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Five new species of entomopathogenic fungi from the Amazon and evolution of neotropical *Ophiocordyceps*

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ABSTRACT

The neotropical biogeographic zone is a 'hot spot' of global biodiversity, especially for insects. Fungal pathogens of insects appear to track this diversity. However, the integration of this unique component of fungal diversity into molecular phylogenetic analyses remains sparse. The entomopathogenic fungal genus *Ophiocordyceps* is species rich in this region with the first descriptions dating to the early nineteenth century. In this study, material from various ecosystems throughout Colombia and Ecuador was examined. Molecular phylogenetic analyses of five nuclear loci including *SSU*, *LSU*, *TEF*, *RPB1*, and *RPB2* were conducted alongside a morphological evaluation. Thirty-five specimens were examined representing fifteen different species of *Ophiocordyceps*, and five new species, *Ophiocordyceps blattarioides*, *Ophiocordyceps tiputini*, *Ophiocordyceps araracuarensis*, *Ophiocordyceps fulgoromorphila*, and *Ophiocordyceps evansii*, were described. An accurate identification of the host allowed us to conclude that host identity and host habitat are positively correlated with phylogenetic species of *Ophiocordyceps* and are probably strong drivers for speciation of neotropical entomopathogenic fungi.

Introduction

The entomopathogenic genus *Ophiocordyceps* was established by Petch (1931) for fungi possessing clavate asci with gradually thickening apices and elongate, fusiform ascospores that do not disarticulate into part-spores. The type species, *Ophiocordyceps blattae* Petch, from Sri Lanka, is rarely collected, but other more commonly collected fungi include *Ophiocordyceps unilateralis* (Tul.) Petch on *Hymenoptera* adults (worker ants) and *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung,

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Hywel-Jones & Spatafora on *Lepidoptera* larvae. In other major taxonomic studies of the genus *Cordyceps* (L.) Link, Kobayasi (1941, 1982) and Mains (1958) both treated *Ophiocordyceps* as a subgenus of *Cordyceps*. With advances in the genetic characterization of fungi, a phylogenetic framework for *Cordyceps* (*Ascomycota*: *Hypocreales*) derived from molecular data has greatly clarified taxonomic systems previously based on morphology alone (Sung *et al.* 2007). *Ophiocordyceps* sensu Petch was resurrected as a genus, and expanded to include the majority of taxa with firm, darkly pigmented stromata based on the placement of *O. unilateralis*, but with a diversity of ascus and ascospore morphologies. Currently there are 184 species recognized in *Ophiocordyceps*, with many additional species described as asexual taxa (Quandt *et al.* 2014), making it the largest of the entomopathogenic genera in *Hypocreales* (Sung *et al.* 2007; Johnson *et al.* 2009). Molecular phylogenetic analyses have incorporated material from Asia, Europe, and North America, highlighting phylogenetic relationships for members of genus *Ophiocordyceps* in the Holarctic and Indo-Malaysian biogeographic zones, but relatively few neotropical specimens of the genus have been sampled (Sung *et al.* 2007). Based on work by mycologists such as Möller (1901), Henning (1904), and Spegazzini (reviewed in Kobayasi 1941), however, the neotropics are known to support a unique assemblage of *Ophiocordyceps*. More recently, Evans (1982) emphasized the importance of systematic collections of entomopathogenic fungi in the Amazon, a subregion within the neotropical zone. Producing a phylogeny of *Ophiocordyceps* inclusive of global diversity requires specimens from the neotropics, particularly considering the high diversity of entomofauna (Lopez & Miranda 2010).

In this study we sampled neotropical *Ophiocordyceps* species from Colombia and Ecuador, including the Imeri and Napo biogeographic provinces (Amazon subregion) and Magdalena province (Caribe subregion) proposed by Morrone (2006). Incorporation of these samples into existing molecular phylogenetic datasets, in combination with host identification to family and genus, allowed the description of new species and the development of hypotheses concerning the role of host association in the evolution and divergence of *Ophiocordyceps* species.

Material and methods

Material collected

Systematic collections were made in three sites of the Amazon region from Colombia with altitudes between 20 and 200 m above sea level, average temperatures of 28 °C, and relative humidity of 90 %. The first site was the Araracuara canyon of the Caquetá River (Puerto Santander, Amazonas) in May of 2009 and September of 2011. The second site was sampled in several expeditions to Uitoto San Francisco community along to the Igara Parana River (La Chorrera, Amazonas), in July of 2010 and March and September of 2011. The third site sampled was the scientific station El Zafire reserve in the Municipality of Leticia (Amazonas) in July of 2011 and January of 2012. Additionally, collections were made in Rio Claro (San Luis, Antioquia) in March of 2012 and November of 2014, a region belonging to Magdalena biogeographic province. Some material collected in July 2004 in the Tiputini Research station (Napo, Ecuador) belonging to Napo biogeographic province was included in this study.

Collecting involved careful examination of leaf litter, downed wood, and elevated plant structures (e.g., leaves, twigs) to detect the emergence of stromata from insect cadavers. Dried specimens were placed in plastic bags with silica gel for transportation to the laboratory for identification. Collections were deposited in Antioquia University Herbarium (HUA), and the collection of Fungi of the Natural History Museum of Andes University (ANDES) in Colombia, and National Herbarium of Ecuador (QCNE). Additionally material provided by the National Herbarium of Colombia (COL) was revised. Fungal fruiting bodies were examined according to Sanjuan *et al.* (2014). Colour descriptions of stromata are according to Kornerup *et al.*, 1984. The host insects were identified to the extent possible (e.g., family, genus, etc.) as allowed by the condition of the specimen.

DNA extraction, PCR amplification, and sequencing

In the field, small pieces of fresh tissue from stromata were placed in 50 μL CTAB extraction buffer (1.4 M NaCl; 100 mM Tris−HCl pH 8.0; 20 mM EDTA pH 8.0; 2 % CTAB w/v) and the DNA extraction process was performed following the method in Kepler *et al.* (2012). Attempts were made to amplify six nuclear loci including the nuclear ribosomal internal transcribed spacer region (*ITS*), small subunit ribosomal RNA (*SSU*) and large subunit ribosomal RNA (*LSU*), elongation factor-1α (*TEF*), and the largest (*RPB1*) and second (*RPB2*) largest subunits of RNA polymerase II. The PCR reaction mixture consisted of 2.5 μL 10 × PCR buffer (Fermentas, Glen Burnie, MA), 2 μL $MgCl_2$ (25 mM), 0.5 μL each primer (10 μM), 0.5 μL dNTP (10 mM each), 1−2 μL template DNA, 0.2 μL *Taq* polymerase (5 U/μL) (Fermentas, Glen Burnie, MA) and dH_2O to a final volume of 25 μL.

Amplification of *ITS* was performed with primers ITS1f and ITS4 (White *et al.* 1990). *SSU* and *LSU* were amplified with NS1/SR7and NS3/NS4 (White *et al.* 1990) and LROR/LR5 primers (Vilgalys & Sun 1994), respectively. Amplification of *TEF* was performed with the primers 983F and 2218R (Rehner & Buckley 2005). Amplification of RPB1 was performed with primers cRPB1-1 aF and cRPB1-CaR (Castlebury *et al.* 2004). Amplification of RPB2 was performed with primers fRPB2-5f2 and fRPB2-7 cR (Liu *et al.* 1999). The PCR reactions were performed in a Thermocycler 1000 (BIORAD, Hercules, CA) programmed as follows: 94 °C for 3 min; ten cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min; 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 2 min; one cycle of 72 °C for 3 min and 4 °C indefinitely (Kepler *et al.* 2011). Sequencing was performed with the amplification primers at the University of Washington (Seattle, USA) and Andes University (Bogotá, Colombia) sequencing centers. The *ITS1* and *LSU* of the several specimens from this study were submitted to the IBOL initiative (http://www.boldsystems.org/).

Sequence alignment and phylogenetic analysis

Sequences from this study were edited using Geneious Pro version 4.8.5 (Drummond *et al.* 2009). A BLAST query of the