Three Novel Entomopathogenic Fungi From China and Thailand

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Entomopathogenic fungi are ubiquitous in tropical rainforests and feature a high level of diversity. This group of fungi not only has important ecological value but also medicinal value. Nevertheless, they are often ignored, and many unknown species have yet to be discovered and described. The present study aims to contribute to the taxonomical and phylogenetic understanding of the genus Paraisaria by describing three new species collected from Guizhou and Yunnan Provinces in China and Krabi Province in Thailand. The three novel species named Paraisaria alba, P. arcta, and P. rosea share similar morphologies as those in the genus Paraisaria, containing solitary, simple, fleshy stroma, completely immersed perithecia and cylindrical asci with thickened caps and filiform ascospores that often disarticulate at maturity. Phylogenetic analyses of combined LSU, SSU, TEF1-α, RPB1, RPB2, and ITS sequence data confirm their placement in the genus Paraisaria. In this study, the three entomopathogenic taxa are comprehensively described with color photographs and phylogenetic analyses. A synopsis table and a key to all treated species of Paraisaria are also included.

Keywords: Insect fungi, Ophiocordycipitaceae, Paraisaria alba, Paraisaria arcta, Paraisaria rosea, taxonomy, Yunnan Province

INTRODUCTION

Entomopathogenic fungi are a group of unicellular or multicellular, heterotrophic, eukaryotic microorganisms that can enter into a parasitic relationship with parasitized insects, killing or otherwise disabling their hosts (Samson et al., 1988). They reproduce via sexual or asexual spores, or both (Mora et al., 2017). It is of global importance to survey and describe insect pathogens (Hyde et al., 2019). Entomopathogenic fungi can act as natural enemies of agricultural pests and
play an important role in maintaining ecological balance (Fernández-Grandon et al., 2020; Sobczak et al., 2020). For example, fungal pathogens such as, *Coeolomomyces*, *Culicinomyces*, and *Lagenidium* have the capacity to kill larvae and adult mosquitoes, reducing their host population (Scholte et al., 2004). Some entomopathogenic fungi, e.g., *Beauveria bassiana*, *Beauveria brongniartii*, *Metarhizium anisopliae*, and *Verticillium lecanii*, have been developed as biocontrol agents usable against agricultural pests like aphids, locusts, grasshoppers and cockchafer in Africa and Europe (Roberts and Hajek, 1992; Shah and Pell, 2003). *Beauveria bassiana* and *B. brongniartii* were found to be especially safe bioinsecticides (Zimmermann, 2007). Additionally, some insect pathogens with pharmacological activities are frequently studied, such as *Cordyceps militaris* extract, which exhibits antitumor properties (Li et al., 2020). *Cordyceps* spp. have been utilized as therapeutic agents for metabolic-related disorders (Cao et al., 2020). *Cordyceps cicadae* has renoprotective effects on hypertensive renal injuries (Huang et al., 2020). Entomopathogenic fungi have important biotechnological applications (Hyde et al., 2019) and *Paraisaria* is no exception. Several studies have explored the importance of *Paraisaria* species, such as their antioxidative activity (Ma et al., 2012), nucleoside components (Suo et al., 2013), intracellular polysaccharide composition (Wang et al., 2019) and AGS gastric cancer cells anti-proliferation effects (Ye et al., 2015). Additionally, *P. heteropoda* reportedly produces anti-bacterial and anti-fungal compounds (Krasnoff et al., 2005). Experiments into optimal cultural conditions and nutritional sources were conducted by Sung et al. (2011). Applications of other species in this genus have been poorly studied.

Entomopathogenic fungi are phylogenetically diverse and taxonomically distributed in Ascomycota, Basidiomycota, Chytridiomycota, Entomophthoromycota, Microsporidia, Oomycota and Zygomycota (Vega et al., 2012; Araújo and Hughes, 2016; Mora et al., 2017). Different groups of entomopathogens usually develop respectively unique strategy to colonize their hosts (Mora et al., 2017). It is worth to mention that entomopathogenic taxa in Entomophthorales (Entomophthoromycota) enter into biotrophic relationships with their insect hosts, while those in Hypocreales (Ascomycota) can be hemibiotrophic at earlier stages and transform into saprophytism (Shah and Pell, 2003). The diversity, taxonomy and phylogeny of entomopathogenic fungi have been extensively studied recently (Aung et al., 2008; Mora et al., 2017; Hyde et al., 2018). Most insect pathogens are known from three families: Clavicipitaceae, Cordycipitaceae, and Ophiocordyptaceae. They are found in the Hypocreales, Hypocreomycetidae, Sordariomycetes, Ascomycota (Sung et al., 2007a; Maharachchikumbura et al., 2016; Wijayarwadene et al., 2018). The generic composition of Ophiocordyptaceae underwent several changes over time (Sung et al., 2007a; Quandt et al., 2014; Maharachchikumbura et al., 2016; Shrestha et al., 2017; Wijayarwadene et al., 2018), and currently ten genera are accepted (Hyde et al., 2020). New combinations of these genera were proposed for *Polystephalomycetes* by Kepler et al. (2013), *Tolypocladium* by Quandt et al. (2014), *Perennicordyceps* by Matočec et al. (2014) and *Drechmeria, Harposporium, Ophiocordyceps*, and *Purpureocillium* by Spatafora et al. (2015). The genus *Paraisaria* was recently recovered in Ophiocordyptaceae (Mongkolsamrit et al., 2019).

The genus *Paraisaria* was established by Samson and Brady (1983), with *P. dubia* as the type species, whose sexual morph was known as *Ophiocordyceps gracilis* (syn. *Cordyceps gracilis*). The sexual morph of this genus is characterized by solitary stromata with a stipe terminating in a globose or ellipsoid fertile head, completely immersed, ostiolate, gregarious perithecia, cylindrical asci and hyaline, filiform, multi-septate ascospores, which break into aseptate fragments when mature. Its asexual morphs are characterized by verticillate branched conidiophores, phalidic, flask-shaped, usually sympodially proliferating conidigenous cells, which terminate in 1–4 necks, and aseptate, hyaline, smooth-walled conidia, which usually aggregate in slimy heads (Samson and Brady, 1983). Li et al. (2004) synonymized *Isaria gracilioides* under *P. gracilioides* and linked its sexual morph to *Ophiocordyceps gracilioides*. Evans et al. (2010) found the asexual morph of *P. myrmicarum* from a red ant host (*Myrmica rubra*) in a natural environment in the United Kingdom. Quandt et al. (2014) have dropped the genus *Paraisaria* and used its sexual genus *Ophiocordyceps* according to the ‘one fungus one name’ principle. Mongkolsamrit et al. (2019) resurrected *Paraisaria* on the basis of three new species, e.g., *P. orthopterorum*, *P. phuwiangensis*, and *P. yodhathaii* as well as eight new combinations, e.g. *P. amazonica* (Sanjuan et al., 2015), *P. biattarioides* (Sanjuan et al., 2015), *P. coenomyiae* (Ban et al., 2015), *P. gracilioides* (Kobayasi, 1941; Pérez-Villamares et al., 2017), *P. gracilis* (Samson and Brady, 1983; Pérez-Villamares et al., 2017), *P. heteropoda* (Sung et al., 2011; Mongkolsamrit et al., 2019), *P. paramyrmicarum* (= *P. myrmicarum*) (Evans et al., 2010) and *P. tettigonia* (Wen et al., 2016). So far, together with the three new species in this study, 14 species are accepted in *Paraisaria*.

This study is part of a larger survey of fungi in the Greater Mekong Subregion where we came across numerous new taxa (Hyde et al., 2018). In this study, three species of entomopathogenic fungi were collected from disturbed forests in China and Thailand, and the typical macro- and micro-morphological characteristics indicate that they are of the *Paraisaria* species. The multigene phylogenetic analysis of LSU, SSU, TEF1-α, RPB1, RPB2, and ITS confirmed their placement within *Paraisaria* as three distinct new species.

**MATERIALS AND METHODS**

**Sample Collection, Isolation, and Morphological Studies**

In this study, a total of four fungal specimens were collected. One specimen (HKAS 102484) was collected from Krabi Province in Thailand on an adult cricket. Two specimens (HKAS 102553 and HKAS 102552) on dead larvae of *Lepidoptera sp.* were collected from Guizhou Province of China. One specimen (HKAS 102546) was collected from Yunnan Province in China on *Coleoptera sp.* larva. Among them, the hosts of specimens...
HKAS 102484, HKAS 102553 and HKAS 102552 were found completely immersed into soil with the stroma protruding from the ground in a forest. Specimen HKAS 102546 was found in a similar condition, but differed in that it was found under a karst stone formation. Macro-morphological characteristics of fresh collections were recorded with a camera (iPhone XS Max) in the field and then the specimens were transported to the laboratory in plastic boxes for subsequent studies. The culture of the specimen HKAS 102546 was created by transferring a small mass of mycelium inside the body of the host into potato dextrose agar (PDA, 1% w/v peptone) using a burned needle and incubated at room temperature (25°C). The pure culture was stored in twice-sterilized water, 15% glycerinum solution and PDA medium, and deposited in the KUMCC culture collection of the Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS). The fruiting bodies were dried with allochroic silica gel and deposited in KUN herbarium of KIB. Facesoffungi numbers were registered as outlined in Jayasiri et al. (2015).

The fresh fruiting bodies were examined and hand-sectioned under an Optec SZ660 stereo dissecting microscope. The key fungal structures viz. ascomata, perithecia, peridium, asci and ascospores were mounted in sterilized water or cotton blue solution slides and observed and photographed using a compound microscope (Nikon ECLIPSE Ni) with a digital camera (Canon EOS 600D) fitted on to the top of the microscope. The important fungal structures were measured with the Tarosoft (R) Image Frame Work program and the images used were processed with Adobe Photoshop CS3 Extended v. 10.0 (Adobe®, San Jose, CA, United States).

### DNA Extraction, PCR Amplification, and Sequencing

The total DNA was extracted from stromal tissue of specimens HKAS 102552, HKAS 102553, HKAS 102484 and from fresh mycelium of KUMCC 20-0001 (ex-type culture of isolate HKAS 102546) using DNA extraction kit (Omega Fungus Genomic DNA Extraction Kit, China), following the protocol of the manufacturer. The obtained DNA was stored at −20°C in a refrigerator. The PCR amplification was performed in 25 µL volumes consisting 12.5 µL PCR mixture (2 × Taq PCR Master Mix, red dye) which contains Taq DNA polymerase, dNTPs, MgCl₂, a reaction buffer, a PCR reaction enhancer, an optimizer and stabilizer, 8.5 µL of twice-sterilized water, 1 µL of each primer and 2 µL of 30 µg/µL DNA template. The internal transcribed spacer (ITS1-5.8S-ITS2, ITS), large subunit ribosomal RNA (LSU rRNA), small subunit ribosomal RNA (SSU rRNA), translation elongation factor 1-alpha gene (TEF1-α) and RNA polymerase II largest subunit (RPB1) and RNA polymerase II second largest subunit (RPB2) were amplified with the primers and procedures mentioned in Table 1. The PCR products were sent to Tsingke company, Yunnan Province, China, for sequencing the above genes. The generated sequences were submitted to GenBank, and the accession numbers have been shown in Table 2.

#### TABLE 1 | Gene and primers used in the phylogenetic analyses.

| Gene (reference) | Primer | Sequences | PCR condition |
|------------------|--------|-----------|---------------|
| LSU (Vigalys and Hester, 1990) | LROR | ACCGCCTGAACTTAAGC | (1) Initialization at 94°C for 3 min. (2) 40 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 50 s, and extension at 72°C for 1 min. (3) Final elongation at 72°C for 10 min. (4) Storage at 4°C. |
| SSU (White et al., 1990) | NS1 | GTAGTCATATGGCTTGTC | |
| | NS4 | CTCCGTCATATCTCGTAA | |
| ITS (White et al., 1990) | ITS4 | TCCTCGCCTATTGATAGC | |
| | ITS5 | GGAAGTAAAAGTCGTAACAAGG | |
| RPB1 (Castlebury et al., 2004) | CPRB1Af | CAYCCWGGYTYATCAAGAA | |
| TEF1-α (Rehner and Buckley, 2005) | 983F | GCYCCYGGHCAVCGTGAAYT | |
| | 2218R | ATGACACCRACRGCRACRGTYTG | |
| RPB2 (Liu et al., 1999; Sung et al., 2007b) | RPB2-5F | GAYGAYMGWAGATAYTGYGG | |
| | RPB2-7cR | CCCATRGGCTTGYRCCCAT |
| Species                | Specimen number | SSU      | LSU      | TEF1-α   | RPB1      | RPB2      | ITS      | References                   |
|------------------------|-----------------|----------|----------|----------|-----------|-----------|----------|-------------------------------|
| Ophiocordyces highlandensis | HKAS 83206      | KMS81282 | –        | –        | KMS81274  | KMS81278  | –        | Yang et al., 2015             |
| Ophiocordyces highlandensis | HKAS 83207      | KMS81284 | –        | –        | KMS81276  | KMS81280  | –        | Yang et al., 2015             |
| Ophiocordyces konnoana   | EFCC 7295        | EF468958 | –        | –        | EF468862  | EF468915  | –        | Araújo et al., 2018           |
| Ophiocordyces konnoana   | EFCC 7315        | EF468969 | –        | EF468753 | EF468861  | EF468916  | –        | Araújo et al., 2018           |
| Ophiocordyces melolonthae | OSC 110993      | DQ522548 | DQ518762 | DQ522331 | DQ522376  | –        | –        | Sung et al., 2007a            |
| Ophiocordyces melolonthae | Ophgrc679       | –        | KC610768 | K610744  | K658666   | –        | –        | Araújo et al., 2018           |
| Ophiocordyces nigrella   | EFCC 9247        | EF468963 | EF468818 | EF468758 | EF468866  | EF468920  | –        | Araújo et al., 2018           |
| Ophiocordyces ravenelli  | OSC 110995       | DQ522550 | DQ518764 | DQ522334 | DQ522379  | DQ522430  | –        | Araújo et al., 2018           |
| Ophiocordyces ravenelli  | OSC 151914       | KJ878932 | –        | KJ878978 | KJ879012  | KJ878950  | –        | Araújo et al., 2018           |
| Ophiocordyces superficialis | MICH 36253      | EF468983 | –        | –        | EF468883  | –        | –        | Sung et al., 2007a            |
| Ophiocordyces variabilis | ARSEF 5365       | DQ522555 | DQ518769 | DQ522340 | DQ522386  | DQ522437  | –        | Araújo et al., 2018           |
| Ophiocordyces variabilis | OSC 110003       | EF468985 | EF468839 | EF468779 | EF468885  | EF468933  | –        | Araújo et al., 2018           |
| Paraisaria alba          | HKAS 102484      | MN943843 | MN943839 | MN92085  | MN92078   | MN92082   | MN947219 | This study                    |
| Paraisaria amazonica     | HUA 186143       | KJ917562 | KJ917571 | KM411989 | KP212902  | KM411982  | –        | Ban et al., 2015              |
| Paraisaria amazonica     | HUA 186113       | KJ917566 | KJ917572 | –        | KP212903  | KM411980  | –        | Ban et al., 2015              |
| Paraisaria arctica       | HKAS 102553      | MN943845 | MN943841 | MN92087  | MN92080   | MN92083   | MN947221 | This study                    |
| Paraisaria arctica       | HKAS 102552      | MN943844 | MN943840 | MN92086  | MN92079   | MN92083   | MN947220 | This study                    |
| Paraisaria blattariae    | HUA186093        | KJ917559 | KJ917570 | KM411992 | KP212910  | –        | –        | Ban et al., 2015              |
| Paraisaria blattariae    | HUA 186108       | KJ917558 | KJ917569 | –        | KP212912  | KM411984  | –        | Ban et al., 2015              |
| Paraisaria coenomyiae    | NBRC 106964      | AB968385 | AB968413 | A968571  | –        | AB968533  | AB968397 | Ban et al., 2015              |
| Paraisaria coenomyiae    | NBRC 108903      | AB968384 | AB968412 | AB968570 | –        | AB968532  | AB968396 | Ban et al., 2015              |
| Paraisaria gracilioides  | HUA 186095       | KJ917556 | –        | KM411994 | KP212914  | –        | –        | Li et al., 2004               |
| Paraisaria gracilioides  | HUA 186092       | KJ917555 | KJ130992 | –        | KP212915  | –        | –        | Mongkolsamrit et al., 2019    |
| Paraisaria gracillus     | EFCC 3101        | EF468955 | EF468810 | EF468750 | EF468858  | EF468913  | –        | Araújo et al., 2018           |
| Paraisaria gracillus     | EFCC 8572        | EF468956 | EF468811 | EF468751 | EF468859  | EF468912  | –        | Araújo et al., 2018           |
| Paraisaria heteropoda    | OSC 106404       | AY489690 | AY489722 | AY489617 | AY489651  | –        | –        | Araújo et al., 2018           |
| Paraisaria heteropoda    | EFCC 10125       | EF468957 | EF468812 | EF468752 | EF468860  | EF468914  | J0049852 | Araújo et al., 2018           |
| Paraisaria orthopteronum | BBC 88305        | –        | MK332583 | MK214080 | MK214084  | –        | MH754742 | Mongkolsamrit et al., 2019    |
| Paraisaria orthopteronum | TBRC 9710        | –        | MK332582 | MK214081 | MK214085  | –        | MH754743 | Mongkolsamrit et al., 2019    |
| Paraisaria phuwiangensis | BBH 43491        | –        | MK192058 | –        | MH211351  | –        | MH188542 | Mongkolsamrit et al., 2019    |
| Paraisaria phuwiangensis | TBRC 9709        | –        | MK192057 | MK214082 | MK214086  | –        | MK192015 | Mongkolsamrit et al., 2019    |
| Species                  | Specimen number | SSU   | LSU   | TEF1-α | RPB1     | RPB2     | ITS           | References                      |
|-------------------------|-----------------|-------|-------|--------|----------|----------|---------------|---------------------------------|
| Paraisaria phuwiangensis| BBH 43492       | –     | MH201169 | MH211355 | MH211352 | –        | MH188541      | Mongkolsamrit et al., 2019     |
| Paraisaria rosea         | HKAS 102546     | MN943846 | MH290088 | MH290081 | MH290084 | MN947222 | This study     |                                 |
| Paraisaria tettigonia    | GZUH CS14062709 | KT345955 | – | KT375440 | KT375441 | – | KT345954 | Wen et al., 2016               |
| Paraisaria yodhatrai     | BBH 43163       | – | MH211353 | MH211349 | – | MH188539 | Mongkolsamrit et al., 2019 |
| Paraisaria yodhatrai     | TBRC 8502       | – | MH211354 | MH211350 | – | MH188540 | Mongkolsamrit et al., 2019 |
| Polycyphalomyces formosus| ARSEF 1424     | KF049615 | KF049634 | KF049689 | KF049651 | KF049671 | KF049661 | Xiao et al., 2018               |
| Polycyphalomyces nipponicus| BCC 2325     | KF049622 | KF049640 | KF049696 | KF049655 | KF049677 | KF049665 | Xiao et al., 2018               |
| Polycyphalomyces ramosopulvinatus | FCC 5566     | KF049627 | KF049640 | KF049696 | KF049655 | KF049677 | KF049665 | Xiao et al., 2018               |
| Polycephalomyces ramosus | MFLU 18-0162   | MK863043 | MK863050 | – | – | – | MK863250 | Xiao et al., 2018               |
| Purpureocillium lilacinum| CBS 284.36     | – | – | – | EF468792 | EF468898 | – | AY624189 | Mongkolsamrit et al., 2019     |
| Purpureocillium lilacinum| CBS 431.87     | – | EF468844 | EF468791 | EF468897 | – | AY624188 | Mongkolsamrit et al., 2019     |
| Purpureocillium takamizusanensis | NHJ 3497   | EU369096 | EU369033 | EU369014 | EU369053 | EU369074 | – | Sung et al., 2007a             |
| Tolypocladium capitatum  | NBRC 106327    | JN941737 | JN941404 | – | JN992471 | – | JN943317 | Mongkolsamrit et al., 2019     |
| Tolypocladium inflatum   | CBS 567.84     | – | MH873477 | – | – | – | MH861779 | Mongkolsamrit et al., 2019     |
| Tolypocladium inflatum   | CBS 127142     | – | MH875875 | – | – | – | MH864435 | Mongkolsamrit et al., 2019     |
| Tolypocladium japonicum  | OSC 110991     | DQ522547 | DQ518761 | DQ522330 | DQ522375 | DQ522428 | JN049824 | Mongkolsamrit et al., 2019     |
| Tolypocladium ophioglossoides | NBRC 106331 | JN941733 | JN941408 | – | JN992467 | – | JN943320 | Mongkolsamrit et al., 2019     |
| Drechmeria gunni         | OSC 76404      | AF339572 | AF339522 | AY489616 | AY489650 | DQ522426 | JN049822 | Mongkolsamrit et al., 2019     |
| Drechmeria balanoides    | CBS 250.82     | AF339588 | AF339539 | DQ522342 | DQ522388 | DQ522442 | MH861495 | Mongkolsamrit et al., 2019     |
| Harposporium anguillae   | ARSEF 5407     | – | AY636080 | – | – | – | – | Mongkolsamrit et al., 2019     |
| Harposporium anguillae   | ARSEF 5593     | – | AY636081 | – | – | – | – | Mongkolsamrit et al., 2019     |
| Harposporium helicoides  | ARSEF 5354     | AF339577 | AF339527 | – | – | – | – | Mongkolsamrit et al., 2019     |
| Perennicordyceps prolifica | NBRC 100744 | JN941709 | JN941432 | – | JN992443 | – | – | Mongkolsamrit et al., 2019     |
| Perennicordyceps prolifica | NBRC 101750 | JN941708 | JN941433 | – | JN992442 | – | JN943340 | Ban et al., 2009               |
| Perennicordyceps prolifica | NBRC 103838 | JN941707 | JN941434 | – | JN992441 | – | JN943339 | Ban et al., 2009               |
| Perennicordyceps cuboides | NBRC 100941 | – | AB378646 | – | – | – | AB378666 | Ban et al., 2009               |
| Perennicordyceps cuboides | NBRC 101742 | – | AB378648 | – | – | – | AB378667 | Ban et al., 2009               |
| Cordyceps militaris      | OSC 93623      | AY184977 | AY184966 | DQ522332 | DQ522377 | – | JN049825 | Kepler et al., 2013            |
| Cordyceps kyusensis      | EFCC 5886      | EF468960 | EF468813 | EF468754 | EF468863 | EF468917 | – | Kepler et al., 2013            |

The new species generated in this study are in black bold.
Sequence Alignment and Phylogenetic Analyses

The generated sequences were assembled with Sequencing Project Management (SeqMan) (Clewley, 1995). The sequences for the combined alignment were selected based on the blast results of LSU, SSU, ITS, TEF, RPB1, and RPB2 as well as the recent references listed in Table 2. The individual gene alignment was aligned in MAFFT v. 7 web server1 (Kuraku et al., 2013; Katoh et al., 2019). The alignments of each locus were improved by manually removing uninformative gaps and ambiguous regions using BioEdit v. 7.0.9.1 (Hall, 1999) and were concatenated in Sequence Matrix v. 1.7.8 (Vaidya et al., 2011). The final combined alignment was converted to a NEXUS file (.nex) with ClustalX2 v. 1.83 (Thompson et al., 1997) and was used for Bayesian inference (BI) analysis and Maximum parsimony analysis (MP). The optimum nucleotide substitution model of each gene was selected by MrModeltest v.2.3 (Nylander, 2004) using the Akaike information criterion (AIC) method and was applied to Bayesian inference (BI) analysis that was performed using MrBayes on XSEDE (2.2.7a) (Ronquist and Huelsenbeck, 2003) on CIPRES Science Gateway2. The Bayesian posterior probability (BYP) was estimated by the Markov Chain Monte Carlo (MCMC) technique. Six simultaneous Markov Chains were run for 2,000,000 generations with sampling every 1,000 generation. The first 25% of sampled trees were discarded during the burn-in period. Maximum likelihood analysis was carried out using RAxML-HPC2 on XSEDE (8.2.10) in CIPRES Science Gateway2. The Bayesian posterior probability (BYP) was estimated by the Markov Chain Monte Carlo (MCMC) technique. Six simultaneous Markov Chains were run for 2,000,000 generations with sampling every 1,000 generation. The first 25% of sampled trees were discarded during the burn-in period. Maximum likelihood analysis was carried out using RAxML-HPC2 on XSEDE (8.2.10) in CIPRES Science Gateway V. 3.3 (Miller et al., 2010), with default algorithm and bootstrap iterations were set to 1,000 and substitution model was set to GTRGAMMA + I. Maximum parsimony analysis was implemented in PAUP v. 4.0b10 (Swofford, 2002) through heuristic search with 1,000 random replicates of stepwise addition and tree-bisection-reconnection (TBR) of branch-swapping algorithm. Gaps were treated as missing data and max trees was set to 1,000. Branches collapsed when minimum branch length was zero. The consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for the maximum parsimony tree. For the delimitation of new species based on nucleotide comparison, we follow the suggestion of Jeewon and Hyde (2016).

The tree topologies were visualized in FigTree v1.4.0 (Rambaut, 2006) and edited in Microsoft power point (2016) and Adobe Photoshop CS3 Extended v. 10.0 (Adobe3, San Jose, CA, United States). The final alignment and trees were submitted to TreeBASE with submission number 256644.

RESULTS

Phylogenetic Analyses

Phylogenetic analyses were constructed with combined LSU, SSU, TEF1-α, RPB1, RPB2, and ITS sequences data of 58 representative taxa in Ophiocordycepipitaceae. Trees were rooted to Cordyceps militaris (OSC 93623) and C. kyusyuensis (EFCC5886) in Cordycipitaceae. The alignment contains 5239 characters, including gaps (LSU: 918, SSU: 1027, TEF1-α: 906, RPB1: 664, RPB2: 1024, ITS: 700). Parsimony analysis of this dataset produced the 20 most parsimonious trees of 4833 steps in length, of which 3436 characters were constant, 380 variable characters parsimony-uninformative and 1423 characters parsimony-informative. The first parsimonious tree was represented as the best tree, with CI = 0.549, RI = 0.777, RC = 0.426 and HI = 0.451. The RAxML analysis of the combined dataset yielded a best scoring tree with a final ML optimization likelihood value of −30766.070218. The matrix had 2305 distinct alignment patterns, with 41.28% undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.236752, C = 0.277080, G = 0.283017, T = 0.203151; substitution rates AC = 1.485223, AG = 3.851975, AT = 0.915108, CG = 1.456245, TC = 0.277080, GC = 0.283017, TG = 0.203151; substitution rates AC = 1.485223, AG = 3.851975, AT = 0.915108, CG = 1.456245, CT = 6.890167, GT = 1.000000; gamma distribution shape parameter α = 0.465094.

In the phylogenetic analyses (Figure 1), eight genera are included in Ophiocordycipitaceae labeled on the tree. With the exception of Ophiocordyceps, the other remaining genera are monophyletic and individually they received strong statistical support. The three novel entomopathogenic fungi grouped with the taxa in Paraisaria with significant statistical support (1.00 PP/100% ML/98% MP). Paraisaria alba (HKAS 102484) constitutes a sister phylogenetic affiliation to P. yodhathaii with 0.96 PP/98% MP statistical support. Paraisaria rosea (HKAS 102546) is closely related to P. amazonica and P. blattariaoides, but this is statistically not supported in all three formats. Two strains of P. arcta grouped as an intermediate clade with close phylogenetic connection to P. coenomyiae, P. gracilioides, and P. heteropoda.

Taxonomy

Paraisaria alba D. P. Wei and K. D. Hyde, sp. nov.

Figure 2

Etymology: alba refers to the white fertile head.

MycoBank number: MB 833999

Facesoffungi number: FoF 07239

Parasitic on an adult cricket (Orthoptera). Sexual morph: Stroma up to 26 mm in tall, single, unbranched, growing from the flank of the host. Fertile head 3.5 mm in diam., globose, white when fresh, yellow brown when dry. Stipe 22.5 × 1.2 mm, slightly flexuous, fleshy, white, glossy, not hollow. Perithecia 200–500 × 100–220 (X̄ = 325 × 145, n = 20) μm, immersed, ovoid. Ascii 160–250 × 2.5–5 (X̄ = 200 × 3.5, n = 10) μm, unitunicate, hyaline, narrow cylindrical, attenuated toward the base, with thickened cap. Peridium 10–40 (X̄ = 20, n = 30) μm in thick, comprising hyaline, thick-walled cell of textura angularis. Apical cap 4.6–7.4 × 3.2–4.9 (X̄ = 6 × 3.8, n = 30) μm, with a narrow tunnel throughout the center. Ascospores filiform, equal to the asci in length, when mature, breaking into numerous secondary ascospores. Secondary ascospores 3–5 × 0.5–1.5 (X̄ = 4 × 1, n = 30) μm, cylindrical, hyaline, smooth, one-celled, straight, with truncated ends.
Figure 1 | Phyllogram generated from maximum likelihood analysis based on combined LSU, SSU, TEF1-α, RPB1, RPB2, and ITS sequence data. Bootstrap values for BI equal to or higher than 95%, ML and MP equal to or greater than 60% are placed on the notes. The newly generated sequences are indicated in blue bold. The host order of Parasaria species and the generic names are labeled in the right side.
Material examined

Thailand, Krabi, Plai Phraya (N: 8°24′410″, E: 98°45′34″). On an adult cricket, 20 December 2018, Deping Wei, 211-1 (HKAS 102484–holotype). We tried to culture *P. alba* by transferring a small piece of inner stroma tissue into a PDA medium using a sterilized needle, but growth was not observed.

Notes

The multigene phylogenetic analysis showed that *P. alba* groups with *P. yodhathaii* with fairly good statistical support (0.96 PP/98% MP, Figure 1). This relationship is, however, not supported by the ML analysis. *Paraisaria alba* differs from *P. yodhathaii* in having solitary stroma, a white fertile head, and smaller perithecia, asci and secondary ascospores, whereas *P. yodhathaii* has paired stromata, grayish yellow fertile head, larger perithecia and larger asci and secondary ascospores (Table 3). The comparison of the nucleotide sequences between *P. alba* and *P. yodhathaii* show 10 (including 6 gaps) out of 410 bp (2.4%), 6 out of 746 bp (0.8%), 5 out of 881 bp (0.56%) and 8 out of 534 bp differences (1.5%) in ITS, LSU, TEF-1α,
### TABLE 3 | Synopsis of *Pараисария* species discussed in this study.

| Species          | Host                   | Distribution               | Stroma (mm) | Fertile part (mm) | Perithecia (µm) | Asci (µm) | Part-ascospores (µm) | Asexual morphs |
|------------------|------------------------|---------------------------|-------------|-------------------|-----------------|------------|----------------------|----------------|
| *P. alba*        | Adult cricket          | Thailand: Krabi Province  | Solitary, 26 long | Globose, white, 3.5 in diam. | Ovoid, 200–500 × 100–220 | 160–250 × 2.5–5 | 3–5 × 0.5–1.5 | Absent |
|                  | Adult or imago of      | Colombia and Ecuador      | Gregarious, 20–45 long | Subglobose to spherical, reddish brown, 2.5–5 | Ovoid-ellipsoidal, 760–1100 × 220–400 | 325–450 × 5 | 9–17 × 0.5–2 | Absent |
| *P. amazonica*   | *P.* alba              | Colombia and Ecuador      | Gregarious, 20–45 long | Subglobose to spherical, reddish brown, 2.5–5 | Ovoid-ellipsoidal, 760–1100 × 220–400 | 325–450 × 5 | 9–17 × 0.5–2 | Absent |
| *P. arcta*       | Larva of Lepidoptera   | China: Guizhou Province   | Solitary, 16 long | Subglobose with constriction at center, white, 2 × 3 | Ampulliform to ellipsoidal, 230–530 × 70–180 | 100–180 × 2–4 | 2.6– 4.2 × 0.5–1.3 | Absent |
| *P. blattioides* | Adult of Blattaria     | Belize, Colombia and Ecuador | Gregarious, 14–20 long | Ovoid, subglobose, chestnut brown, 2–3 × 1.5–2.5 | Ellipsoidal, 650–800 × 220–300 | 180–250(–300) × 4–5 | 6–16 × 1.5 | Absent |
| *P. coenomyiae*  | Larva of Coenomyia     | Japan                      | Solitary, 30–35 long | Ovoid, subglobose, chestnut brown, 8 × 10 | Lanceolate, 700–750 × 200–220 | 500–750 × 7.8–8.0 | 8–15 × 1.8–2.5 | Absent |
| *P. graciloides* | Larva of Elateridae    | Bolivia, China,            | Usually solitary, 20–90 long | Spherical, pale rufous, 4–5.5 | Ellipsoidal to naviform, 680–900 × 200–280 | 450–700 × 5 × 6.5 | 7–12 × 1–2 | Present |
|                  |                        | Colombia, Japan and Mexico|            |                    |                |           |                      |                |
| *P. gracilis*    | Larva of Hespaliae     | Africa, America, Asia,     | Usually solitary, 40– 90 long | Globose to ellipsoidal, red ochreous to pale orange, 4–9 × 4–7 | Elongate to oviform, (320–)560–840 × 200–360 | (200–)400–528 × 5–8 | 5–9 × 1.5–2 | Present |
|                  |                        | Europe, and Oceania       |            |                    |                |           |                      |                |
| *P. heteropoda*  | Nymph of Cicadidae     | Australia, Japan           | Solitary, 120 long | Ovoid, cinnamon buff, 7–9 × 6–7 | Ellipsoidal, 610–660 × 210 | 250–300 × 5.2–7 | 6–7.7 × 0.9–1 | Present |
| *P. myrmicarum*  | Myrmica rubra          | United Kingdom             | –           | –                 | –               | –          | –                    | Present |
| *P. orthopterorum* | Nymph of Orthoptera   | Thailand: Trat Province    | Solitary, 10–45 long | Globose, gray orange, 2–4 × 3 | Obclavate, 520–650 × 150–250 | 400 × 5 | 5–10 × 1–1.5 | Present |
| *P. phuwiangensis* | Larva of Elateridae   | Thailand: Khon Kaen Province | Solitary, 30–50 long | Globose to subglobose, light brown, 4–8 × 4–7 | Oleniglycose, 800–1200 × 300–380 | 500 × 3–5 | 5–10 × 1–2 | Present |
| *P. rosea*       | Larva of Coleoptera    | China: Yunnan Province     | Solitary, 14.5 long | Subglobose, pale pink, 4.5 × 4 | Ampulliform, 500–900 × 150–350 | 230–390 × 3.5–6 | 4–11 × 1.5–2.5 | Present |
| *P. tettigonia*  | Adult of Tettigonia    | China: Guizhou Province    | Paired, 32.5–37.5 long | Globose, white, 2–2.5 | Elongated to ampulliform, 520–680 × 205–275 | 530–615 × 6.5–9.3 | 6.7–9.4 × 1.5–2.3 | Absent |
| *P. yodhathai*   | Larva of Elateridae    | Thailand: Khon Kaen        | Gregarious, 20–35 long | Globose to subglobose, grayish yellow, 2–4 × 2–5 | Obclavate, 650–800 × 160–250 | 480 × 5–6 | 5–10 × 1–2 | Present |

The new species generated in this study are in **bold**.'
Paraisaria arcta D. P. Wei and K. D. Hyde, sp. nov.

Etymology: *arcta* refers to the constricted fertile head.

*Paraisaria arcta* (HKAS 102553, holotype).

**Parasitic** on larva of Lepidopteran larva. **Sexual morph:**

*Stroma* 16 mm long, single, arising from the mouth of host larva. *Fertile head* 2 mm long, 3 mm wide, white, nearly globose, constricted at the center, with sticky and crystal-like substance on the surface. *Stipe* 14 mm long, 2 mm wide, straight, fleshy, white, glossy. *Perithecia* 230–530 × 70–180 (\( \bar{x} = 387 \times 113, n = 20 \)) \( \mu \)m, completely immersed, ampulliform to ellipsoid. *Peridium* 14–20 (\( \bar{x} = 17, n = 30 \)) \( \mu \)m wide, composed of hyaline, thick-walled, smooth-walled cells of *textura angularis*. *Asci* 100–180 × 2–4 \( \mu \)m (\( \bar{x} = 137 \times 2.9, n = 15 \)), unitunicate, hyaline, narrow cylindrical,
tapering toward the base, 8-spored, with thickened cap. Apical cap 3.5–4.5 × 2–3.6 μm thick (X = 4 × 2.8, n = 20), with a narrow tunnel throughout the center. Ascospores hyaline, narrow filiform, equal to the asci in length, when mature, breaking into numerous secondary ascospores. Secondary ascospores 2.6–4.2 × 0.5–1.3 μm (X = 3.3 × 0.9, n = 60), cylindrical, with truncated ends, hyaline, smooth, one-celled, straight.

Material examined
China, Guizhou Province, Qianxinan Buyei and Miao Autonomous Prefecture, Ceheng County, Gaofeng Village (N: 24°57′33″, E: 105°50′1″), on dead larva of Lepidoptera sp., 6 August 2018, Deping Wei, GFC604 (HKAS 102553 – holotype); GFC603 (HKAS 102552 – paratype). The culturing of P. arcta was tried by transferring a mass of mycelium found inside body of the larva host to a PDA medium using a sterilized needle. However, mycelium growth was not observed.

Notes
Parasaria arcta resembles P. alba found in Krabi Province, Thailand and P. tettigonia discovered in Guizhou Province, China in having white fertile heads but differs from P. alba in its associated host and number of stromata are distinct from P. tettigonia (Wen et al., 2016). Parasaria arcta can also be distinguished from the other species in Parasaria by the color and shape of its fertile head. A conspicuous ravine throughout the center of the fertile head is present in P. arcta, which is lacking in the other species in this genus. The detailed comparisons are shown in Table 3. Multigene phylogenetic analysis showed P. arcta constitutes a distant clade from other species in Parasaria, with strong statistical support (100% ML, 100% MP, 1.00 PP, Figure 1). Herein, we introduce this collection as a new species of Parasaria.

Parasaria rosea D. P. Wei and K. D. Hyde, sp. nov. Figures 4, 5
Etymology: rosea refers to its pink fertile head.
MycoBank number: MB834001
Facesoffungi number: FoF 07241
Parasitic on a larva of Coleoptera. Host buried in the soil, with the stroma erumpent from the ground. Sexual morph: Stroma up to 14.5 mm long, laterally emerging from the middle part of the larva body, simple, erect. Fertile head 4.5 × 4 mm, subglobose, pale pink at top and paler toward the base when fresh, pale yellow-brown when dry. Stipe 10 × 1.5 mm, white, straight, unbranched, glossy, cylindrical, inside not hollow. Perithecia 500–900 × 150–350 (X = 762 × 256, n = 30) μm, completely immersed, ampulliform, ostiolate. Peridium 9–15 (X = 12, n = 30) μm wide, composed of hyaline, thick-walled cells of textura angularis to textura globulosa to textura prismatica. Asci 230–390 × 3.5–6 (X = 280 × 5, n = 15) μm, hyaline, cylindrical, unistinate, eight-spored, possessing a prominent apical cap. Apical cap 5–7 × 2–6 (X = 6 × 4, n = 20) μm, with a conspicuous tunnel throughout the center. Ascospores filiform, hyaline, breaking into secondary ascospores when mature. Secondary ascospores 4–11 × 1.5–2.5 (X = 7.5 × 2, n = 30) μm, hyaline, cylindrical with truncate ends, smooth-walled, asceptate. Asexual morph: Hyphomycetous.

Synnemata producing from the center of culture after 16 months incubation in dark environment, composed of loose, septate hyphae, white, filamentous, aerial, straight, branched, fasciculate, bearing shining droplets and conidiophores. Mycelium 2.4–3.7 (X = 3, n = 10) μm in wide, septate, hyaline, smooth-walled. Conidiophores 33–48 (X = 41, n = 10) μm in height, irregularly differentiate from the synnemata, sparse, gregarious, branched. Phialides 5.8–11.5 × 3–5.5 (X = 8.6 × 4, n = 30) μm, ampulliform, 1-necked, hyaline, asceptate, enteroblastic, phialidic, monophialidic. Conidia 8–12 × 2–2.6 (X = 9.8 × 2.3, n = 50) μm, hyaline, cylindrical, smooth-walled, asceptate, with round ends.

Culture characteristics
Culture was made from mycelium inside body of the host larva, slowly growing on PDA, reaching 1.3 cm in diam after incubated at room temperature (25°C) for 50 days, convex, dense, with undulate edges, smooth surface become filamentous after forming aerial synnemata. The shooting conidia land on the surrounding culture and develop new colonies.

Material examined
China, Yunnan Province, Kunming, Western hill Park (N: 24°57′28″, E: 102°38′17″), on larva of Coleoptera sp. buried in soil, 27 July 2018, Deping Wei, XS2712 (HKAS 102546 – Holotype); (KUMCC 20-0001 – ex-type living culture).

Notes
Parasaria rosea is closely related to P. amazonica and P. blattarioides, without any statistical support (Figure 1). However, P. rosea can be distinguished from these related species based on the number of stromata, the color of the fertile head and the size of asci and secondary ascospores (Table 3). The ITS sequence of P. amazonica and P. blattarioides are not available in GenBank database; the nucleotide differences in the TEF1-α, RPB1 and RPB2 region between P. rosea and the two above species are greater than 1.5% (Table 4). Thereby, we introduced P. rosea as a new species in this genus based on the distinctive morphology and molecular support.

DISCUSSION
The sexual morph of Parasaria species phenotypically share an erect or slightly flexuous, cylindrical, colorless, fleshy stipe that terminates in a subglobose to globose fertile head and completely immersed perithecium. Asci are cylindrical with a thickened apical cap. Ascospores are hyaline, multi-septate and usually break into numerous cylindrical, truncated fragments at maturity. However, they can be distinguished according to their associated host, the number of stroma and the color of the fertile head. Species in this genus usually infect several stages of insects, such as larvae of Coleoptera, Diptera, and Lepidoptera; nymphs of Hemiptera and Orthoptera; or adults of Dictyoptera, Hymenoptera (ant) and Orthoptera (Evans et al., 2010; Sanjuan et al., 2015; Mongkolsamrit et al., 2019). According to the number of stromata, species of Parasaria can be divided into three groups: solitary stroma, paired stromata and multiple stromata (see the key below). The shape of their fertile head features little variation,
though differing in color, ranging from white, pale pink, pale rufous, red ochreous to pale orange, chestnut, cinnamon buff, grayish, reddish brown to dark brown (see Table 3).

The asexual morphs of this genus are known in eight species, viz. *P. myrmicarum* (Evans et al., 2010), *P. gracilis* (Samson and Brady, 1983), *P. gracioloides* (Li et al., 2004), *P. rosea* (this study), *P. heteropoda*, *P. orthopterorum*, *P. phuwiangensis*, and *P. yodhathaii* (Mongkolsamrit et al., 2019). Their conidiophores are irregularly branched and generally develop from white, rope-like synnemata. Their phialides are flask-shaped, with a swollen base and narrow neck. Most species produce only one neck from the terminal phialides. Some species, e.g., *P. gracilis*, *P. gracioloides*, *P. myrmicarum* and *P. orthopterorum* produce 1–4 necks per phialides. Their conidia are cylindrical or ellipsoid or fusiform. Some species, e.g., *P. orthopterorum* and *P. yodhathaii* have both cylindrical and fusiform forms of conidia (Mongkolsamrit et al., 2019).

Sung et al. (2007a) have concluded that multi-gene phylogeny gave more deeper understanding of phylogenetic relationships of *Cordyceps* and Clavicipitaceae than that of single gene. Recently,
FIGURE 5 | Asexual morph of Paraisaria rosea (KUMCC 20-0001, ex-type). (a,d) Upper and lower views of cultures on PDA after 50 days. (b,e) Upper and lower views of cultures on PDA after 16 months incubation in dark environments. (c,f) Enlargement of aerial synnemata produced on culture. (g) Synnema bearing conidiophores. (h–l) Phialides. (m) Conidia. (n,o) Irregularly aggregated conidia. Scale bars: (g) 100 µm, (h–l) 30 µm, (m–o) 5 µm. (h–k,m mounted in cotton blue reagent.).

However, individual gene phylogenies are rarely utilized for identification of species in Paraisaria.

TABLE 4 | The comparison of nucleotide sequences between Paraisaria rosea and two close species.

| Species                  | TEF1-α (bp) | RPB1 (bp) | RPB2 (bp) |
|--------------------------|-------------|-----------|-----------|
| Paraisaria amazonica     | 4.4% (38/862) | 5.7% (37/642) | 4.3% (31/711) |
| Paraisaria blattarioides | 1.6% (14/862) | 2.5% (16/629) | –         |

Key to the Accepted Species in Paraisaria

1. Host belong to Hymenoptera..............................P. myrmicarum
2. Fertile part colorless....................................3
   3. Fertile part pigmented.....................................4
   4. Fertile part constrict at the center....................P. arcta
   5. Fertile part is not constricted at the center...........5
5. Stromata gregarious...........................................6

the combined LSU-TEF1-α-RPB1 datasets (Mongkolsamrit et al., 2019), combined SSU-LSU-TEF-RPB2 datasets (Ban et al., 2015), and combined SSU-LSU-TEF1-α-RPB1-RPB2 datasets (Quandt et al., 2014; Sanjuan et al., 2015) were allowed for intraspecific and intergeneric identification within Ophiocordycipitaceae.
DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MN943843, MN943839, MN929085, MN929078, MN929082, and MN947219; https://www.ncbi.nlm.nih.gov/genbank/, MN943845, MN943841, MN929087, MN929080, and MN947221; https://www.ncbi.nlm.nih.gov/genbank/, MN943844, MN943840, MN929086, MN929079, MN929083, and MN947220.

AUTHOR CONTRIBUTIONS

D-PW, DW, and SK: conceptualization. D-PW: data curation. D-PW and DW: formal analysis, methodology, and writing – original draft. SL, ST, and SK: funding acquisition. D-PW and DW: investigation. ST and SK: project administration. KH, J-CX, and PM: supervision. CT-a, AE, SM, ST, SK, KH, J-CX, PM, NS, and SL: writing – review and editing. All authors: contributed to the article and approved the submitted version.

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