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Higher classification of mealybugs (Hemiptera: Coccomorpha) inferred from molecular phylogeny and their endosymbionts

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Abstract

Mealybugs (Hemiptera: Pseudococcidae, Rhizoecidae and Xenococcidae) are important organisms for understanding processes of evolution, especially microbial symbiotic systems and interactions with host plants. Molecular phylogenetic trees were reconstructed for 128 species of mealybug using DNA fragments of eight loci, namely a mitochondrial (*COI*), nuclear ribosomal RNA (*18S* and *28S D2* and *D10*) and nuclear protein-encoding genes (*EF-1α 5′* and *3′*, *Dynamin* and *wingless*). In addition, data on the types of obligate endosymbionts were used to test the monophyly of major groups resulting from this molecular phylogeny. Based on the data from DNA sequences, morphology and obligate endosymbionts, we present a phylogeny supporting the families Rhizoecidae and Xenococcidae separate from Pseudococcidae, and the separation of *Rastrococcus* Ferris from Phenacoccinae and Pseudococcinae. Consequently, *Rastrococcus* is excluded from Phenacoccinae and elevated to subfamily Rastrococcinae **subfam. nov.** We also found support for Putoidae as a family distinct from the true mealybugs. *Phenacoccus rubicola* Kwon, Danzig & Park is transferred to *Coccura* Šulc.

KEYWORDS
Phenacoccinae, Pseudococcinae, Rastrococcinae, Rhizoecidae, Xenococcidae

INTRODUCTION

Pseudococcidae, commonly known as 'mealybugs', represent the second-largest group of scale insects with ca. 2000 described species in 250 genera worldwide (as listed in ScaleNet; García Morales et al., 2019). Many species are considered economic pests of agriculture because they directly or indirectly damage a variety of plants by sap-sucking and virus transmission (Kondo et al., 2008; McKenzie, 1967; Tsai et al., 2010). Scale insects, including mealybugs, have been used as important organisms for diverse evolutionary studies, such as symbioses with endosymbionts (Bublitz et al., 2019; Downie & Gullan, 2005; Garber et al., 2021; Gruwell et al. 2007, 2010; Husnik et al., 2013; Husnik & McCutcheon, 2016; Thao et al., 2002; von Dohlen et al., 2001), interactions with host plants (Hardy, 2017; Hardy et al., 2015, 2016), ants (Quek et al., 2016; Ueda et al., 2008) or parasitoid wasps (Deng et al., 2013) and evolution of genetic and reproductive systems (Mongue et al., 2021; Normark, 2003; Ross et al., 2010; Ross, Hardy, et al., 2012; Ross, Shuker, et al., 2012).

Previous phylogenetic works for mealybugs

The higher classification of Pseudococcidae has been revised several times based on phylogenetic studies (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008; Kaydan et al., 2015). These studies have shown congruent phylogenetic hypotheses for the major diversification of evolutionary lineages in Pseudococcidae, namely Phenacoccinae and Pseudococcinae (Planococcini, Pseudococcini and Trabutinini) as well as a separation of Putoidae. Although the group, named Rhizoecini/Rhizoecinae/Rhizoecidae, is considered a distinct lineage, its phylogenetic placement was not constant in all analyses.

Previously, five subfamilies had been suggested in Pseudococcidae, namely Pseudococcinae, Phenacoccinae, Rhizoecinae, Trabutininae and Sphaerococcinae (Danzig, 1980; Koteja, 1974a, 1974b, 1988; Tang, 1992; Williams, 1985). However, Downie and Gullan (2004) showed that the clade of Trabutininae was placed as a tribe within Pseudococcinae. In addition, Sphaerococcinae was polyphyletic within Pseudococcinae, but the authors could not strongly suggest a change in its taxonomic affiliation because the type genera and species were not included. In addition, a family identity of Putoidae was supported when *Icerya* was used as the outgroup. The Rhizoecinae clade was sister to the clade of Phenacoccinae + Pseudococcinae. In the phylogenetic analyses of Downie and Gullan (2004), support for major clades was poor or unresolved.

The phylogenetic reconstruction of Hardy, Gullan, and Hodgson (2008) based on the combined dataset (molecular + morphology) produced a tree supported Phenacoccinae and Pseudococcinae, and three tribes of Pseudococcinae: Pseudococcini, Planococcini and Trabutinini. However, Rhizoecinae was recovered as a tribe within Phenacoccinae. Pseudococcinae was rendered paraphyletic by Sphaerococcinae, with the latter transferred to Pseudococcinae. In Hardy, Gullan, and Hodgson (2008), species of *Rastrococcus* Ferris were included for the first time in the phylogenetic analyses, and were placed in Phenacoccinae as sister to Rhizoecini. Although nodal confidence for the major clades in the Pseudococcidae was improved in this study by adding taxa and morphological data, species of Phenacoccinae were still undersampled compared to Pseudococcinae.

The latest study, Kaydan et al. (2015), supported Pseudococcidae forming two clades of Phenacoccinae and Pseudococcinae using various Palaearctic species. They recovered a well-resolved tree based on partial DNA sequences of only two loci, *COI* and *28S*, and the taxa limited to the Palaearctic region. Rhizoecidae were used to root the tree, although there had been no phylogenetic study to confirm Rhizoecidae as a separate family from Pseudococcidae. Hodgson (2012) had previously raised Rhizoecini to family rank based on the morphology of adult males. In Rhizoecidae, Xenococcinae was excluded and erected to the family Xenococcidae because of its unique morphology (Danzig & Gavrilov-Zimin, 2014).

Use of obligate endosymbionts in phylogeny

The types of obligate endosymbionts in scale insects have potential use as indicators of the diversification of major lineages. Information on obligate endosymbionts can be used to test and update the higher classification of scale insects (see Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008). In Coccomorpha, some families have distinctive lineages of obligate endosymbionts: Coelostomidiidae (harbouring *Hoataupuhia coelostomidicola* Dhami et al.) (Dhami et al., 2012, 2013), Coccidae (*Ophiocordyceps* Petch) (Deng et al., 2021), Dactylopiidae (*Dactylopiibacterium carminicum* Ramírez-Puebla et al.) (Ramírez-Puebla et al., 2010; Vera-Ponce de León et al., 2017), Diaspididae (*Uzinura diaspidicola* Gruwell et al.) (Gruwell et al., 2007), Monophlebidae (*Walczuchella monophlebidarum* Rosas-Pérez et al.) (Matsuura et al., 2009; Rosas-Pérez et al., 2014) and Pseudococcidae (*Tremblaya princeps* Thao et al. in Pseudococcinae and *T. phenacola* Gruwell et al. in Phenacoccinae) (Downie & Gullan, 2005; Gruwell et al. 2010; Husnik & McCutcheon, 2016). In addition, several lineages of Flavobacteriia were detected in species belonging to Cryptococcidae, Lecanodiaspididae and Ortheziidae (Rosenblueth et al., 2012), although extensive studies are needed to confirm these bacteria as obligate endosymbionts in each family. Furthermore, different types of obligate endosymbionts were identified from (γ-Proteobacteria) Putoidae and Rhizoecidae and Xenococcidae (*Brownia rhizoecola* Gruwell et al.) based on phylogenetic evidence (Gruwell et al., 2010, 2014). Notably, *Rastrococcus* species in Pseudococcidae harbour endosymbionts affiliated to Flavobacteriia (Gruwell et al., 2010), which is a different lineage from the usual obligate endosymbionts of the Phenacoccinae and Pseudococcinae that belong to β-Proteobacteria. The above findings have shown that bacterial symbionts exhibit considerable fidelity with their hosts that may be phylogenetically and taxonomically informative.

In this study, we present an updated molecular phylogeny using improved taxon and data sampling of molecular loci to hypothesize the evolutionary relationships among higher groups. The specific aims of this research were to: (i) reconstruct the molecular phylogeny using DNA fragments of a mitochondrial and nuclear ribosomal RNA and protein-encoding genes, (ii) identify the obligate endosymbionts of ingroup species based on 16S rDNA sequences, (iii) review the major lineages of mealybugs based on the molecular phylogeny and their obligate endosymbionts, and (iv) propose a revised classification based on molecular and morphological evidence as well as data of obligate endosymbionts.

MATERIALS AND METHODS

Taxon sampling

A total of 128 taxa were used for the molecular analyses, including 123 ingroup and five outgroup species (Table S1). The ingroup comprised the families Putoidae (four species of one genus), Rhizoecidae (eight species of three genera), Xenococcidae (one species of one genus) and Pseudococcidae (55 species of 15 genera within Phenacoccinae and 55 species of 24 genera within Pseudococcinae). We included the type species or at least type genera of families and subfamilies as follows: *Phenacoccus aceris* (Signoret) for Phenacoccinae, *Pseudococcus longispinus* (Targioni Tozzetti) for Pseudococcinae, *Puto* for Putoidae and *Rhizoecus K*ünckel d'Herculais for Rhizoecidae. However, the type species was unavailable for Xenococcidae. Outgroups were selected from Matsucoccidae, Monophlebidae, Kuwaniidae and Ortheziidae according to previous hypotheses of the phylogenetic relationships among scale families (Cook et al., 2002; Gullan & Cook, 2007; Hodgson & Hardy, 2013; Vea & Grimaldi, 2016). Many taxa (68 species) were newly obtained for this study. All samples from Korea, Laos, Malaysia, Myanmar, the Philippines and Vietnam were collected by the first author (J. Choi) and others from Japan, Indonesia and United States were provided by colleagues (see Acknowledgements).

A sample of each species was preserved in 99% ethanol and stored at −20°C. For species identification and morphological analyses, several individuals from the same sample were mounted on glass microscope slides following the methods of Danzig and Gavrilov-Zimin (2014). Samples of each species were dissected into the following two parts: (i) body cuticle for molecular analyses of the host and (ii) the body contents for detection of the obligate endosymbionts. All vouchers are deposited in the College for Agriculture and Life Science, Seoul National University (SNU). DNA sequences for the remaining ingroup taxa (60 species) were retrieved from National Centre for Biotechnology Information (NCBI) (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008) and the electronic supplement files of Kaydan et al. (2015) to supplement with groups that were under-sampled. Among the taxa that were used in the molecular analyses of Downie and Gullan (2004) and Hardy, Gullan, and Hodgson (2008), we selectively chose the species (33 species) that revealed the lineages of obligate endosymbionts in Thao et al. (2002) and Gruwell et al. (2010, 2014). An *EF-1α 5′* sequence of *Dysmicoccus brevipes* (Cockerell) and a *28S D10* sequence of *Pseudococcus viburni* (Signoret) were downloaded from NCBI. Although there is no information about the obligate endosymbionts of each species and the molecular data were limited to only two loci (*COI* and *28S D2*), 27 species of Phenacoccinae were chosen to obtain representatives of this subfamily, which were used in the phylogenetic analysis of Kaydan et al. (2015).

DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted from the dissected parts (body cuticle) of each sample using the DNeasy Blood & Tissue kit (Qiagen, Inc., Dusseldorf, Germany) according to the manufacturer's instructions. We selected eight loci, such as *COI*, *18S*, *28S D2* and *D10*, *Dynamin*, *EF-1α 5′* and *3′* and *wingless*, for collecting molecular data. Among them, *Dynamin* and *wingless* were newly included in the phylogenetic analyses of mealybugs because of their potential usefulness in phylogenetics (Hardy, 2007; Hardy, Gullan, Henderson, & Cook, 2008). The remaining markers were used in the previous phylogenetic studies (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008; Kaydan et al., 2015). Polymerase chain reaction (PCR) amplification of these loci was performed following primers and their PCR protocols given in Table S2. The PCR was conducted with AccuPower PCR PreMix (Bioneer, Daejeon, Korea) in 20 ml, including 0.4 μM of each primer, 20 μM of dNTPs, 20 μM of $MgCl_2$ and 0.05 μg of DNA template. PCR products were assessed in 1.5% agarose gel electrophoresis and purified using a QIAquick PCR purification kit (Qiagen), and then sequenced at Bionics Inc. (Seoul, Korea) and Macrogen Inc. (Seoul, Korea).

Alignments and sequence editing

After DNA fragments were assembled using SEQMAN PRO v.7.1.0 (DNASTAR, Inc., Madison, WI, USA), each assembled sequence was compared with nucleotides in NCBI using Basic Local Alignment Search Tool (BLAST) search to check for contamination. Unproblematic sequences were aligned with MAFFT v.7 (Katoh & Standley, 2013). We edited the aligned sequences from eight loci using the following procedures: (i) introns of *Dynamin*, *EF-1α 5′* and *3′* sequences were detected using the GT-AG rule (Rogers & Wall, 1980) and deleted to prevent inclusion of ambiguous alignments (Talavera & Castresana, 2007); (ii) protein-coding sequences (*COI*, *Dynamin*, *EF-1α 5′* and *3′* and *wingless*) were screened for stop codons through the amino acid translation of the alignments to exclude pseudogenes and (iii) remaining ambiguous and poorly aligned parts were removed using GBLOCKS 0.91b (Castresana, 2000; Talavera & Castresana, 2007) with relaxed parameter settings (minimum numbers of sequences for a conserved position and a flank position: both minimum; maximum number of contiguous non-conserved positions and minimum length of a block: both default; allowed gap positions: with half). The edited sequences of each locus were concatenated using SEQUENCEMATRIX v.1.7.8. (Vaidya et al., 2011). Missing characters and gap regions were filled as '?' and '-' in the dataset, respectively. All sequences acquired in this study were submitted to GenBank (accession numbers are in Table S1).

Phylogenetic analyses

The final molecular dataset was analysed with Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP). The best partition scheme was selected by PARTITION-FINDER2 v.2.1.1 (Lanfear et al., 2016) for BI and ML analyses (Table S3). BI analysis was conducted with MRBAYES v.3.2.6 (Ronquist et al., 2012) under substitution models for each partition from PARTITION-FINDER2 v.2.1.1 (Table S3). BI analyses were conducted in two independent runs each with four chains (one cold and three heated) for 10 million generations with a burn-in of 25% of the samples. Trees were sampled every 1000 generations. The average standard deviation of split frequencies below 0.01 and the average potential scale reduction factor for parameter values reaching 1.00 were examined in MRBAYES v.3.2.6 to assess convergence of the two runs. A 50% majority-rule consensus tree was obtained from the remaining trees to assess posterior probabilities. ML analysis was performed with IQ-tree (Nguyen et al., 2015) under the best-fit models automatically determined for each partition using ModelFinder (Kalyaanamoorthy et al., 2017) available in IQ-tree (Table S3). Branch support was assessed with 1000 replicates of ultrafast bootstrap approximation (Hoang et al., 2018). MP analysis was carried out using PAUP* v.4.0 (Swofford, 2003). Heuristic search was performed with 1000 random sequence addition replicates (10 trees held at each step) using tree-bisection-reconnection branch swapping. All characters were equally weighted and unordered, and gaps were treated as missing data. Branch support values were calculated with 1000 bootstrap replicates and branches with less than 50% bootstrap were collapsed. Clades with BI posterior probability (PP) values and ML ultrafast bootstraps (UFBoot) ≥ 95% and MP bootstraps (MPBoot) ≥ 70% were considered well supported

(Hillis & Bull, 1993; Huelsenbeck & Rannala, 2004; Trifinopoulos & Minh, 2018). All trees from BI, ML and MP analyses in molecular phylogeny were visualized with FIGTREE v.1.4.3 (Rambaut, 2009).

Molecular analyses of obligate endosymbionts

Obligate endosymbionts of each species were surveyed based on molecular evidence of 16S rRNA. Following the procedures as above, genomic DNA was extracted from the dissected parts (body contents) of each sample. PCR amplification of 16S rRNA was conducted with universal and/or specific primers (primers and their PCR protocols given in Table S4). If PCR products using universal primers resulted in no band of DNA on the gel electrophoresis or failed sequencing (showing mixed picks), we sequentially used specific primers to amplify 16S rRNA fragments of Flavobacteriia, β-Proteobacteria and γ-Proteobacteria, respectively (most endosymbionts of sternorrhynchan insects belong to these bacterial lineages; Sudakaran et al., 2017). We failed to get the DNA sequence of endosymbionts from the original population of *Rastrococcus rubellus* Williams, so we used another population that was collected from Laos (Table S1). The 16S rRNA sequences were identified by BLAST (Altschul et al., 1997) searches. To determine whether the detected endosymbionts are obligate or facultative endosymbionts, each assembled sequence was compared with molecular data published from several mealybug endosymbiotic studies based on molecular analyses and/or fluorescence in situ hybridization (Downie & Gullan, 2005; Gruwell et al. 2010; Husnik & McCutcheon, 2016; Koga et al., 2013; Michalik et al., 2019; Thao et al., 2002; von Dohlen et al., 2001). The dataset for the phylogenetic analysis of endosymbionts included 16S rRNA sequences of endosymbionts detected from 83 ingroup species (Table S5). The obligate endosymbionts of *Rastrococcus iceryoides* (Green) and *Puto albicans* McKenzie were excluded for this analysis because their sequences possibly had some errors. The sequences of 17 other endosymbionts and free-living bacteria were used for outgroups (Table S6). The ML analysis was conducted under the best-fit model (TVMe + I + G4), following the methods of sequence alignment and editing, and phylogenetic analysis suggested above.

RESULTS

The total length of the concatenated sequence that was used for molecular phylogenetic analyses was 3543 bp (410 bp for *COI*, 258 bp for *Dynamin*, 259 bp for *EF-1α 5′*, 351 bp for *EF-1α 3′*, 453 bp for *wingless*, 543 bp for *18S*, 609 bp for *28S D2* and 660 bp for *28S D10*). In the MP analysis, among 3543 total characters, 1905 characters were constant, 272 variable characters were parsimony-uninformative and 1366 characters were parsimony-informative. The combined molecular dataset yielded 6720 most parsimonious trees (MPTs) of 10,287 steps, with a consistency index of 0.28 and a retention index of 0.66. The strict consensus tree of 6720 equally parsimonious trees with bootstrap (MPBoot) for each node is shown in Figure S3.

The phylogenetic trees from BI (Figure S1), ML (Figure S2) and MP (Figure S3) analyses showed largely congruent tree topologies for higher groups. In both the BI and ML analyses (Figures S1 and S2), each of the Pseudococcidae, Putoidae and Rhizoecidae were recovered as monophyletic, showing separate phylogenetic placements. The MP analysis (Figure S3) agreed with these results from the BI and ML analyses except for the inclusion of *Neochavesia caldasiae* (Balachowsky) (Xenococcidae) within the major clade of Pseudococcidae. Among BI, ML and MP analyses, the following major differences were shown: (i) *N. caldasiae* was sister to all other species of Rhizoecidae on the BI and ML trees (Figures S1 and S2), whereas it formed a clade with species of *Rastrococcus* on the MP tree (Figure S3) and (ii) two species of *Heliococcus* Šulc formed a clade sister to other species of Pseudococcidae (minus species of *Rastrococcus*) on the BI and ML trees (Figures S1 and S2), whereas these were placed within the clade of Phenacoccinae (minus species of *Rastrococcus*) on the MP tree (Figure S3). Other minor differences among tree topologies of the BI, ML and MP analyses are indicated by a dash in Figure 1.

Putoidae (four representatives) formed a clade (PP = 1.0, UFBoot = 100, MPBoot = 100) that was placed outside the clade, including representative species of Pseudococcidae, Rhizoecidae and Xenococcidae (Figures S1–S3). The putoid clade was clustered with a clade, including species of *Matsucoccus* Cockerell and *Orthezia* Bosc d'Antic that were used as outgroups.

Rhizoecidae (eight representatives) and Xenococcidae (one representative) formed a clade (PP = 0.95) on the BI and ML trees, which was sister to the clade, including representative species of Pseudococcidae. In the clade of Rhizoecidae (PP = 1.0, UFBoot = 98) on the BI, ML and MP trees (Figures S1–S3), *Rhizoecus* (three representatives) and *Ripersiella* Tinsley (four representatives) were non-monophyletic, clustered together with the *Geococcus* Green.

Pseudococcidae (110 representatives) formed a clade (PP = 0.97), sister to the clade of the Rhizeocidae species on the BI and ML trees (Figures S1 and S2). The pseudococcid clade on the BI and ML trees (Figures S1 and S2) consisted of the four major clades, including representative species of *Rastrococcus* (PP = 1.0, UFBoot = 100), *Heliococcus* (PP = 1.0, UFBoot = 100), other Phenacoccinae (poorly supported) and Pseudococcinae (PP = 1.0, UFBoot = 100). On the MP tree, the clades of *Rastrococcus* (MPBoot = 100), *Heliococcus* (MPBoot = 100) and Pseudococcinae (MPBoot = 78) also were supported. The Phenacoccinae species (minus species of *Heliococcus* and *Rastrococcus*) formed a clade with the Pseudococcinae species on the BI and ML trees (Figures S1 and S2). The *Rastrococcus* clade was placed outside the clade of Phenacoccinae + Pseudococcinae on the BI, ML and MP trees (Figures S1–S3). In the clade of *Rastrococcus*, *R. iceryoides* was sister to the other species.

Phenacoccinae (55 representatives) was non-monophyletic because the clades of *Heliococcus* (two representatives) and/or *Rastrococcus* (10 representatives) were separated from that of the remaining Pseudococcidae species on the BI, ML and MP trees (Figures S1–S3). Among the remaining Phenacoccinae species, each

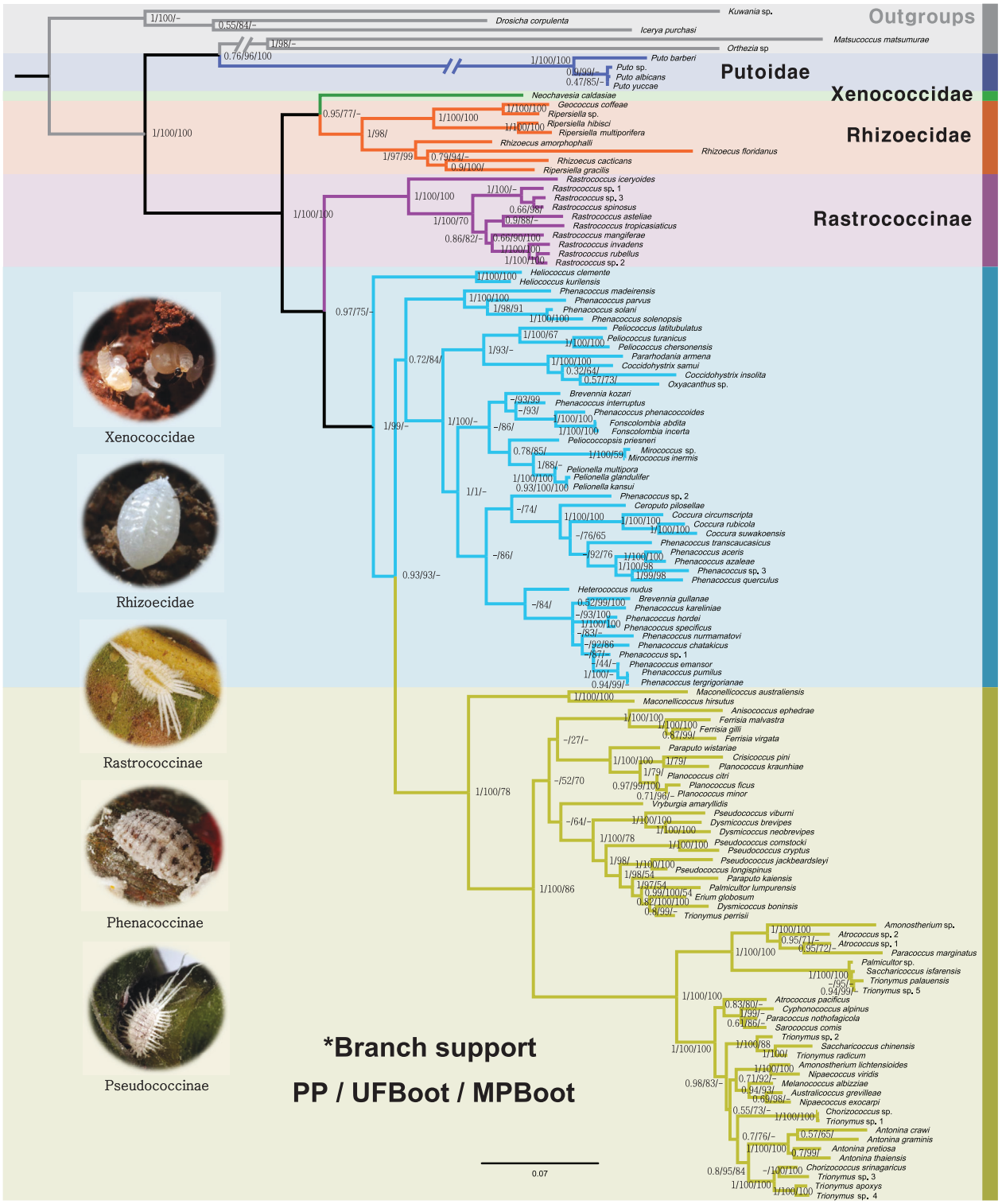

FIGURE 1 Molecular phylogeny of mealybugs (128 taxa) obtained from maximum likelihood (ML) in IQ-tree combined with support values from Bayesian inference (BI) in MRBAYES and maximum parsimony (MP) in PAUP (clades with different tree topologies in the BI and MP trees are indicated by '–'). A total of 3543 bp of concatenated DNA of *COI*, *18S*, *28S D2* and *D10*, *EF-1α 5′* and *3′*, *Dynamin*, and *wingless* was used for these analyses. Three numbers at each node represent support values: BI posterior probability (PP)/ML ultrafast bootstrap (UFBoot)/MP bootstrap (MPBoot). The affiliation of each clade is written on the right-hand side of the trees and is depicted by different colours according to the classification of mealybugs suggested in this study

representative of *Coccura* Šulc (three representatives, PP = 1.0, UFBoot = 100, MPBoot = 100), *Fonscolombia* Lichtenstein (two representatives, PP = 1.0, UFBoot = 100, MPBoot = 100), *Heliococcus* (two representatives, PP = 1.0, UFBoot = 100, MPBoot = 100), *Mirococcus* Borchsenius (two representatives, PP = 1.0, UFBoot = 100), *Peliococcus* Borchsenius (three representatives, PP = 1.0, UFBoot = 100) and *Pelionella* Kaydan (three representatives, PP = 1.0, UFBoot = 100, MPBoot = 100) formed a clade. However, *Brevennia* (Goux) (two representatives), *Coccidohystrix* (Lindinger) (two representatives) and *Phenacoccus* Cockerell (21 representatives) were non-monophyletic. At the basal node of the Phenacoccinae clade (minus species of *Heliococcus* and *Rastrococcus*), the clade, including *Phenacoccus madeirensis* Green, *Phenacoccus parvus* Morrison, *Phenacoccus solani* Ferris and *Phenacoccus solenopsis* Tinsely (PP = 1.0, UFBoot = 100, MPBoot = 100) was placed as sister to the remaining Phenacoccinae species on the BI, ML and MP trees (Figures S1–S3).

Pseudococcinae (55 representatives) formed a clade (PP = 1.0, UFBoot = 100, MPBoot = 78) on the BI, ML and MP trees (Figures S1–S3). In this clade, each representative of *Antonina* Signoret (four representatives, PP = 1.0, UFBoot = 100, MPBoot = 100), *Ferrisia* Fullaway (three representatives, PP = 1.0, UFBoot = 100, MPBoot = 100) and *Maconellicoccus* Ezzat (two representatives, PP = 1.0, UFBoot = 100, MPBoot = 100) formed a clade, whereas *Amonostherium* Morrison & Morrison (two representatives), *Atrococcus* Goux (two representatives), *Chorizococcus* McKenzie (two representatives), *Dysmicoccus* Ferris (three representatives), *Nipaecoccus* Šulc (two representatives), *Palmicultor* Williams (two representatives), *Paracoccus* Ezzat & McConnell (two representatives), *Paraputo* Laing (two representatives), *Planococcus* Ferris (four

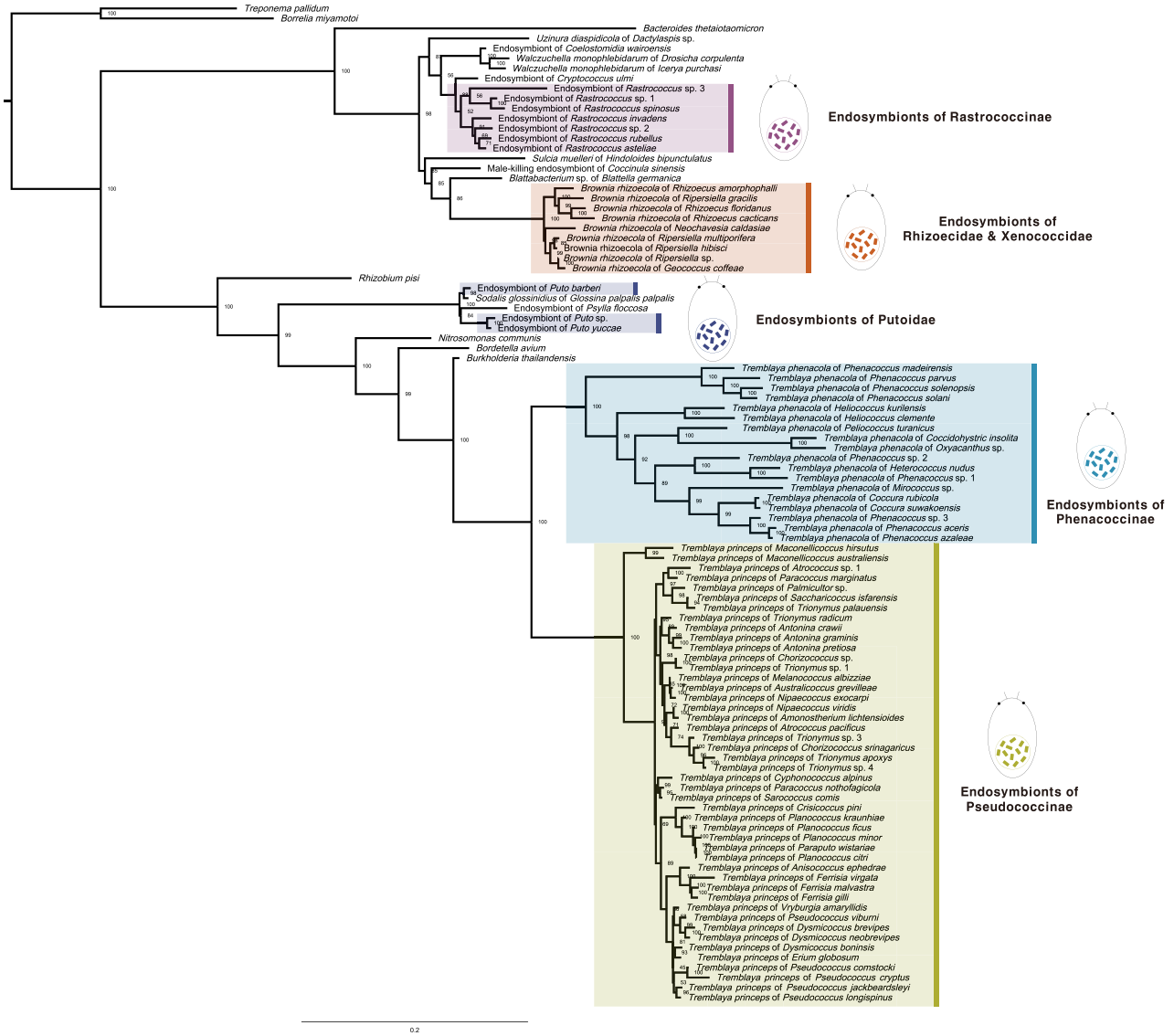

FIGURE 2 Phylogenetic tree of endosymbionts from 83 ingroup taxa of mealybugs and putoids, inferred from maximum likelihood (ML) analysis using IQ-tree. A total of 1229 bp of 16S rRNA sequences was used for this analysis. Endosymbionts of other organisms and free-living bacteria (17 sequences) were included for outgroups. ML ultrafast bootstrap (UFBoot) values are shown at each node

representatives), *Pseudococcus* Westwood (five representatives), *Saccharicoccus* Ferris (two representatives) and *Trionymus* (Berg) (eight representatives) were non-monophyletic. On the BI, ML and MP trees (Figures S1–S3), the *Maconellicoccus* clade (PP = 1.0, UFBoot = 100, MPBoot = 100) was placed as sister to the clade of the remaining Pseudococcinae species.

Obligate endosymbionts

In total, five lineages of obligate endosymbionts were found from 85 ingroup species (Table S5) based on 16S rRNA sequences that were obtained from this study (52 spp.) and previous studies (33 spp.) (Gruwell et al., 2010, 2014; Thao et al., 2002). Among them, the obligate endosymbionts of 36 species were newly determined in this study (see asterisks in Table S5). These endosymbionts belonged to either phylum Bacteroidetes (Flavobacteriia) or Proteobacteria (β-Proteobacteria or γ-Proteobacteria). The Pseudococcidae species (64 spp.) (minus species of *Rastrococcus*) showed two species of *Tremblaya* (β-Proteobacteria) as obligate endosymbionts: *T. phenacola* from the species of Phenacoccinae (18 spp.) and *T. princeps* from the species of Pseudococcinae (46 spp.). An undefined lineage of Flavobacteriia was detected from the species of *Rastrcococcus* (8 spp.). In the Rhizoecidae (8 spp.) and Xenococcidae species (1 spp.), their endosymbionts were identified as *Brownia rhizoecola* (Flavobacteriia). The endosymbionts of Putoidae species (4 spp.) were undefined lineages of γ-Proteobacteria. The obligate endosymbionts of 38 ingroup species were not be determined. Among the newly acquired samples of ingroups (63 spp.) in this study, we failed to get 16S rRNA sequences of obligate endosymbionts from 11 species (listed under Table S5).

The ML tree of obligate endosymbionts was reconstructed based on 1229 bp of 16S rRNA sequences from 100 samples of symbionts and free-living bacteria (Figure 2). The detected obligate endosymbionts of mealybugs were placed in five separate clades. The symbionts (γ-Proteobacteria) of *Puto* were non-monophyletic because a symbiont of *Puto barberi* (Cockerell) was separated from a clade of symbionts from other species of *Puto*. These symbionts of *Puto* were related to *Sodalis glossinidius* of a tsetse fly and a symbiont of a psyllid. *B. rhizoecola* of Rhizoecidae and Xenococcidae was monophyletic (UFBoot = 100), sister to *Blattabacterium* of a cockroach. The Flavobacteriia of *Rastrococcus* formed a clade but it was poorly supported (UFBoot = 52). This clade was sister to a symbiont of a felt scale. *Tremblaya* from the rest of Pseudococcidae species was monophyletic (UFBoot = 100), sister to a free-living bacteria (*Burkholderia thailandensis*). The clade of *Tremblaya* formed two main subclades for *T. phenacola* (UFBoot = 100) and *T. princeps* (UFBoot = 100).

DISCUSSION

The current subfamily classification of Pseudococcidae with a basal dichotomoy of Phenacoccinae and Pseudococcinae was well supported in this study based on molecular phylogenies of hosts and their obligate endosymbionts. This is consistent with the results of the previous phylogenetic studies (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008; Kaydan et al., 2015). However, the present molecular phylogeny implies that the families Putoidae, Rhizoecidae and Xenococcidae and the genus *Rastrococcus*, are distinct serial paraphyletic lineages that do not share a recent common ancestor. Among them, Rhizoecidae, Xenococcidae and *Rastrococcus* are inconsistent with the previous classifications and/or phylogenetic hypotheses. Each of these three groups (Putoidae, Rhizoecidae + Xenococcidae and *Rastrococcus*) showed a different lineage of obligate endosymbionts in this study, which also are different from those of Pseudococcidae species.

Rastrococcus

The separation of *Rastrococcus* from Phenacoccinae is well supported (Figure 1). The representatives of each group (including type species of the two Pseudococcidae subfamilies and *Rastrococcus*) formed separate monophyletic groups with high support values. The *Rastrococcus* clade was sister to a clade, including other Phenacoccinae and Pseudococcinae species (Figure 1). In the phylogenetic tree of Hardy, Gullan and Hodgson (2008) based on the combined dataset (molecular + morphology), a clade with *Rastrococcus* species was nested within the Phenacoccinae clade, which is inconsistent with the results in this study. Differences in topology might result from different compositions of sampled species and their sequence data for phylogenetic analyses, or effect of morphological data in the dataset of Hardy, Gullan, and Hodgson (2008).

In the molecular evidence from 16S rRNA, the species of *Rastrococcus* showed a lineage of obligate endosymbionts belonging to Flavobacteriia of Bacteroidetes, which is different from those of Pseudococcidae that belong to β-Proteobacteria (Figure 2) (Gruwell et al., 2010). In other words, *Rastrococcus* is a distinct lineage with an infection by a different endosymbiont from those of other Pseudococcidae. Buchner (1957) suggested that this genus should be considered a separate group because the endosymbionts of several *Rastrococcus* species were distinct from those of *Macrocerococcus* Leonardi (*Puto* of today) and *Phenacoccus*. This suggestion by Buchner (1957) was supported by Tremblay (1989), who found that their endosymbiotic structures had no resemblance to the types found in *Pseudococcus* or *Puto*. The diagnostic character states that *Rastrococcus* mostly shares with species of Phenacoccinae, including the presence of quinquelocular pores, antennae with nine segments, short conical or lanceolate dorsal setae and claw with a denticle (sometimes absent) (Williams, 2004b; Table 1). However, this group can be considered a separate subfamily having an apomorphic morphological feature if priority is given to a particular trait instead of assigning high value to their synapomorphic traits with Phenacoccinae.

The adult females of *Rastrococcus* have a peculiar formation of the cerarian setae that are likely to support lateral wax filaments (Cox, 1987). The structure and number of cerarii are taxonomically

TABLE 1 Diagnostic character states of *Rastrococcus* based on 31 described species and comparison with type species of Putoidae, Rhizoecidae, Xenococcidae and Pseudococcidae (Phenacoccinae and Pseudococcinae)

Species	Type of cerarian setae	Number of cerarian setae on anal lobe	Number of cerarii	Auxiliary setae on cerarii	Sizes of cerarian trilocular pores and those on the rest dosum	Antennal segments	Quinquelocular pores (no. of size)	Dorsal setae	Claw with a denticle	Reference
Rastrococcus adinandrae Williams	Truncated conical	8–11	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (2004b)
Rastrococcus asteliae (Maskell)	Truncated conical	7	16 pairs	Absent	Different	9	Present (one size)	Conical or lanceolate	Absent	Williams (1989)
Rastrococcus banksiae Williams	Truncated conical	14	17 pairs	Absent	Similar	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1985); Williams (1989)
Rastrococcus biggeri Williams & Watson	Truncated conical	18	17 pairs	Absent	Similar (uncertain)	9	Present (two sizes)	Conical or lanceolate	Unknown	Williams (1989)
Rastrococcus chinensis Ferris	Truncated conical	About 12	15 or 16 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Absent	Williams (1989); Williams (2004b)
Crisicoccus enterprise Williams	Truncated conical	15	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989); Williams (2004b)
Rastrococcus iceryoides (Green)	Truncated conical	22	17 pairs	Absent	Different	9	Present (one size)	Conical or lanceolate	Present	Williams (1989); Williams (2004b); this study
Rastrococcus invades Williams	Truncated conical	18	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989); Williams (2004b); this study
Rastrococcus jabadiu Williams	Truncated conical	About 12	17 pairs	Absent	Similar	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989); Williams (2004b)
Rastrococcus kendariensis Gavrilov-Zimin	Truncated conical	About 10	17 pairs	Absent	Different	9	Present (three sizes)	Conical or lanceolate	Present	Gavrilov - Zimin (2013)
Rastrococcus lamingtoniensis Williams	Truncated conical	About 20	17 pairs	Absent	Similar	9	Present (two sizes)	Conical or lanceolate	Unknown	Williams (1985); Williams (1989)
Rastrococcus mangiferae (Green)	Truncated conical	16	15 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Absent/ present	Williams (1989); Williams (2004b); this study
Rastrococcus matileae Williams & Watson	Truncated conical	10	14 pairs	Absent	Different	9	Present (one size)	Conical or lanceolate	Absent	Williams (1989)
Rastrococcus melaleucae Williams	Truncated conical	Fewer than 15	15 or 16 pairs	Absent	Similar	9	Present (one size)	Conical or lanceolate	Absent	Williams (1985); Williams (1989)
Rastrococcus monk Williams	Truncated conical	About 12	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989); Williams (2004b)
Rastrococcus namartini Williams & Henderson	Truncated conical	About 10	10 pairs	Absent	Different	9	Present (one size)	Conical or lanceolate	Absent	Williams and Henderson (2005)
Rastrococcus neoguineensis Williams & Watson	Truncated conical	About 15	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989)
Rastrococcus nepalicus Williams	Truncated conical	About 14	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (2004b)

(Continues)

TABLE 1 (Continued)

Species	Type of cerarian setae	Number of cerarian setae on anal lobe	Number of cerarii	Auxiliary setae on cerarii	Sizes of cerarian trilocular pores and those on the rest dosum	Antennal segments	Quinquelocular pores (no. of size)	Dorsal setae	Claw with a denticle	Reference
Rastrococcus nivalis (Maskell)	Truncated conical	About 10	17 pairs	Absent	Similar	9	Present (one size)	Conical or lanceolate	Present	Williams (1985); Williams (1989)
Rastrococcus rubellus Williams	Truncated conical	With 15–20	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989); Williams (2004b); this study
Rastrococcus spinosus (Robinson)	Truncated conical	10–12	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989); this study
Rastrococcus stolatus (Froggatt)	Truncated conical	9–17	15–17 pairs	Absent	Similar (uncertain)	9	Present (one size)	Conical or lanceolate	Present	Williams (1985); Williams (1989)
Rastrococcus taprobanicus Williams	Truncated conical	About 16	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989); Williams (2004b)
Rastrococcus tropicasiaticus Williams	Truncated conical	About 13	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (2004b); this study
Rastrococcus truncatispinus Williams	Truncated conical	About 20	17 pairs	Absent	Similar (uncertain)	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1985); Williams (1989)
Crisicoccus winding Williams & Watson	Truncated conical	About 9	17 pairs	Absent	Similar	9	Present (two sizes)	Conical or lanceolate	Unknown	Williams (1989); Williams (2004b)
Rastrococcus viridarii Williams	Truncated conical	About 11	17 pairs	Absent	Different	9	Present (two sizes)	Conical or lanceolate	Present	Williams (1989)
Rastrococcus wilsoni Williams	Truncated conical	About 15	17 pairs	Absent	Different	9	Present (one size)	Conical or lanceolate	Present	Williams (2004b)
Rastrococcus balinensis Buchner	—	—		—	—	—	—	—	—	Without morphological information. These species were named only based on the different structure of the endosymbionts from those of other *Rastrococcus* species (Williams, 2019).
Rastrococcus fransenii Buchner	—	—		—	—	—	—	—	—	
Rastrococcus pseudospinosus Buchner	—	—		—	—	—	—	—	—	
Phenacoccus aceris (Signoret) (Phenacoccinae)	Conical	2–6 (mostly 2 or 3)	18 pairs	Absent	Similar	9	Present (one size)	Conical or lanceolate	Present	Danzig & Gavrilov-Zimin (2014); this study
Pseudococcus longispinus (Targioni Tozzetti) (Pseudococcinae)	Conical	2	17 pairs	Present (3 or 4 on anal lobe cerarii)	Similar	8	Absent	Flagellate	Absent	McKenzie (1967)
Puto antennatus (Signoret) (Putoidae)	Conical	10	20 pairs	Absent	Similar	9	Absent	Conical or lanceolate	Present	Danzig & Gavrilov-Zimin (2014)
Rhizoecus falcifer Künckel d'Herculais (Rhizoecidae)	Entirely absent	—	Absent	—	(Cerarius absent)	5	Absent	Flagellate	Absent	McKenzie (1967); Kozár & Konczné Benedicty (2007)
Xenococcus annandalei Silvestri (Xenococcidae)	Entirely absent	—	Absent	—	(Cerarius absent)	4	Absent	Flagellate	Absent	Williams (1978)

significant for classifying the mealybugs at various levels (Danzig & Gavrilov-Zimin, 2014; Gavrilov-Zimin & Danzig, 2012; Williams et al. 2011). In general, *Rastrococcus* species have multiple truncated conical setae (ca. 10–20 setae) on each cerarius (in total 14–17 pairs, usually 17) accompanied by trilocular pores but without auxiliary setae (Williams, 1985, 1989, 2004b; Table 1). In contrast, the adult females of typical species of Phenacoccinae and Pseudococcinae have two or a few more 'conical' setae (usually 2) on each cerarius, the latter numbering at most 18 pairs (usually 17 or 18, but some species have more or less than this number) with accompanying trilocular pores and often with slender auxiliary setae (Cox, 1987; Danzig, 1986; Danzig & Gavrilov-Zimin, 2014; McKenzie, 1967; Williams, 1985, 2004b). Among the features of *Rastrococcus* that are associated with cerarii, the truncated conical shape of cerarian setae is an almost unique characteristic across Pseudococcidae and other families in Coccomorpha (except in *Ripersia leptospermi* Maskell [Eriococcidae]). In sort, it can be used as an apomorphic characteristic to support *Rastrococcus* as belonging to a separate subfamily. It is also worth mentioning that the size and structure of trilocular pores on cerarii are different from those on the rest of derm in most species of *Rastrococcus* (Williams, 1989). In particular, their cerarian trilocular pores are larger than normal dorsal trilocular pores (Table 1). On the other hand, trilocular pores on cerarii are typically similar in size to those on the remaining derm in Phenacoccinae and Pseudococcinae, although their dorsal and ventral pores occasionally are different in size (Williams, 2004b). Further study of their ultrastructure is needed to examine the exact appearance of trilocular pores of *Rastrococcus*. In studies using scanning electron microscopy, the wax-exuding loculi were different in structure among species of Phenacoleachiidae, Putoidae and Pseudococcidae (Cox, 1984; Foldi, 1983).

Based on morphology, *Rastrococcus* is also supported by a certain structure on the abdomen of their adult males, which is different from those of Phenacoccinae (Hodgson, 2020; Williams, 1989). The structure and number of glandular pouches have been considered important characteristics in adult male taxonomy of scale insects (Hodgson, 2020; Hodgson & Hardy, 2013). The adult male of the type species of *Rastrococcus*, *R. iceryoides*, is different from those of Phenacoccinae in having a single pair of glandular pouches on abdominal segment VIII. In constrast, the adult males of the Phenacoccinae possess two pairs of glandular pouches, one pair on segment VII and another on VIII. However, this difference between *Rastrococcus* and Phenacoccinae is not constant, because *R. invadens* and *R. vicorum* Williams & Watson each have two pairs of glandular pouches (Williams, 1989). Although this characteristic can separate the type species of *Rastrococcus* from Phenacoccinae, it cannot distinguish *R. iceryoides* from Pseudococcinae because the adult males of the latter have either no glandular pouches or only a single pair on segment VIII (Hodgson, 2020).

Based on the molecular and morphological evidence plus the type of obligate endosymbionts, *Rastrococcus* is excluded from Phenacoccinae and elevated to the subfamily *Rastrococcinae* **subfam. nov**.

Rhizoecidae and Xenococcidae

Our molecular phylogeny (Figure 1) placed the Rhizoecidae + Xenococcidae clade as sister to Rastrococcinae **subfam. nov.** + Phenacoccinae + Pseudococcinae. This result is largely congruent with those of Hodgson (2002) and Downie and Gullan (2004). The obligate endosymbionts of Rhizoecidae and Xenococcidae are different from those of Phenacoccinae and Pseudococcinae (Figure 2). Although there has been no morphological study on their endosymbiotic systems, Gruwell et al. (2010) found that the obligate endosymbionts of Rhizoecidae and Xenococcidae (as Rhizoecini) belonged to Flavobacteriia and named the lineage *B. rhizoecola* based on the 16S rRNA sequences from six species. This endosymbiont group was sister to *Blattabacterium* (endosymbionts of cockroach) rather than to those of *Rastrococcus* on the phylogenetic tree of endosymbionts (Figure 2) (Gruwell et al., 2010). Although preliminary, these results imply that Rhizoecidae + Xenococcidae is an independent lineage infected by a different endosymbiont from those in Pseudococcidae.

Historically, the morphology of rhizoecine mealybugs was considered to be the 'primitive' form of mealybugs. Koteja (1974b) mentioned that the adult females of Rhizoecinae have the primitive shape of labium (narrow, longer than wide) among the other Pseudococcidae (wide, length as long as wide). After examination of the adult males of the type species, *Rhizoecus falcifer* Künckel d'Herculais, Beardsley (1962) concluded that *Rhizoecus* is likely to show the most primitive shape of mealybugs, lacking a cervical constriction between the head and thorax. The morphological differences in the adult males between Rhizoecidae (Rhizoecinae and Xenococcinae) and Pseudococcidae (Phenacoccinae and Pseudococcinae) were emphasized by Hodgson (2012). In addition, the morphological diagnoses for the adult females of Pseudococcidae and Rhizoecidae were provided in Hodgson (2012), although these might have been inferred from previous literature. In the key to the subfamilies by Kozár and Konczné Benedicty (2007), the morphological separation of the adult females of Pseudococcidae and Rhizoecidae also was presented based on examination of extensive species of rhizoecine females.

In order to obtain even stronger evidence in support of the Rhizoecidae and Xenococcidae, additional species and, in particular, type species should be included in the molecular analyses and the morphological analyses for males. Especially, the suggested family Xenococcidae needs to be reviewed after further taxon sampling because the type species of *Neochavesia* Williams, *N. caldasiae*, was only used in the phylogenetic analyses (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008; this study). Additional sampling of Xenococcidae may show it to be part of Rhizoecidae because Hodgson (2020) noted that it seems likely to be non-monophyletic. The position of *N. caldasiae*, sister to species of *Rastrococcus*, on the MP tree might be an artefact of the phylogenetic analysis because the other results (Figures 1 and 2) implicated a close relationship with Rhizoecidae. Here, we tentatively support the families Rhizoecidae and Xenococcidae

based on the molecular and morphological evidence plus the possession of a particular/different type of endosymbionts. Currently, the Rhizoecidae and Xenococcidae include 16 genera and 3 genera, respectively (Table 2).

Putoidae

Our phylogeny (Figure 1) recovered the clade of *Puto* species placed outside Xenococcidae + Rhizoecidae + Rastrococcinae **subfam. nov.** + Phenacoccinae + Pseudococcinae. In most previous phylogenetic analyses, Putoidae consistently formed a separate clade outside the main cluster of Pseudococcidae (Cook et al., 2002; Downie & Gullan, 2004; Gullan & Cook, 2007; Hodgson, 2002; Hodgson & Hardy, 2013; Vea & Grimaldi, 2016). Here, the type species of *Ceroputo* Šulc, *C. pilosellae* Šulc, was placed within Phenacoccinae instead of Putoidae and supports the transfer of *Ceroputo* to Phenacoccinae from Putoidae (Hardy, Gullan, & Hodgson, 2008; Williams et al. 2011).

The lineages of endosymbionts from the putoid species differ from those of Pseudococcidae (Figure 2). The 16S rRNA sequences from the four species of *Puto* were determined as γ-Proteobacteria (Gruwell et al., 2010, 2014). Buchner (1965) mentioned that *Puto* and *Macrocerococcus* (currently synonymized with *Puto*) have greatly divergent endosymbionts from those of other Pseudococcidae, which was supported by Tremblay (1989). It was hard to say that these are obligate endosymbionts of *Puto* because they did not form a monophyletic group (Figure 2) and limited *Puto* species were investigated. The lineages of endosymbionts from other Pseudococcidae (Flavobacteriia and β-Proteobacteria, named as *T. phenacola* and *T. princeps*), have never been detected in *Puto* species. In contrast, *T. phenacola* was detected from *C. pilosellae* (Michalik et al., 2019), which implied that *Ceroputo* was part of Phenacoccinae. These results suggest that Pseudococcidae and Putoidae are independent lineages with different endosymbionts.

The status of Putoidae (separated from *Ceroputo* and *Phenacoccus*) was well reviewed by Williams et al. (2011) based on the comparison of the morphology of most *Puto* species with those of *Ceroputo* and *Phenacoccus*. This study also supported Putoidae being a separate family from the genus *Ceroputo* and other Pseudococcidae based on the molecular and morphological evidence plus the possession of a particular/different type of endosymbionts. Currently, the Putoidae includes two genera (Table 2).

Phenacoccinae

We recovered Phenacoccinae (minus species of *Heliococcus* and *Rastrococcus*) as monophyletic in all the analyses, although support values for the clade were relatively low. This might result from the inclusion of some phenacoccine taxa that had many missing gene regions. The separation of *Heliococcus* from the Phenacoccinae was unclear because their representatives were clustered with other species of Phenacoccinae on the MP tree (Figure S3). In addition, endosymbionts of *Heliococcus* species formed a clade with those of other Phenacoccinae species (Figure 2). Previous phylogenetic studies highly supported the Phenacoccinae having a sister relationship with Pseudococcinae (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008; Kaydan et al., 2015) (although Rhizoecinae were included within Phenacoccinae clade in the tree of Hardy, Gullan, and Hodgson (2008)). These constant phylogenetic results suggest that Phenacoccinae is an independent evolutionary sub-lineage in Pseudococcidae, sharing a common ancestor with Pseudococcinae.

The endosymbionts of Phenacoccinae were determined by Gruwell et al. (2010) as a lineage of β-Proteobacteria and identified as *T. phenacola* based on 16S rRNA sequences. In our study, *T. phenacola* was constantly detected from 11 species of Phenacoccinae, thus supporting the previous results (Table S5). *Phenacoccus* (type genus of Phenacoccinae) has an unpaired mycetome, and their endosymbionts are in direct contact with the cytoplasma of the bacteriocyte due to the lack of mucous spherules (Buchner, 1965; Tremblay, 1989).

Within Phenacoccinae, there is no formal classification except for some subgroupings suggested by previous authors (Danzig & Gavrilov-Zimin, 2014, 2015; McKenzie, 1967; Williams & Gullan, 2010). At the generic level, this subfamily possibly includes 57 genera (Table 2). Hardy, Gullan, and Hodgson (2008) placed 69 genera in this subfamily, with 16 genera transferred from Pseudococcidae to Rhizoecidae or Xenococcidae (Hodgson, 2012). In our study, two of the genera were newly assigned to Phenacoccinae based on morphological traits of their adult females or males (see Table 2). In addition, *Rastrococcus* was excluded in this study (transferred to Rastrococcinae **subfam. nov.**). Kaydan et al. (2015) stated that *Artemicoccus* Balachowsky, *Heterobrevennia* Kaydan and possibly *Euripersia* Borchsenius could be included in Phenacoccinae. Among the genera of Phenacoccinae, 18 were considered synonyms of other existing genera in Phenacoccinae or Pseudococcinae (Danzig, 2001, 2007; Danzig & Gavrilov-Zimin, 2012, 2014; Koçak & Kemal, 2009) (see Table 2).

In this study, we observed a clear acetabuliform ovisac of *Phenacoccus rubicola* Kwon, Danzig & Park, which is accordant with one of the major characteristics of *Coccura*. Other morphological characteristics of *P. rubicola* are similar to those of *Coccura*, especially having numerous tubular ducts around body margin (Kwon et al., 2003). In the phylogenetic analyses, *P. rubicola* formed a clade with other *Coccura* species (Figure 1). Consequently, *P. rubicola* is transferred to *Coccura*.

Pseudococcinae

In all previous phylogenetic studies (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008; Kaydan et al., 2015) and the present analyses (Figure 1), Pseudococcinae is supported as a monophyletic group sister to Phenacoccinae. Based on 16S rRNA sequences, *T. princeps* (β-Proteobacteria) was constantly detected from the extended samples of Pseudococcinae (Thao et al., 2002). Here, *T. princeps* was also

TABLE 2 Generic composition of Pseudococcidae (Phenacoccinae, Pseudococcinae and Rastrococcinae **subfam. nov.**), Rhizoecidae, Xenococcidae and Putoidae

Family (subfamily)	Genus
Pseudococcidae (Phenacoccinae)	57 genera: *Annulococcus* James; *Antoninella* Kiritchenko; *Artemicoccus* Balachowsky[a]; *Asphodelococcus* Morrison[a]; *Australiputo* Williams[a]; *Bessenayla* Goux[a]; *Boreococcus* Danzig; *Bouhelia* Balachowsky[a]; *Calyptococcus* Borchsenius[a]; *Ceroputo* Šulc; *Coccidohystrix* Lindinger; *Coccura* Šulc; *Cucullococcus* Ferris; *Dawa* Williams; *Eastia* De Lotto; *Erimococcus* Ezzat; *Eriocorys* De Lotto; *Euripersia* Borchsenius[a]; *Giraudia* Goux[a]; *Heliococcus* Šulc; *Heterobrevennia* Kaydan[a]; *Heterococcopsis* Borchsenius[a]; *Heterococcus* Ferris; *Lacombia* Goux; *Laingiococcus* Morrison; *Lankacoccus* Williams; *Longicoccus* Danzig[a]; *Madacanthococcus* Mamet; *Malekoccus* Matile-Ferrero; *Mammicoccus* Balachowsky; *Mirococcus* Borchsenius; *Oxyacanthus* De Lotto; *Paramococcus* Foldi & Cox; *Parapedronia* Balachowsky[a]; *Pararhodania* Ter-Grigorian; *Peliococcopsis* Borchsenius; *Peliococcus* Borchsenius; *Pelionella* Kaydan[b]; *Pellizzaricoccus* Kozár[a]; *Perystrix* Gavrilov; *Phenacoccus* Cockerell; *Polystomophora* Borchsenius[a]; *Prorhizoecus* Miller & McKenzie; *Pseudorhodania* Borchsenius[a]; *Rhodania* Goux; *Ritsemia* Lichtenstein; *Scaptococcus* McKenzie; *Seabrina* Neves[a]; *Seyneria* Goux; *Sinococcus* Wu & Zheng; *Spinococcus* Borchsenius[a]; *Stachycoccus* Borchsenius[a]; *Stemmatomerinx* Ferris; *Stipacoccus* Tang; *Synacanthococcus* Morrison; *Trimerococcus* Balachowsky; *Williamsicoccus* Vea & Grimaldi (fossil)[b]
Pseudococcidae (Pseudococcinae)	211 genera: *Acaciacoccus* Williams & Matile-Ferrero; *Acinicoccus* Williams; *Acrochordonus* Cox; *Adelosoma* Borchsenius; *Aemulantonina* Williams; *Agastococcus* Cox; *Albertinia* De Lotto; *Allomyrmococcus* Takahashi; *Allotrionymus* Takahashi[b]; *Amonostherium* Morrison & Morrison; *Anaparaputo* Borchsenius[a]; *Anisococcus* Ferris; *Antonina* Signoret; *Antoninoides* Ferris; *Apodastococcus* Williams; *Archeomyrmococcus* Williams; *Asaphococcus* Cox; *Asteliacoccus* Williams; *Atriplicicoccus* Williams & Granara de Willink; *Atrococcus* Goux; *Australicoccus* Williams; *Balanococcus* Williams; *Bimillenia* Matile-Ferrero & Ben-Dov; *Bolbococcus* Williams; *Boninococcus* Kawai; *Borneococcus* Williams; *Brasiliputo* Williams & Granara de Willink; *Brevennia* Goux; *Bromusicoccus* Kaydan[b]; *Callitricoccus* Williams; *Cannococcus* Borchsenius; *Chaetococcus* Maskell; *Chaetotrionymus* Williams; *Chileputo* Williams & Granara de Willink; *Chloeoon* Anderson; *Chlorococcus* Beardsley; *Chnaurococcus* Ferris; *Chorizococcus* McKenzie; *Chryseococcus* Cox; *Cintococcus* Goux[a]; *Circaputo* McKenzie; *Cirnecoccus* Mamet; *Clavicoccus* Ferris; *Coleococcus* Borchsenius; *Colombiacoccus* Williams & Granara de Willink; *Conicosoma* De Lotto; *Conulicoccus* Williams; *Coorongia* Williams; *Cormiococcus* Williams; *Crenicoccus* Williams; *Criniticoccus* Williams; *Crisicoccus* Ferris; *Crocydococcus* Cox; *Cryptoripersia* Cockerell's; *Cyperia* De Lotto; *Cypericoccus* Williams; *Cyphonococcus* Cox; *Delococcus* Ferris; *Delottococcus* Cox & Ben-Dov; *Dicranococcus* Williams; *Discococcus* Ferris; *Distichlicoccus* Ferris; *Diversicrus* De Lotto; *Dorsoceraricoccus* Dong & Wu[b]; *Doryphorococcus* Williams; *Drymococcus* Borchsenius[a]; *Dysmicoccus* Ferris; *Epicoccus* Cockerell; *Erioides* Green; *Erium* Cockerell; *Eucalyptococcus* Williams; *Eurycoccus* Ferris; *Exallomochlus* Williams; *Exilipedronia* Williams; *Extanticoccus* Williams; *Farinococcus* Morrison; *Ferrisia* Fullaway; *Fijicoccus* Williams & Watson; *Fonscolombia* Lichtenstein; *Formicococcus* Takahashi; *Gallulacoccus* Beardsley; *Glycycnyza* Danzig; *Grewiacoccus* Brain; *Hadrococcus* Williams; *Hippeococcus* Reyne; *Hopefoldia* Foldi; *Hordeolicoccus* Williams; *Humococcus* Ferris; *Hypogeococcus* Rau; *Iberococcus* Gómez-Menor Ortega; *Idiococcus* Takahashi & Kanda; *Inopicoccus* Danzig; *Ityococcus* Williams; *Kalaginella* Danzig & Gavrilov-Zamin[b]; *Kenmorea* Williams; *Kermicus* Newstead; *Kiritshenkella* Borchsenius; *Komodesia* Gavrilov-Zimin[b]; *Lachnodiella* Hempel; *Laminicoccus* Williams; *Lanceacoccus* Williams; *Lantanacoccus* Williams & Granara de Willink; *Lenania* De Lotto; *Leptococcus* Reyne; *Lomatococcus* Borchsenius[a]; *Londonia* De Lotto; *Maconellicoccus* Ezzat; *Macrococcus* Morrison; *Maculicoccus* Williams; *Madagasia* Mamet; *Madangiacoccus* Williams; *Madeurycoccus* Mamet; *Malaicoccus* Takahashi; *Mascarenococcus* Mamet; *Maskellococcus* Cox; *Mauricoccus* Mamet; *Melanococcus* Williams; *Metadenopsis* Matesova[b]; *Metadenopus* Šulc; *Miconicoccus* Williams & Miller; *Mirococcopsis* Borchsenius; *Miscanthicoccus* Takahashi; *Misericoccus* Ferris; *Mizococcus* Takahashi; *Mollicoccus* Williams; *Moystonia* Williams; *Mutabilicoccus* Williams; *Natalensia* Brain; *Neoclavicoccus* Cohic; *Neoripersia* Kanda; *Neosimmondsia* Laing; *Neotrionymus* Borchsenius; *Nesococcus* Ehrhorn; *Nesopedronia* Beardsley; *Nesticoccus* Tang; *Nipaecoccus* Šulc; *Nudicauda* Gavrilov; *Octococcus* Hall; *Odacoccus* Williams & Watson; *Ohiacoccus* Beardsley; *Oracella* Ferris; *Orbuspedum* Gavrilov-Zimin[b]; *Orococcus* De Lotto; *Orstomicoccus* Mamet; *Palaucoccus* Beardsley; *Palmicultor* Williams; *Paludicoccus* Ferris; *Pandanicola* Beardsley; *Papuacoccus* Williams & Watson; *Paracoccus* Ezzat & McConnell; *Paradiscococcus* Williams; *Paradoxococcus* McKenzie; *Paraferrisia* Williams & de Boer; *Paramonostherium* Williams; *Paramyrmococcus* Takahashi; *Parapaludicoccus* Mamet; *Paraporisaccus* Lu & Wu[b]; *Paraputo* Laing; *Paraserrolecanium* Wu[b]; *Paratrionymus* Borchsenius; *Paulianodes* Mamet; *Pedrococcus* Mamet; *Pedronia* Green; *Penthococcus* Danzig[a]; *Peridiococcus* Williams; *Phyllococcus* Ehrhorn; *Pilococcus* Takahashi; *Planococcus* Ferris; *Pleistocerarius* Matile-Ferrero; *Poecilococcus* Brookes; *Porisaccus* Hendricks & Kosztarab; *Porococcus* Cockerell; *Promyrmococcus* Williams; *Prorsococcus* Williams; *Pseudantonina* Green; *Pseudococcus* Westwood; *Pseudoferrisia* Kaydan & Gullan[b]; *Pseudoripersia* Cockerell; *Pseudotrionymus* Beardsley; *Quadrigallicoccus* Williams & Miller; *Renicaula* Cox; *Rosebankia* De Lotto; *Saccharicoccus* Ferris; *Sarococcus* Williams & de Boer; *Serrolecanium* Shinji; *Spartinacoccus* Kosztarab; *Sphaerococcus* Maskell; *Spilococcus* Ferris; *Strandanna* De Lotto; *Stricklandina* Matile-Ferrero & Le Ruyet; *Strombococcus* Williams; *Syrmococcus* Ferris; *Tangicoccus* Kozár & Walter; *Tasmanicoccus* Williams; *Telocorys* De Lotto; *Thaimyrmococcus* Williams; *Tibetococcus* Tang; *Tomentocera* Beardsley; *Trabutina* Marchal; *Tridiscus* Ferris; *Trionymus* Berg; *Trochiscococcus* Williams & Pellizzari; *Turbinococcus* Beardsley; *Tylococcus* Newstead; *Tympanococcus* Williams; *Ventrispina* Williams; *Villosicoccus* Williams; *Volvicoccus* Goux; *Vryburgia* De Lotto; *Wapoacoccus* Ben-Dov[b]; *Yudnapinna* Williams

(Continues)

TABLE 2 (Continued)

Family (subfamily)	Genus
Pseudococcidae (Rastrococcinae **subfam. nov.**)	1 genus: *Rastrococcus* Ferris
Pseudococcidae *Incertae sedis*	10 genera: *Anthelococcus* McKenzie; *Archanginella* Danzig & Gavrilov-Zamin; *Ehrhornia* Ferris; *Eupeliococcus* Savescu[a]; *Gomezmenoricoccus* Kozár & Walter; *Marendellea* De Lotto; *Marmyan* Koteja (fossil); *Mombasinia* De Lotto; *Nairobia* De Lotto; *Ripersia* Signoret
Rhizoecidae	16 genera: *Benedictycoccina* Kozár & Foldi; *Brevicoccus* Hambleton; *Capitisetella* Hambleton; *Coccidella* Hambleton; *Electromyrmococcus* Williams; *Geococcus* Green; *Hambletonrhizoecus* Kozár & Konczné Benedicty; *Ishigakicoccus* Tanaka; *Kissrhizoecus* Kozár & Konczné Benedicty; *Leptorhizoecus* Williams; *Marottarhizoecus* Kozaár & Konczne Benedicty; *Pseudorhizoecus* Green; *Pygmaeococcus* McKenzie; *Rhizoecus* Kunckel d'Herculais; *Ripersiella* Tinsley; *Williamsrhizoecus* Kozár & Konczné Benedicty
Xenococcidae	3 genera: *Eumyrmococcus* Silvestri; *Neochavesia* Williams & Granara de Willink; *Xenococcus* Silvestri
Putoidae	2 genera: *Palaeotupo* Koteja & Azar (fossil); *Puto* Signoret

[a]Considered synonyms of other genera.
[b]Newly assigned in this study.

constantly detected from 31 species of Pseudococinae (Table S5). In contrast to Phenacoccinae, the endosymbionts of *Pseudococcus* and related genera are not directly in contact with the cytoplasma because they are embedded in mucous spherules (Buchner, 1965; Tremblay, 1989). In particular, the endosymbionts of Pseudococcinae show a 'nested symbiotic system' in which several lineages of γ-Proteobacteria live symbiotically inside β-Proteobacteria, named *T. princeps* (McCutcheon & von Dohlen, 2011; von Dohlen et al., 2001).

Within Pseudococcinae, a stable classification has not been established, however, three tribes (Planococcini, Pseudococcini and Trabutinini) have been constantly supported in phylogenetic studies (Downie & Gullan, 2004; Hardy, Gullan, & Hodgson, 2008; Kaydan et al., 2015). This subfamily possibly includes 211 genera (Table 2). After Hardy, Gullan, and Hodgson (2008) placed 201 genera in this subfamily, *Euripersia* was transferred from Pseudococcinae to Phenacoccinae (Kaydan et al., 2015). Here, 11 genera including three genera that were omitted in the list of Hardy, Gullan, and Hodgson (2008) are newly assigned to Pseudococcinae based on morphological traits of their adult females (Table 2). Among the genera of Pseudococcinae, five were considered synonyms of other genera in Phenacoccinae or Pseudococcinae (Borchsenius, 1949; Danzig & Gavrilov-Zimin, 2015) (see Table 2).

Proposed classification

Subfamily Rastrococcinae ***subfam. nov***.

Type genus: *Rastrococcus* Ferris, 1954: 55.

Diagnostic characteristics of adult females. (i) cerarian setae truncated conical in shape, about 7–20 setae on each anal lobe; (ii) cerarii 10–17 pairs in number (mostly 17 pairs); (iii) cerarian trilocular pores larger than those on the rest of derm except for some species; (iv) antenna 9-segmented; (v) quinquelocular pores present, with one or two sizes; (vi) dorsal setae conical or lanceolate; (vii) claw with a denticle but sometimes absent.

Remarks. This subfamily is monotypic. The obligate endosymbionts of this group belong to a lineage of Flavobacteriia (Bacteroidetes).

Key to families/subfamilies of mealybugs based on morphology (adult stage) and biological traits of females

1. Body generally more than 1.5 mm long; cerarii at least 1 pair present (rarely entirely absent); dorsal anterior and posterior ostioles usually present or represented by posterior pair only; antennae rarely less than 6-segmented; legs present or absent; if legs present, claw often stout, with or without a denticle; bitubular and tritubular ducts absent; frontal sclerotised plate absent; internal genitalia genital chamber) not very distinct, usually membranous or lightly sclerotised; rarely hypogaeic, living on various parts of plant (e.g. leaves, stems, twigs, crowns and roots).....2

- Body smaller, usually less than 1.5 mm long; cerarii entirely absent or represented by anal lobes with long flagellate setae; dorsal anterior and posterior ostioles sometimes entirely absent; antennae with 6 or fewer segments; legs present; claw slender and needle-like, without a denticle; bitubular or tritubular ducts present in many species; frontal sclerotised plate often present; internal genitalia often well sclerotised and obvious; hypogaeic, typically living on plant roots.....4

2. Cerarii, if present, with cerarian setae conical in shape, typically with 2 setae on each anal lobe, non-anal lobe cerarii usually without a sclerotised plate; cerarii with or without auxiliary setae; cerarian trilocular pores of similar size to those on rest of derm; quinquelocular pores, if present, only of one size.....3

- Cerarian setae truncated conical in shape, generally located on a sclerotised plate, each anal lobe usually with about 7–22 setae; cerarii without auxiliary setae; cerarian trilocular pores usually larger than those on rest of derm (but sometimes similar in size); quinquelocular pores of 2 sizes usually present..... Rastrococcinae **subfam. nov.**

3. Legs present; claw usually with a denticle; tarsal digitules setose; antennae mostly 9-segmented (occasionally 6–8 segmented);

dorsal setae usually conical; quinquelocular pores present or absent……Phenacoccinae

- Legs rarely reduced or entirely absent; claw without a denticle, if present, poorly developed near claw apex; tarsal digitules usually knobbed; antennae with fewer than 9 segments, usually 6–8 segmented (rarely 1–4 segmented); dorsal setae usually flagellate; quinquelocular pores entirely absent……Pseudococcinae

4. Body usually elongate-oval or broadly oval, without head and thorax dilated (except *Leptorhizoecus* Williams); ostioles and wax pores/ducts usually present; circuli internally flat or slightly bulbous; eyes sometimes present; nymphal stages of female normally without a pupal stage……Rhizoecidae

- Body usually with head and thorax dilated; ostioles and wax pores/ducts absent (except *Neochavesia caldasiae* (Balachowsky)); circuli internally cylindrical or cup-shaped at the centre of the distal end; eyes absent; nymphal stages of female usually including a pupal stage in the third-instar (with a cuticle sac enclosing the female before final moult)……Xenococcidae

Note. The diagnostic character states of Phenacoccinae, Pseudococcinae, Rhizoecidae and Xenococcidae used in this key followed Williams (2004a, 2004b), Kozár & Konczné Benedicty (2007), Hardy, Gullan, and Hodgson (2008), Schneider & LaPolla (2011) and Hodgson (2012). The diagnostic features of Rastrococcinae **subfam. nov**. were chosen among character states in Table 1.

CONCLUSION

We show that the higher classification of mealybugs includes the three families Rhizoecidae, Xenococcidae and Pseudococcidae, the latter divided into three subfamilies Phenacoccinae, Pseudococcinae and Rastrococcinae **subfam. nov.** Except for Xenococcidae, the present molecular analyses of mealybugs and their endosymbionts support the monophyly of the major lineages. Some internal nodes on the phylogenetic tree were poorly supported. In order to develop the mealybug phylogeny, further study is needed using more extensive DNA sequences from a number of genes and additional samples.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at http://www.ncbi.nlm.nih.gov, reference numbers available in supplementary Tables S1, S5 and S6

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1. Molecular phylogenetic tree (BI) of mealybugs (128 taxa) obtained from Bayesian inference analysis in MRBAYES. Numbers close to each node are the values of posterior probability (PP).

Figure S2. Molecular phylogenetic tree (ML) of mealybugs (128 taxa) obtained from maximum likelihood analysis in IQ-tree. Numbers close to each node are the values of bootstrap (UFBoot).

Figure S3. Molecular phylogenetic tree (MP) of mealybugs (128 taxa) obtained from maximum parsimony analysis in PAUP. The strict consensus tree of 6720 equally most parsimonious trees (10,287 steps) is presented. Numbers close to each node are the values of bootstrap (MPBoot).

Table S1. Taxa used in this study with GenBank accession numbers.

Table S2. Primers used in this study.

Table S3. Optimal partition scheme and best-fitting models selected by PartitionFinder2 and ModelFinder for molecular analyses.

Table S4. Primers for 16S rRNA gene sequences of endosymbionts.

Table S5. Endosymbionts detected from 85 ingroup taxa with GenBank accession numbers (*, endosymbionts newly determined in this study).

Table S6. Outgroups used for molecular analysis of endosymbionts with GenBank accession numbers.

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