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Molecular Species Delimitation Using COI Barcodes of Mealybugs (Hemiptera: Pseudococcidae) from Coffee Plants in Espírito Santo, Brazil

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Abstract: Mealybugs are insects belonging to the family Pseudococcidae. This family includes many plant-pest species with similar morphologies, which may lead to errors in mealybug identification and delimitation. In the present study, we employed molecular-species-delimitation approaches based on distance (ASAP) and coalescence (GMYC and mPTP) methods to identify mealybugs collected from coffee and other plant hosts in the states of Espírito Santo, Bahia, Minas Gerais, and Pernambuco, Brazil. We obtained 171 new COI sequences, and 565 from the BOLD Systems database, representing 26 candidate species of Pseudococcidae. The MOTUs estimated were not congruent across different methods (ASAP-25; GMYC-30; mPTP-22). Misidentifications were revealed in the sequences from the BOLD Systems database involving *Phenacoccus solani* × *Ph. solenopsis*, *Ph. tucumanus* × *Ph. baccharidis*, and *Planacoccus citri* × *Pl. minor* species. Ten mealybug species were collected from coffee plants in Espírito Santo. Due to the incorrect labeling of the species sequences, the COI barcode library of the dataset from the database needs to be carefully analyzed to avoid the misidentification of species. The systematics and taxonomy of mealybugs may be improved by integrative taxonomy which may facilitate the integrated pest management of these pests.

Keywords: biodiversity; agricultural pests; DNA barcoding; species identification; coffee plant; phylogenetic analysis; entomology; systematics; integrative taxonomy

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1. Introduction

Coffee (*Coffea* spp.) is the second-most valuable commodity in the world and a crop that drives a multi-billion-dollar worldwide market [1,2]. From coffee planting and harvest to the consumer's table, the production chain faces numerous challenges, such as climate change, price volatility, post-harvest storage, and mycotoxins [3–6], as well as insect pests and diseases [7–10]. Mealybugs (Hemiptera: Pseudococcidae) are phytophagous insects with a global distribution [11–14]. They are vectors of plant pathogens that harm agricultural crops, and they are significant pests of coffee [15–17].

Because mealybugs are typically small and cryptic, it is challenging to identify and control them. They also secrete wax, which creates a coating that protects them from pesticides [18–21]. The effective prevention and management of pests such as mealybugs depends on the accurate identification of species, which is often difficult using traditional methods based on morphological characteristics [22,23]. Mealybug identification based on morphology is a challenge because it requires the microscopic examination of mature females; it is especially difficult to differentiate closely related species using this method [24]. The other limitations of using morphological methods for species identification include immature specimens, specimens with physical deterioration, and multiple species with overlapping morphological characteristics that inhibit accurate identification [25].

These taxonomic issues have recently been investigated using molecular methods [26–31]. DNA barcoding is a genetic technique that uses a portion of the mitochondrial gene cytochrome c oxidase I (COI), which has approximately 658 base pairs, to identify species [32–34]. DNA barcoding has contributed to the discovery of new species, assessment of biodiversity, clarification of taxonomic ambiguities, and correction of misidentifications in studies of diversity [35–37]. Taxonomic and systematic investigations have also examined the delimitation of species based on the COI gene, with DNA barcoding used with genetic distance or tree-based methods for species delimitation to increase the accuracy of identifications [38–44].

Mealybugs are a group of insects that have been subjected to relatively little research in Brazil, despite significant recent advancements in the study of species delimitation. A search in the BOLD Systems database [45] in April 2022 using the term "Pseudococcidae" produced 3417 published records and 276 BINs, whereas the phrase "Pseudococcidae Brazil" produced 69 records and 11 BINs. In other words, just 2% of the world records are from Brazil, despite the fact that Brazil is a country with high species richness [37]. This percentage demonstrates the lack of awareness about the biodiversity of mealybugs in Brazil.

Because of the economic importance of coffee, mealybugs, as coffee pests, and the challenges involved in their identification based on morphology, information on the species of mealybugs present in the coffee crop in state of Espírito Santo is essential. Thus, the aims of this study were to develop a DNA barcode library for mealybug species collected in Espírito Santo, Brazil, based on morphological analysis, to assess the accuracy of COI barcodes, and to perform phylogenetic analyses for species delimitation in the family in Brazil.

2. Materials and Methods

2.1. Sample Collection and Morphological Identification

Adult female mealybugs were collected from coffee plants in the Brazilian states of Espírito Santo and Bahia (Figure 1), with three or more specimens collected at each sample location when possible. Specimens were preserved in 95% ethanol and stored at −30 °C after collection. Mealybug specimens were also collected from other host plants to investigate mealybug species composition in the state.

Collected mealybugs, including DNA voucher specimens, were slide-mounted and identified by light microscopy using methods described by Sirisena et al. (2013) [46]. Mealybugs were examined with a phase-contrast compound microscope (Ernst Leitz GmbH Biomed, Wetzlar, Germany) and identified using taxonomic keys [47–52].

Planococcus minor (Maskell, 1897) from Minas Gerais, *Ferrisia dasylirii* (Cockerell, 1896), *Phenacoccus solenopsis* (Tinsley, 1898), and *Planococcus citri* (Risso, 1813) from Pernambuco, Brazil, were included in the research as reference samples. This research did not include any endangered or protected insect species and consent from landowners was acquired for collection of the mealybugs. A total of 226 mealybug samples from four states were used in this study. Information on host plants from which specimens were collected and geographic coordinates of collection locations are available in Table S1 (Supplementary Materials).

2.2. DNA Extraction, Amplification, and Sequencing

The methodology described by Arseneau, Steeves, and Laflamme (2017) [53] was used to extract genomic DNA from each individual mealybug specimen separately, without

crushing. The original technique was adjusted to include a minimum of 8 h of incubation, with no RNase A in this stage, and an elution step using 40 µL of DNAse-free water. A NanoDropTM 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to verify DNA yield and purity. DNA extracts were preserved in a freezer at −30 °C.

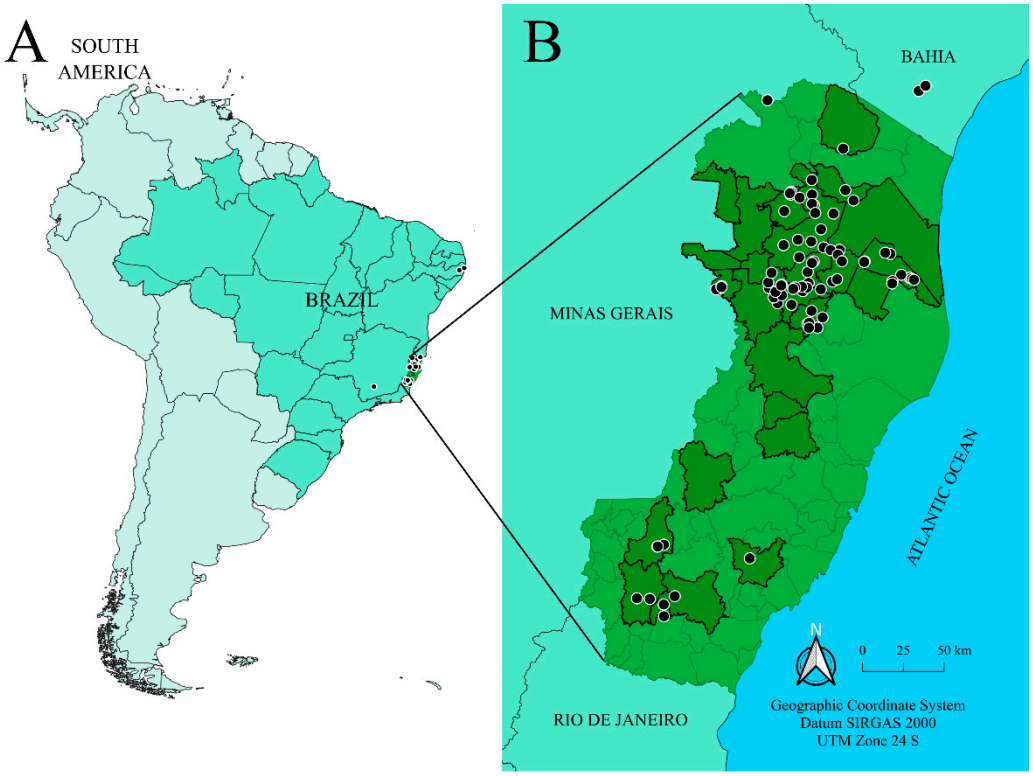

Figure 1. Sampling locations in Brazil for mealybugs (Hemiptera: Pseudococcidae) collected in the present study. (**A**) Map of South America indicating the geographical location of samples collected in the states of Minas Gerais (MG) and Pernambuco (PE). (**B**) Map of Espírito Santo indicating the geographical location of sampling sites (black dots) and municipalities sampled without specific geographical location (dark green with black borders). Specific collection locations and additional information for samples are available in Table S1 (Supplementary Materials). The map was created using QGIS Geographic Information System v3.16 (https://qgis.org accessed on 12 November 2022) by P.V. Oliveira.

COI gene amplicons (~670 bp) were amplified and sequenced using PCO-F1 and LEP-R1 primers [24]. The reaction master mix comprised 1X PCR Buffer, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.2 µM forward and reverse primers, 1 unit of Platinum™ Taq DNA Polymerase (Invitrogen), 20 ng of DNA and ultrapure DNase/RNase-Free distilled water up to a final volume 12.5 µL. The following PCR procedures were modified from Park et al. (2010) [24]: A 2-min initial denaturation at 94 °C, followed by 5 cycles of 40 s at 94 °C, 40 s at 45 °C, and 1 min at 72 °C. This preceded 35 cycles of 40 s at 94 °C, 40 s at 51 °C, and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C, which ended at 4 °C.

Successful PCR products were confirmed using electrophoresis on a 1.5% agarose gel stained with SYBRTM Safe DNA Gel Stain (Invitrogen). Primer residues and unincorporated nucleotides were eliminated using ExoSAP enzymes. Sequencing reactions were carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit in accordance with the manufacturer's instructions. Samples were precipitated with EDTA/NaOAc/ethanol following the sequencing procedure, dried at 50 °C for 30 min, and then sequenced using an ABI 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

2.3. Data Assembly and Analysis

MEGA X was used to analyze, edit, and align raw sequences [54]. The MUSCLE algorithm was used for multiple sequence alignment [55]. All sequences translated into amino acids were examined for the presence of stop-codons and indels to ensure there were no nuclear pseudogenes [56].

Our mealybug COI-sequence dataset was created by combining 171 new COI sequences obtained in the present study, all sequences from other countries classified to mealybug species level by BOLD methods, and all publicly available records of mealybugs restricted to Brazil. To prevent missing data, sequences of less than 580 bp were not included in the analysis. The 736 sequences that made up the final dataset are provided in SM1 (Supplementary Materials). DNA sequences generated in this study are available in the GenBank database under accession numbers OP381504-OP381598, OP391569-OP391593, OP391594-OP391608, OP425673-OP425695, and OP450828-OP450839.

2.4. Phylogenetic Reconstruction and Species-Delimitation Methods

This study employed a haplotype-based species delimitation method, with each haplotype represented by a single sequence (except for *Phenacoccus solani* × *Ph. solenopsis* and *Planococcus citri* × *Pl. minor* species) to simplify computing requirements. The haplotype sequences used to infer species delimitation (n = 110) were determined using DnaSP 6 software [57]. The aligned COI haplotypes used for the species-delimitation study are available in SM2 (Supplementary Materials). The numbers of sequences used for *Phenacoccus solani* × *Ph. solenopsis* and *Planococcus citri* × *Pl. minor* species were different because these closely related species shared haplotypes, with three sequences selected for *Phenacoccus solani* × *Ph. solenopsis* species and two for *Planococcus citri* × *Pl. minor* species.

Maximum likelihood (ML) and Bayesian inference (BI) were used to evaluate the phylogenetic connections among the studied specimens. The Bayesian Information Criterion (BIC) was used to infer the substitution model for phylogenetic-tree reconstruction using jModelTest v.2.1.10 [58]. GTR+I+G was used as the best-fit model in ML analysis with RAxML v 8.2.12 [59] and 1000 bootstrap replicates. *Ripersiella emarai* and *R. multiporifera* sequences were employed as outgroups [60]. The San Diego Supercomputer Center's CIPRES Science Gateway (https://www.phylo.org/index.php accessed on 12 November 2022) was used for the ML analysis.

To derive an ultrametric tree, BI analysis was carried out in BEAST v.1.8.4 [61] utilizing the GTR+I+G as a substitution model, the speciation birth–death model as a tree prior, and the lognormal relaxed clock model. Three independent runs of 10 million generations, sampled every 10,000 generations, and 25% burn-in were conducted. Convergence was evaluated with Tracer v.1.7.1 [62], the trees were summarized in TreeAnnotator v.2.4.8 [63], and the resultant tree was constructed using FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/ accessed on 12 November 2022)

To estimate the molecular operational taxonomic units (MOTUs), species-delimitation techniques using distance- and tree-based methodologies were used. Assemble Species by Automatic Partitioning (ASAP), a webserver for species delimitation (https://bioinfo.mnhn.fr/abi/public/asap/ accessed on 12 November 2022) was used with the K2P model and default settings for the distance-based method [64]. Bayesian Poisson Tree Processes (bPTP; https://species.h-its.org/ptp/ accessed on 12 November 2022) [65] and multi-rate Poisson Tree Processes (mPTP; https://mptp.h-its.org accessed on 12 November 2022) with default settings were used for the tree-based method [66].

A non-ultrametric tree estimated by RAxML version 8.2.12 was used to calculate MOTUs for both methods. The Generalized Mixed Yule Coalescent model (GMYC; https://species.h-its.org/gmyc/ accessed on 12 November 2022) made use of an ultrametric tree that BEAST v.1.8.4 had reconstructed using a single-threshold approach with default settings [67]. When at least two of the three methods were in agreement, a consensus species delimitation was chosen. The Kimura-2-parameter (K2P) model was used to compute overall mean distance,

intraspecific distances, and interspecific distances among MOTUs [68]. MEGA X was used to determine mean pairwise distances using pairwise deletion [54].

3. Results and Discussion

A total of 171 novel COI barcode sequences from 7 genera and 10 species were recovered, with the identifications confirmed morphologically. Furthermore, 565 publically available BOLD sequences were added to our dataset of 736 sequences without outgroups, including 219 from Brazil and 517 from other countries, representing the mealybug species recorded in Brazil (SM1, Supplementary Materials). This study included 10 morphologically identified candidate species and 16 candidate species from the database. There were 328 variable sites (53.33%), of which 257 (78.35%) were parsimony-informative and 71 (21.65%) were singletons.

In the public databases, conflicting findings were observed for two genera: three sequences of "*Phenacoccus solani*" and three of *Phenacoccus solenopsis*; and two sequences of "*Planococcus citri*" and two of *Planococcus minor*, which shared the same haplotype. In the cases in which species identification was questionable, the name of the species was noted with quotation marks. The final haplotype alignment included 118 sequences (without outgroups), representing 110 different haplotypes (Supplementary Materials). The estimated molecular operational taxonomic units were not consistent among the methods: 25 for ASAP, 30 for GMYC, and 22 for mPTP (Figure 2).

For species delimitation, the use of multiple approaches may be more reliable than a single method [69]. In the present study, three approaches were studied and ASAP was found to be the best species-delimitation model for mealybugs. *Planococcus citri* and *Pl. minor* were recognized as a single species, suggesting an inability to delimit these two closely related species. The weaker genetic divergence between them can explain this clustering [70–72]. This method identified 25 candidate species, near to the number used initially (26 species). Thus, ASAP has better biological significance (delimiting species concordantly) and a lower number of singletons than GMYC.

With a strong theoretical basis, GMYC was created to delimit species using single-locus data. However, compared to other approaches, it often produces more OTUs. [73]. *Dysmicoccus brevipes*, *M. hirsutus*, and *Ps. longispinus*, for example, were each separately split into two, two, and three candidate species by GMYC, respectively. This could mean cryptic species, but it is necessary to be cautious. More gene sequences are necessary to perform a reliable species delimitation in these cases. For this to be definitive, an integrated taxonomic strategy and more thorough sampling are required [71,74].

Although mPTP has been regarded as a useful technique for species delimitation, in the present study, mPTP was combined with well-known taxa such as *Ferrisia virgata* (Cockerell), *F. dasylirii*, *Pseudoccoccus jackbeardsleyi* (Gimpel & Miller), and *Ps. annonae* (Pacheco da Silva & Kaydan) [38,75]. The bPTP approach was eliminated from our study because it estimated too many singletons, which might have led to an incorrect interpretation of the data [73]. As input, bPTP does not require an ultrametric tree or a sequence-similarity threshold [65]. Instead, it adds Bayesian support values to the input tree to delimit species. The greatest Bayesian support value at a node suggests that all of that node's descendants are most likely species [42,76,77].

Due to character loss or poor specimen quality, it can be challenging to distinguish closely related mealybug species based on morphology. In the present study, sequences from the BOLD Systems database that were deposited as *Ph. solani* were noted. However, our results strongly suggest that the sequences are from *Ph. solenopsis*. All 14 specimens collected and identified as *Ph. solenopsis* based on their morphology were sequenced and matched to *Ph. solenopsis* in BOLD to support this conclusion.

Similar circumstances apply to the *Pl. citri* sequences from BOLD, which apparently belonged to the *Pl. minor* species. These findings may help to explain why *Ph. solani* x *Ph. solenopsis* and *Pl. citri* × *Pl. minor* have inconsistent species identifications based on morphology, since they share geographic regions and have similar morphologies [78]. In

addition, *Phenacoccus tucumanus* (Granara de Willink, 1983) sequences were matched to *Ph. baccharidis* (Williams; 99.77% likelihood of placement).

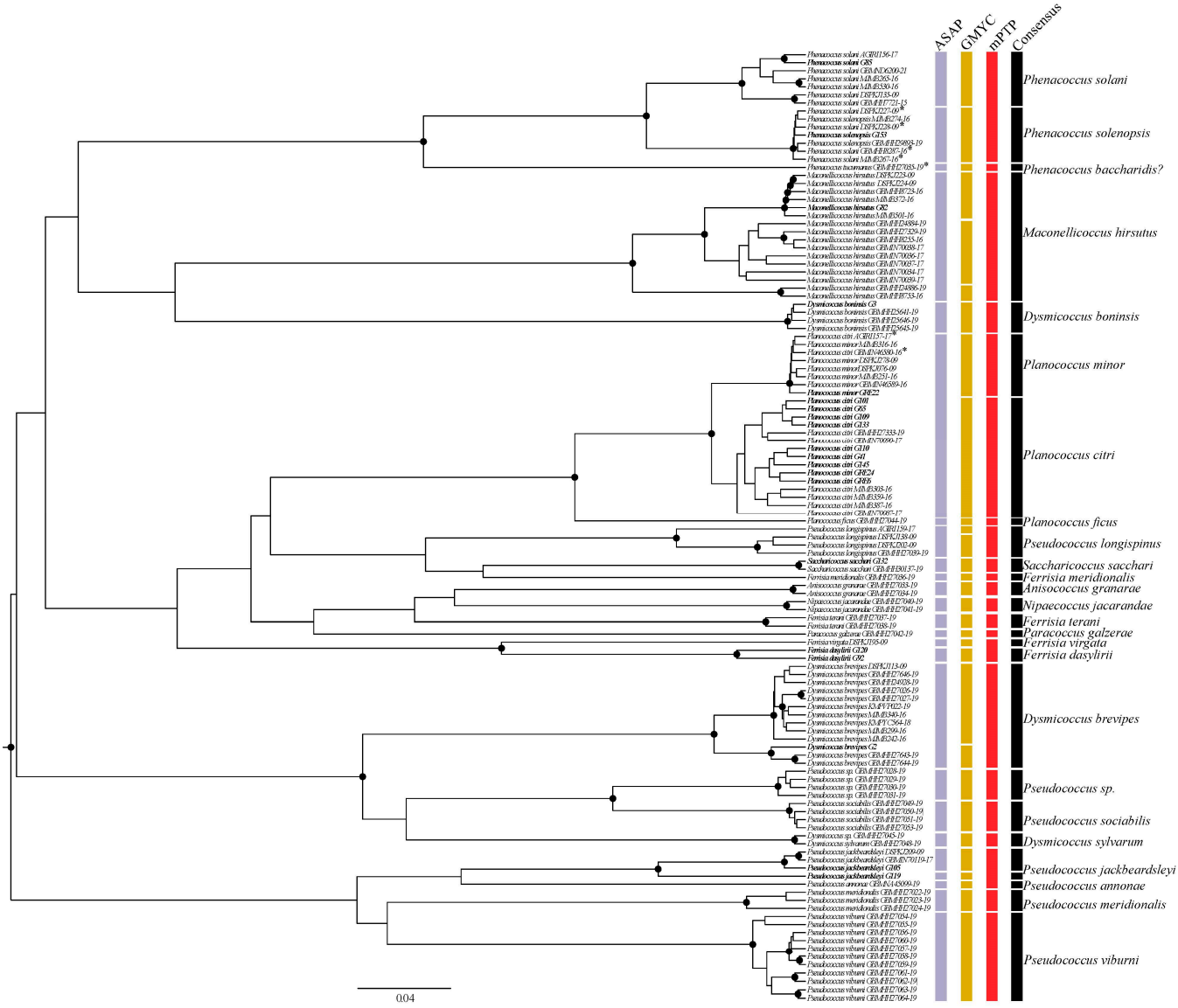

Figure 2. Phylogenetic analysis of mealybugs (Hemiptera: Pseudococcidae) based on COI-gene haplotypes and results of species-delimitation approaches. This tree was estimated using Bayesian inference. Black dots in the nodes indicate statistical support of posterior probability (>0.9). Nominal species in bold indicate new sequences obtained in the present study and asterisks (*) indicate errors in the species identification using the BOLD Systems database. Colored bars on the right show the putative species inferred by ASAP, GMYC, and mPTP. The last bar (far right) indicates species consensus through the approaches.

The use of genetic/molecular databases has been shown to increase the accuracy of identification [79]. Nevertheless, many sequences obtained from databases may result in incorrect identifications and create taxonomic confusion. To prevent problems with accuracy in biodiversity data, care must be taken when taxonomic information is obtained from repositories [80,81].

The genera *Maconellicoccus*, *Phenacoccus*, and *Planococcus* were sampled with a significant number of haplotypes, according to the BI phylogeny (Figure 2). These monophyletic groups have PP values over 0.9. Although only one or two haplotypes were examined, we also found monophyletism for the genera *Anisococcus*, *Nipaecoccus*, *Paracoccus*, and

Saccharicoccus. However, the delimitation of putative species may be biased because of this underrepresentation of sequences [82].

The paraphyletic groups for the genera *Dysmicoccus* and *Pseudococcus* are shown. These taxonomic groups had comparable phylogenetic relationships in the ML phylogeny, with high support values (Figure S1, Supplementary Materials). Despite the fact that only one marker was utilized for defining the species, our results are comparable to those of other publications that used multilocus approaches [60].

In the present study, we discovered 25 candidate species for Brazil using the consensus reached by the methodologies, which included the ASAP, GMYC, and mPTP approaches. In the state of Espírito Santo, 10 species of mealybugs were identified. With the exception of *Saccharicoccus sacchari* (Cockerell), the other nine mealybug species were previously identified on *Coffea* spp. However, in this study, one specimen of *Dysmicoccus boninsis* (Kuwana) and two of *S. sacchari* (Cockerell) were the only species collected from sugarcane, and one specimen of *Ph. solenopsis* was collected from okra.

The rapid screening and identification of mealybugs of commercial significance and quarantine concern is made possible by COI barcodes [83]. Our findings suggest a successful identification strategy that can facilitate pest management in the Brazilian coffee industry. The closely related species *Pl. citri* and *Pl. minor*, which are both common and abundant in Brazil, were identified. By using known natural enemies of the identified species, coffee growers may also gain from species identification. For instance, the citrus mealybug (*Pl. citri*) is attacked by a variety of natural enemies that have been identified around the world, including a large number of hymenopteran parasitoids, predatory fly larvae, lacewings, and ladybug beetles [84–88].

4. Conclusions

Because mealybugs are common and serious pests of many agriculture crops, the accurate identification of mealybug species is essential to enable the integrated pest management (IPM) of these insects. In addition, the accurate identification of mealybugs is necessary for quarantine interceptions to prevent the spread of these pests to uninfested regions. In the present study, a DNA barcode library for mealybug species was produced, using mealybug species obtained mainly in Espírito Santo, Brazil, a major coffee-producing region.

Ten mealybug species were collected and identified from coffee plants in Espírito Santo, and our results demonstrate that the molecular delimitation of species is useful to ascertain the diversity of the species in this crop. Despite possible limitations in the mealybug database, our results shows that two of the three delimitation strategies examined were reliable.

Accurate species identification is essential for integrated pest management. The Pseudococcidae COI barcode library for the state of Espírito Santo obtained in the present study provides an advantageous reference for the reliable and rapid identification of mealybug species, particularly for closely related and cryptic species. In addition, coffee farming will benefit from the ability to use recognized natural enemies to manage mealybugs in coffee plantations identified by integrative taxonomy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15020305/s1, Figure S1: Phylogenetic relationships of mealybugs (Hemiptera: Pseudococcidae) based on COI-gene haplotypes. This tree was estimated using Maximum Likelihood with RaxML. Black dots in the nodes indicate bootstrap values (>50%). Nominal species in bold indicate new sequences obtained in the present study and asterisks (*) indicate errors in the species identification using the BOLD Systems database; Table S1: List of specimen samples used in this study, geographic coordinates and Best ID in the BOLD Systems database; The alignments of COI sequences of all specimens and haplotypes are available in fasta file.

Author Contributions: Conceptualization, P.V.O., M.J.F., J.A.V., M.P.C. and G.G.P.; methodology, P.V.O., E.L.O., K.B.B., F.A.N.d.A. and G.G.P.; validation, C.B.M.; formal analysis, P.V.O. and C.B.M.; investigation, P.V.O., E.L.O., K.B.B., F.A.N.d.A., V.C.P.d.S. and C.B.M.; resources, A.R.d.S., V.C.P.d.S., M.J.F., J.A.V. and M.P.C.; data curation, P.V.O., E.L.O., K.B.B., F.A.N.d.A., V.C.P.d.S. and C.B.M.; writing—original draft preparation, P.V.O.; writing—review and editing, K.B.B., V.C.P.d.S., C.B.M.,

M.P.C. and G.G.P.; visualization, P.V.O., C.B.M. and G.G.P.; supervision, A.R.d.S. and G.G.P.; project administration, A.R.d.S., J.A.V. and G.G.P.; funding acquisition, G.G.P. All authors have read and agreed to the published version of the manuscript.

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