technology Project (ZDKJ2021016).

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding authors.

Acknowledgments: We thank Rui Meng (Animal and Plant Quarantine Center, Haikou Customs District, China) for providing the mealybug specimens.

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

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