

AN ABSTRACT OF THE DISSERTATION OF

Ryan M. Kepler for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on November 24, 2010.

Title: Advances in Molecular Systematics of Clavicipitaceous Fungi
(Sordariomycetes: Hypocreales)

Abstract approved: _____
Joseph W. Spatafora

Historical concepts of Clavicipitaceae have included a broad range of species that display diverse morphologies, ecological modes and host associations. When subjected to multigene phylogenetic investigation of evolutionary history, the family was found to be polyphyletic, largely driven by diversity in the genus *Cordyceps*, previously containing over 400 taxa. The majority of *Cordyceps* sensu lato now resides in the families Cordycipitaceae, for which the genus *Cordyceps* has been retained owing to the placement of the type *C. militaris*, and the genera *Ophiocordyceps* and *Elaphocordyceps* in Ophiocordycipitaceae. The genus *Metacordyceps* was defined for species of *Cordyceps* remaining in Clavicipitaceae sensu stricto and contains relatively few species owing to convergent morphologies. Clavicipitaceae remains considerably diverse and its members attack hosts across three kingdoms of life, including insects and rotifers, plants and other fungi. There remains a significant number of *Cordyceps* species for which molecular or morphological data are insufficient, and are therefore considered incertae sedis with

regards to family until new material is available for examination. This work expands the sampling of taxa in Clavicipitaceae sensu lato for inclusion in phylogenetic reconstruction, with particular emphasis on residual species of *Cordyceps*. The resulting phylogenies were then used to refine concepts morphological features that define boundaries between taxa and explore the evolution of host association, morphology and ecology with ancestral character-state reconstruction.

Taxon sampling was increased by extensive field collections and collaborations with other researchers. The diversity present in newly acquired specimens represents all three pathogen groups. The inclusion of these taxa in a five-gene dataset (nrSSU, nrLSU, TEF, RPB1, RPB2) advances understanding of systematic relationships within Clavicipitaceae. Molecular data supports the movement of seven species from *Cordyceps* sensu lato to *Metacordyceps*, doubling the number of species known for the genus. The addition of new species reveals significant structure within the genus and allows for a reevaluation and strengthening of morphological concepts attributed to the genus. A clade of species sister to Clavicipitaceae was identified as *Veterocordyceps* gen. nov. This finding provides clarity to the results of previous workers who considered species in the genus as members of *Ophiocordyceps* based on morphological data and a more limited molecular dataset. *Cordyceps fratriscida*, a pathogen of ergot, and the rice pathogen *Ustilaginoidea virens* were placed phylogenetically within Clavicipitaceae. The genus *Tyrranocordyceps* gen. nov. was established for fungal pathogens that attack closely related species of ergot. *Tyrranocordyceps* represents a sexual state for a clade of

fungus pathogens known only from asexual states. *Ustilaginoidea virens* was found to occupy a separate lineage of plant pathogens, representing a third clade of clavicipitaceous fungi deriving their nutrition from a plant source. Ancestral character-state reconstruction reveals plant pathogens/endophytes have arisen multiple times during the history of the family and *T. fraticida* evolved from a plant pathogenic ancestor. The switch to a plant based nutritional mode appears to be facilitated by an ancestral host association with scale insects (Hemiptera: Sternorrhyncha:Coccoidea). These insects are sessile in their adult stage and insert their mouthparts directly into living plant tissues, a characteristic that might mediate the acquisition of plant nutrients by their fungal pathogens and eventually result in bypassing the insect host all together. Finally, ancestral character-state reconstructions were performed on a phylogeny representing teleomorphic taxa from throughout Hypocreales for the characters of stipe (absence or presence) and habitat (terrestrial or arboreal). Hypocreales was found to have evolved from a terrestrial, astipitate ancestor. Stipitate morphologies were derived multiple times over the evolution of the order. This observation accounts for the phylogenetic signal in the stipe characters of color and texture. The characters of stipe and habitat were found to be highly correlated. Species occurring in arboreal habitats tend to be astipitate. This was inferred to be the retention of the ancestral state for early diverging members of Cordycipitaceae. However, all other astipitate, arboreal species were inferred to have lost the stipe after moving from a terrestrial habitat. This is likely an evolutionary

response to release from evolutionary pressures to elevate reproductive tissues above a substrate.

©Copyright by Ryan M. Kepler
November 24, 2010
All Rights Reserved

Advances in Molecular Systematics of Clavicipitaceous Fungi (Sordariomycetes:
Hypocreales)

by
Ryan M. Kepler

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented November 24, 2010
Commencement June 2011

Doctor of Philosophy dissertation of Ryan M. Kepler presented on November 24, 2010.

APPROVED:

Major Professor, representing Botany and Plant Pathology

Chair of the Department of Botany and Plant Pathology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Ryan M. Kepler, Author

ACKNOWLEDGEMENTS

The work contained in these pages has been a highly collaborative effort and would be significantly diminished were it not for the generous support and assistance offered by the following people: Research in Japan would not have been possible without the tireless assistance of Tsuyoshi Hosoya and his staff at the National Museum of Nature and Science in Tsukuba during the summers of 2007 and 2008. The exposure he provided to Japanese culture and life is every bit as significant to me as his contribution to my research and I owe him a great debt of thanks. Hiroki Sato provided images for the *Cordyceps* Electronic Monograph, as well as the most amazing version of Bohemian Rhapsody I have ever heard during karaoke. Every member of the Japanese Society for Vegetable Wasps and Plant worms (Tochukaso No Kai) provided endless entertainment and assistance during collecting trips. Yasumasa Okuzawa in Kyoto is owed a special debt of thanks for sharing his beautiful city with me as well as specimens from his personal collection. Takeshi Sakane also contributed specimens that feature prominently in this work. Inoue-san in Yamagata took me to some of the sites collected by the great Japanese *Cordyceps* researchers Yosio Kobayasi and Daisuke Shimizu.

During the Asian Mycology Congress in 2007, Andy Wilson helped fight back the sewer system of Penang, Malaysia and has been turning up in random places ever since. Jennifer Luangsa-Ard and the crew at Biotec in Bangkok, Thailand contributed specimens to this work and thoughtful discussion on the relationships of clavicipitaceous fungi. In addition, they looked out for me when I was laid up by

Mefloquin in a hotel room during a visit there December of 2007. I am thankful for that. Siril Wijesundara at the Royal Botanic Gardens in Peradeniya, Sri Lanka allowed access to the herbarium in search of the elusive collections of Thomas Petch, who described many species of hypocrealean fungi while director there.

Yi-Jian Yao at the Chinese Academy of Science in Beijing helped negotiate travel and networking throughout China with great humor and cheer. He and his lab provided a home away from home for three months during the summer of 2009. Xiao Liang Wang fearlessly tried to teach me some Mandarin on our twelve-hour train rides from Beijing to Fujian and found the teleomorph of what will eventually be a new species (Chapter 1). Wenjing Wang and I may have traveled into North Korea together, depending on how you draw the border at the Yalu River. Chuck Cannon at the Xishuangbanna Botanical Garden kept me dry and well feed while collecting in Yunnan. Sheng-Hua Wu at the National Museum of Natural Science in Taichung, Taiwan provided access to herbarium specimens and has been a great resource for East Asian mycology in general.

A great number of people in Corvallis, OR are owed thanks and any attempt to list them all here would be futile. However, the members of my committee, Aaron Liston, Chris Marshall, Pat Muir and Dan Rosenburg, have provided a real sense community in academia, and not just an academic community. The graduate students in the Department of Botany and Plant Pathology past, present (and likely future) have been an inspiration in countless ways. Dianne Simpson has been an ally since day one of my time in BPP. Chris Sullivan at the Center for Genome Research and

Biocomputing has shown a phenomenal amount of patience with me while I banged my head against the wall of computational biology.

It has been an honor to work in the lab of Joseph Spatafora. I will always be grateful for the opportunities to pursue *Cordyceps* to the ends of the earth. There has been plenty of challenge associated with obtaining and synthesizing the research contained in this dissertation. However, Joey has constantly been a source of guidance, support and rebuke when necessary throughout the process.

Past members of the lab have also been invaluable for advice and camaraderie throughout my stay here. The work previously conducted by Desiree Johnson and Gi-Ho Sung provided the foundation from which this work was possible. Desiree also caught me when I fell, literally, during climbing trips to Smith Rock State Park. Collecting with Gi-Ho was always an adventure, whether it was negotiating dry counties in North Carolina or bullet trains in Japan. The friendship and experience of Conrad Schoch and Barbara Robbertse made life easier in the lab. My fellow graduate students, Cedar Hesse, Alija Mujic and Alisha Owensby, are all amazing sources of comic relief, in addition to brilliant mycologists. The contributions of several student workers, especially Ryan Woolverton and Ryan Yodder (The “other” Ryans) John Reeves, Giang Phan, and Alex Boyd proved essential for some of the lab work and programming tasks contributing to this dissertation.

Finally, I wish to express my undying amazement at Dana McCoskey for not only putting up with, but embracing the fir needles and bits of mushrooms strewn about the house in the fall, spying on ducks in the winter, digging gardens in the

spring, tending them in the summer, and always being ready for the next adventure, no matter how big or small. You have been both a rock and a moving target all at once and I love you for it.

Funding for research was provided by a National Science Foundation Partnership for Enhancing Expertise in Taxonomy (PEET) grant to Joseph Spatafora. Additional funding was provided by NSF for two East Asia and Pacific Summer Institutes (EAPSI) fellowships to Ryan Kepler supporting research in Japan (2007) and China (2009). Deep Hypha provided funding for travel to the Asian Mycology Congress in Penang, Malaysia and visit to the Royal Botanic Gardens in Peradeniya, Sri Lanka. Attendance to the Mycological Society of America annual meeting (2010) was supported in part by a Denison Mentor Travel Award from the society. Travel to the 9th International Mycology Congress in Edinburgh, Scotland was assisted by a bursary provided by the Congress and funding from the Department of Botany and Plant Pathology in the form of an Anita Summers Travel Grant, and money from the Graduate Student Association.

CONTRIBUTION OF AUTHORS

Dr. Joseph W. Spatafora was involved in the writing and editing of each manuscript. Sayaka Ban and Dr. Akira Nakagiri performed DNA extraction for some of the specimens used in Chapter 3. Drs. Gi-Ho Sung and Eiji Tanaka assisted in some of the writing and editing for Chapter 4, and Dr. Tanaka also contributed images. Dr. Tsuyoshi Hosoya performed microscopy on the culture of *T. fraticida*.

TABLE OF CONTENTS

	<u>Page</u>
CHAPTER 1. Introduction.....	1
Taxonomic History of the Clavicipitaceous Fungi.....	1
Ecological and Morphological Characteristics of Clavicipitaceous Fungi.....	3
Human Uses Of Clavicipitaceous Fungi.....	6
Premise and Timeline of the Study.....	8
References.....	9
CHAPTER2. New Teleomorph Combinations in the Entomopathogenic Genus <i>Metacordyceps</i>	12
Abstract.....	13
Introduction.....	15
Materials and Methods.....	19
Specimen Collection.....	19
DNA extraction, PCR and sequencing.....	19
Phylogenetic analyses.....	21
Results.....	23
Taxonomy.....	24
Discussion.....	26
Phylogenetic structure of <i>Metacordyceps</i>	27
Morphology of the core <i>Metacordyceps</i>	27
Morphology of species outside of the core <i>Metacordyceps</i>	30
Anamorphic relationships of <i>Metacordyceps</i>	30

TABLE OF CONTENTS (CONTINUED)

	<u>Page</u>
Host associations of <i>Metacordyceps</i>	33
Implications for biological control.....	34
Conclusion.....	35
References.....	37
CHAPTER3. <i>Veterocordyceps</i> gen. nov. represents a unique clade of diversity in Clavicipitaceae s.l.....	
Abstract.....	53
Introduction.....	55
Materials and Methods.....	60
Specimen Collection.....	60
DNA extraction, PCR and sequencing.....	60
Phylogenetic analyses.....	61
Results.....	63
Taxonomy.....	65
Discussion.....	69
References.....	74
CHAPTER4. Host jumping onto close relatives and across kingdoms by <i>Tyrannocordyceps fraticida</i> gen. nov. and <i>Ustilaginoidea</i>	
Abstract.....	87
Introduction.....	89
Materials and Methods.....	93
Specimen Collection.....	93
DNA extraction, PCR and sequencing.....	94

TABLE OF CONTENTS (CONTINUED)

	<u>Page</u>
Phylogenetic analyses.....	95
Ancestral Character-State Reconstruction.....	97
Morphological Examination.....	98
Results.....	98
Gene sampling and phylogenetic analyses.....	98
Ancestral character-state reconstructions.....	99
Morphological Examination.....	100
Taxonomy.....	101
Discussion.....	103
References.....	109
CHAPTER5. The Evolution of Stipe Morphology in Hypocreales.....	121
Abstract.....	122
Introduction.....	123
Materials and Methods.....	128
Specimen Collection.....	128
DNA extraction, PCR and sequencing.....	128
Phylogenetic analyses.....	129
Ancestral Character-State Reconstruction and correlation analyses.....	130
Results and Discussion.....	132
Phylogenetic analyses.....	132
Relationships and ancestral character-state reconstructions of Hypocreales.....	133
Hypocreaceae.....	133
Cordycipitaceae.....	134
Ophiocordycipitaceae.....	136
Clavicipitaceae.....	139
Character Correlations.....	140
Conclusions.....	142

TABLE OF CONTENTS (CONTINUED)

	<u>Page</u>
References.....	145
CHAPTER 6. Conclusion.....	161
References.....	166

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
2.1 Clade for the family Clavicipitaceae extracted from a larger maximum likelihood tree obtained from the analysis of the 135 species concatenated, five-gene dataset.	42
2.2 A. Examples of <i>Metacordyceps</i> from this study	44
3.1 Maximum Likelihood tree obtained from analysis in RAxML of a concatenated 5 gene dataset (SSU, LSU, TEF, RPB1, RPB2) showing placement of <i>Veterocordyceps</i>	78
3.2 <i>Veterocordyceps prolifica</i> subg. <i>Veterocordyceps</i> and <i>Veterocordyceps pleuricapitata</i> subg. <i>Pulvinata</i> . A-C <i>Veterocordyceps prolifica</i>	79
4.1 Strict consensus tree produced by Bayesian analysis of a concatenated dataset of 5 genes (SSU, LSU, TEF, RPB1, RPB2) showing the placement of <i>T. fratricida</i> and <i>U. virens</i>	113
4.2 Macroscopic and microscopic features of <i>U. virens</i> and <i>T. fratricida</i>	115
5.1 Maximum Likelihood mirror tree of the concatenated 5 gene data set.....	148
5.2 Detail of the families Hypocreaceae and Cordycipitaceae from the tree depicted in Fig. 5.1.....	149
5.3 Detail of the family Ophiocordycipitaceae from the tree depicted in Fig. 5.1.....	150
5.4 Detail of the family Clavicipitaceae from the tree depicted in Fig. 5.1.....	151
5.5 Species representative of the diversity of morphological types present in clavicipitaceous fungi.....	152

LIST OF TABLES

<u>Table</u>	<u>Page</u>
2.1 Information for primers used for PCR in this study.....	45
2.2 Specimen information for materials used in this study.....	46
2.3 Morphological characteristics of <i>Metacordyceps</i> species.....	51
3.1 Information for primers used for PCR in this study.....	80
3.2 Taxon, specimen voucher and sequence information for specimens used in this study.....	81
4.1 Information for primers used to amplify sequences used in this study.....	116
4.2 Taxon, specimen voucher and sequence information for specimens used in this study.....	117
4.3 Likelihood scores and posterior probabilities from Maximum Likelihood and Bayesian ancestral character-state reconstructions.....	120
5.1 Information for primers used in this study.....	153
5.2 Voucher information, character coding and genbank number for taxa used in this study.....	154
5.3 Results of ancestral character state reconstructions.....	160

CHAPTER 1 – INTRODUCTION

TAXONOMIC HISTORY OF THE CLAVICIPITACEOUS FUNGI

The historical reliance of mycologists on gross morphological features for classification, which as Miller (1949) pointed out “fluctuate with varying environments or ecological habitats”, resulted in largely artificial groupings of taxa. The family Clavicipitaceae (Sordariomycetes: Hypocreales) was first recognized as a subfamily of the Hypocreaceae by Lindau (in Engler and Prantl 1897) centered on the plant pathogenic genus *Claviceps* Tul. This is a morphologically and ecologically diverse family characterized by the production of cylindrical asci bearing a thickened apical cap within perithecia. Ascospores are filiform, usually forming partspores that disarticulate at maturity. The taxonomic status for the family has fluctuated dramatically over the years. Nannfeldt (1932) recognized these fungi as a unique group and established them as the order Clavicipitales. Based on characteristics of the thickened ascus tip and the well-defined perithecia Miller (1949) considered them to be a family in the Xylariales (=Sphaeriales sensu Miller). In a review of the hypocrealean fungi by Rogerson (1970), the order Clavicipitales, containing only the family Clavicipitaceae, was reinstated and recognized as closely related to Hypocreales.

In spite of the attention and study given to the group, the higher relations of clavicipitaceous fungi remained unresolved until the introduction of molecular

phylogenetic methods by Spatafora and Blackwell (1993). Using small subunit ribosomal DNA sequence data, they resolved Hypocreales, not Xylariales, as sister to Clavicipitales. Over time, the ordinal classification of Clavicipitales gave way to the recognition of the family Clavicipitaceae as a family within Hypocreales, e.g., Rossman et al. (1999). Using a dataset containing species across the entire spread of the class Sordariomycetes, Zhang et al. (2006) confirmed placement of Clavicipitaceae within Hypocreales and supported the sister relationship with Hypocreaceae recovered by Spatafora and Blackwell (1993).

Following on the work of previous researchers, e.g., Gauman (1926), Diehl (1950) attempted to organize relationships within Clavicipitaceae, establishing three subfamilies: Oomycetoidea, containing *Ascopolyporus* and *Hypocrella*. The Clavicipitoidea was established for plant associated species and included three tribes, Clavicipiteae for the genus *Claviceps*, Balansiae for *Atkinsonella*, *Balansia* and *Epichloë*. The tribe Ustilaginoidea was established for *Munkia* and *Ustilaginoidea*. The majority of the insect associated genera including *Cordyceps* and *Torrubiella* were included in Cordycipitoidea. These distinctions relied heavily on characters of conidia, the spores produced during asexual reproduction.

As with the higher-level relationships of Clavicipitaceae, molecular phylogenetic investigation of the family revealed contradictions to morphology based classifications. Initial investigations with ribosomal DNA exposed a high degree of diversity within the family. However limited taxon sampling and resolving power of the genes used prevented further reclassification (Artjariyasripong et al. 2001, Sung et

al. 2001, Stenstrup et al. 2005). Inclusion of a greater breadth of species, particularly the diverse and species rich genus *Cordyceps*, as well as the introduction of protein coding genes finally rejected the subfamilial classification of Diehl (Spatafora et al. 2007, Sung et al. 2007) resulting in the recognition of three families of clavicipitaceous fungi (Sung et al. 2007). Cordycipitaceae was erected to contain the clade of fungi surrounding the type for *Cordyceps*, *C. militaris* (L:Fr.) Link, as well as *Torrubiella* and the enigmatic genera *Ascopolyporus* and *Hyperdermium*. The family Ophiocordycipitaceae contains *Cordyceps* species transferred to the resurrected genus *Ophiocordyceps* sensu Petch, owing to the placement of *O. unilateralis* (Tul.) Petch. Ophiocordycipitaceae also contain species pathogenic on false truffles and cicada nymphs in the genus *Elaphocordyceps*. Clavicipitaceae are now recognized in a much more restricted sense, and includes the genera *Hypocrella*, and *Regiocrella*. The plant-associated genera of the subfamily Clavicipitoidea are also included, although this taxon is no longer recognized. A handful of species previously placed in *Cordyceps* has remained in the family, and are now classified as *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora. Although the work of Sung et al. (2007) was the most extensive investigation of clavicipitaceous fungi to date, the majority of taxa from *Cordyceps* and other genera remain unsampled and are considered incertae sedis.

ECOLOGICAL AND MORPHOLOGICAL CHARACTERISTICS OF CLAVICIPITACEOUS FUNGI

Clavicipitaceous fungi are globally distributed, and can be found on every continent, including Antarctica (Roddam and Rath 1997, Hughes and Lawley 2003). They are predominantly forest species and may be found in tropical or temperate deciduous and coniferous habitats. However notable exceptions exist, such as *O. sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, which inhabits grasslands of the Tibetan plateau. East Asia and Southeast Asia is the center of known diversity, however other areas of the world are still poorly sampled. They exploit a wide range of niches, and may be buried in soil or rotten wood, in the leaf litter, or exposed on the underside of leaf surfaces or stems. They are reliant on wet humid conditions, and valleys or stream corridors are especially good habitats.

All species exhibit a pathogenic lifestyle that encompasses a broad range of affiliations across plant, arthropod and fungal hosts. Although any one species is generally restricted to a single host species or set of closely related species, arthropod hosts span twelve orders (Kobayasi 1941, 1982). Arthropod pathogens can be found in all three families of clavicipitaceous fungi, and host affiliation generally lacks phylogenetic signal. However, host may be informative between closely related taxa.

Plant associated species most commonly infect grasses (Poales: Poaceae) or sedges (Poales: Cyperaceae), however *Shimizuomyces paradoxus* Kobayasi infects seeds of the genus *Smilax* (Liliales: Smilacaceae). All plant-associated fungi are restricted to Clavicipitaceae, however species infecting grasses and sedges share a common ancestor, while *S. paradoxus* derived the lifestyle independently within the family (Sung et al. 2007). Fungal hosts include false truffles in the genus

Elaphomyces, as well as sclerotia of *Claviceps*. Pathogens of *Elaphomyces* are restricted to *Elaphocordyceps* in Ophiocordycipitaceae, however *Elaphocordyceps* also attacks cicada nymphs and beetle larvae and represent recent host jumps from truffles to below ground and wood-inhabiting immature stages of arthropods (Spatafora et al. 2007). *Cordyceps* pathogens of *Claviceps* sclerotia are currently unexamined and of uncertain phylogenetic placement.

The diversity of morphologies exhibited by clavicipitaceous fungi is comparable to the range of hosts exploited. The majority of species produce perithecia on an elevated stroma or stipe, although astipitate forms are also common. The presence or absence of a stipe was considered a phylogenetically informative character for separation of the genera *Cordyceps* and *Torrubiella*, however work by Johnson et al. (2009) determined the astipitate species to be distributed throughout all three families of clavicipitaceous fungi. The presentation of perithecia for both stipitate and astipitate species may be superficial, pseudoimmersed in a mass of fluffy hyphae, or fully immersed in the stroma. Coloration may range from pale (white, cream or pallid) to bright (yellow, orange or red) to dark (black, brown or olive green).

Sung et al. (2007) determined that the genera of *Cordyceps*-like fungi to emerge from phylogenetic analyses could be determined in part by combinations of habitat/niche and stromatal characters. Species in *Cordyceps* s.s. display pallid to brightly colored stromata that are fleshy in texture and are most common in leaf litter or shallow soil habitats. In contrast, *Ophiocordyceps* species typically have firm to pliant or wiry stromata that are darkly colored and typically occur deeply buried in soil

or rotten wood. Members of *Elaphocordyceps* are distinguished from *Ophiocordyceps* by predominantly infecting *Elaphomyces* false truffles. Characters able to identify *Metacordyceps* are relatively. Stromatal color is typically white to lilac, purple or green, often turning black or dark purple when bruised or dried. These characters are often convergent and morphological characters able to unambiguously place taxa are still lacking.

The morphology of the anamorph (asexual reproductive state), is also phylogenetically informative. However, as with other morphological characters exceptions are known. For Cordycipitaceae, *Beauveria*, *Engyodontium*, *Gibellula*, *Isaria*, *Lecanicillium*, and *Simplicillium* anamorphs are most common. In Ophiocordycipitaceae *Haptocillium*, *Hirsutella*, and *Hymenostilbe* are the major anamorph types. Clavicipitaceae was found to be associated with *Aschersonia*, *Ephelis*, *Metarhizium*, *Pochonia*, and *Sphacelia* anamorph types. Anamorphic forms are able to persist on a much broader range of substrates than their sexual counterparts and are commonly isolated from soil lacking any obvious host.

HUMAN USES OF CLAVICIPITACEOUS FUNGI

The species *O. sinensis* has been used for centuries in Traditional Chinese Medicine. Although considered a general tonic to improve health, specific uses of this fungus include treatment of pulmonary ailments as well as renal conditions. *Ophiocordyceps sinensis* has recently been investigated for an anti-tumor activity. It

is thought the nucleotide analogue cordycepin blocks polyadenylation thereby limiting tumor growth (Im 2003). The global market for *O. sinensis* has expanded rapidly in recent years. This has increased the collection pressure in the grasslands of the Tibetan Plateau. As a result there is growing concern as to the sustainability of current collection practices, and efforts are underway to examine these effects (Stone 2008).

Tolypocladium inflatum (W. Gams) is another species useful as a source of medicine. Commonly occurring globally as a soil fungus, *T. inflatum* is the biological source of cyclosporin A, a compound used as an immune suppressant for recipients of organ transplants. *Tolypocladium inflatum* has been identified as the asexual state of *Elaphocordyceps subsessilis* (Petch) G.H. Sung, J.M. Sung, and Spatafora (Hodge et al. 1996, Sung et al. 2007).

In addition to their importance in medicine, clavicipitaceous fungi have received attention as biological control agents against a wide range of pests. The most commonly used species are those of *Metarhizium anisopliae* (Metchn.) Sorokin and *Beauveria bassiana* (Bals.-Criv.) Vuill., of Clavicipitaceae and Cordycipitaceae respectively. Both species are relatively easy to grow and can be found world wide. *Metarhizium anisopliae* and *B. bassiana* have been investigated for their biological control potential owing to their expansive host ranges. However, recent molecular investigations have revealed a higher degree of phylogenetic structure than previously suspected and the expansive host range may in fact be a case of cryptic speciation (Bischoff et al. 2009, Rehner and Buckley 2005).

PREMISE AND TIMELINE OF THE STUDY

This research was conducted as part of a Partnership for Enhancing Expertise in Taxonomy (PEET) program of The National Science Foundation. The purpose of the study was to study the evolution and relationships of clavicipitaceous fungi to aid in identification and delineation of taxonomic boundaries through the use of molecular and morphological data. Specimens were obtained through intensive field collection efforts, as well as collaborations with researchers' throughout the world, but focused primarily on East and Southeast Asia. Fieldwork was conducted over four seasons from 2006-2009. An initial collecting trip was conducted over two weeks in August of 2006 in the Southeastern United States. The summers of 2007 and 2008 were spent in Japan for field collecting and herbarium work at the National Museum of Science and Nature in Tsukuba. The summer of 2009 was spent in China, with work conducted at the Chinese Academy of Sciences in Beijing

References

- ARTJARIYASRIPONG, S., J.I. MITCHELL, N.L. HYWEL-JONES, AND E.B. GARATH JONES. 2001. Relationships of the genus *Cordyceps* and related genera, based on parsimony and spectral analysis of partial 18S and 28S ribosomal gene sequences. *Mycoscience* 42: 503-517.
- BAN, S., T. SAKANE, K. TOYAMA, AND A. NAKAGIRI. 2009. Teleomorph-anamorph relationships and reclassification of *Cordyceps cuboidea* and its allied species. *Mycoscience* 50: 261-272.
- BISCHOFF, J.F., S.A. REHNER, AND R.A. HUMBER. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* 101: 512-530.
- DIEHL, W.W. 1950. *Balansia* and the *Balansiae* in America. Agricultural monograph No. 4. U. S. D. A., Washington.
- GAUMANN, E. 1926. *Vergleichende morphologie der pilze*. 626 pp., illus, Jena.
- HODGE, K.T., S.B. KRASNOFF, AND R.A. HUMBER. 1996. *Tolypocladium inflatum* is the anamorph of *Cordyceps subsessilis*. *Mycologia* 88: 715-719.
- HUGHES, K.A., AND B. LAWLEY. 2003. A novel Antarctic microbial endolithic community within gypsum crusts. *Environmental Microbiology* 5: 555-565.
- IM, D. 2003. Linking Chinese medicine and G-protein-coupled receptors. *Trends in Pharmacological Sciences* 24: 2-4.
- JOHNSON, D. ET AL. 2009. Systematics and evolution of the genus *Torrubiella* (Hypocreales, Ascomycota). *Mycological Research* 113: 279-289.
- KOBAYASI, Y. 1981. Revision of the genus *Cordyceps* and its allies 1. *Bulletin of the National Science Museum, Tokyo*, series B 7: 1-13.
- KOBAYASI, Y. 1941. The genus *Cordyceps* and its allies. *Science reports of the Tokyo Bunrika Daigaku* 84: 53-260.
- LINDAU, G. 1897. Hypocreales. In A. Engler, and K. Prantl [eds.], *Die natürlichen Pflanzenfamilien*, 343-372. Verlag W. Engleman, Leipzig.
- MILLER, J.H. 1949. A revision of the classification of the ascomycetes with special emphasis on the pyrenomycetes. *Mycologia* 41: 99-127.

- NANNFELDT, J.A. 1932. Studien uber die morphologie und systematik der nicht-lichenisierten, inoperculaten Discomyceten. *Nova Acta R. Soc. Scient. upsal. ser 6* 8: 1-368.
- REHNER, S.A., AND E. BUCKLEY. 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1- α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97: 84-98.
- RODDAM, L.F., AND A.C. RATH. 1997. Isolation and Characterization of *Metarhizium anisopliae* and *Beauveria bassiana* from Subantarctic Macquarie Island. *Journal of Invertebrate Pathology* 69: 285-288.
- ROGERSON, C.T. 1970. The hypocrealean fungi (Ascomycetes, Hypocreales). *Mycologia* 62: 865-910.
- ROSSMAN, A.Y., G.J. SAMUELS, C.T. ROGERSON, AND R. LOWEN. 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). *Studies in Mycology* 1-248.
- SPATAFORA, J.W., AND M. BLACKWELL. 1993. Molecular systematics of unitunicate perithecial ascomycetes: The Clavicipitales-Hypocreales connection. *Mycologia* 85: 912-922.
- SPATAFORA, J.W., G.-. SUNG, J.-. SUNG, N.L. HYWEL-JONES, AND J.R. WHITE. 2007. Phylogenetic evidence for an animal pathogen origin for ergot and the grass endophytes. *Molecular Ecology* 16: 1701-1711.
- STENSRUD, O., N.L. HYWEL-JONES, AND T. SCHUMACHER. 2005. Towards a phylogenetic classification of *Cordyceps*: ITS nrDNA sequence data confirm divergent lineages and paraphyly. *Mycological research* 109: 41-56.
- STONE, R. 2008. MYCOLOGY: Last Stand for the Body Snatcher of the Himalayas? *Science* 322: 1182.
- SUNG, G., N.L. HYWEL-JONES, J. SUNG, J.J. LUANGSA-ARD, B. SHRESTHA, AND J.W. SPATAFORA. 2007. Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud Mycol* 57: 5-59.
- SUNG, G., J.W. SPATAFORA, R. ZARE, K.T. HODGE, AND W. GAMS. 2001. A revision of *Verticillium* sect. *Prostrata*. II. Phylogenetic analyses of SSU and LSU nuclear rDNA sequences from anamorphs and teleomorphs of the Clavicipitaceae. *NOVA HEDWIGIA* 72: 311-328.

- WENGER, R.M. 1984. Synthesis of cyclosporine. Total syntheses of 'cyclosporin A' and 'cyclosporin H', two fungal metabolites isolated from the species *Tolypocladium inflatum* GAMS. *Helvetica Chimica Acta* 67: 502-525.
- ZHANG, N. ET AL. 2006. An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia* 98: 1076-1087.

Chapter 2

New teleomorph combinations in the entomopathogenic genus *Metacordyceps*

Ryan Kepler and Joseph W. Spatafora

Department of Botany and Plant Pathology
Oregon State University
Corvallis, OR 97331
USA

To be submitted to *Mycologia*

ABSTRACT

The genus *Metacordyceps* was erected to accommodate arthropod pathogenic species in Clavicipitaceae (Hypocreales) that were formerly classified in *Cordyceps* sensu Kobayasi *et* Mains. Of the current arthropod pathogenic genera of Hypocreales, the genus *Metacordyceps* remains one of the most poorly understood and contains a number of teleomorphic morphologies convergent with species of *Cordyceps* s.s. (Cordycipitaceae) and *Ophiocordyceps* (Ophiocordycipitaceae). Importantly, the anamorph genera *Metarhizium* and *Pochonia* were found to be associated only with *Metacordyceps* and demonstrated to be phylogenetically informative for the clade. Several species of *Cordyceps* considered incertae sedis in the current taxonomic framework of clavicipitaceous fungi were collected during field expeditions with an emphasis in East Asia. Species confirmed to be members of *Metacordyceps* include *Cordyceps atrovirens* Kobayasi & Shimizu, *Cordyceps indigotica* Kobayasi & Shimizu, *Cordyceps khaoyaiensis* Hywel-Jones, *Cordyceps kusanagiensis* Kobayasi & Shimizu, *Cordyceps martialis* Speg., *Cordyceps owariensis* Kobayasi, *Cordyceps owariensis* var. *viridescens* Uchiy. & Udagawa, and *Cordyceps pseudoatrovirens* Kobayasi & Shimizu.

Incorporation of these species in a multigene phylogenetic framework of the major clades of clavicipitaceous fungi more than doubled the number of species in *Metacordyceps* and allowed for refinement of morphological concepts for the genus consistent with the phylogenetic structure. Based on these findings we discuss evolution of the genus, subgeneric relationships, anamorph connections, and highlight

additional species that should be sampled for inclusion in *Metacordyceps* when appropriate field material is discovered.

Key words: anamorph-teleomorph connection, arthropod pathogen, Clavicipitaceae, *Metarhizium*, morphological plasticity, *Pochonia*

INTRODUCTION

The genus *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora was defined for a handful of *Cordyceps* species remaining in Clavicipitaceae s.s. after molecular phylogenetic investigations revealed that *Cordyceps* sensu Kobayasi (1941, 1982) was polyphyletic, resulting in taxonomic revision at the levels of family and genus (Sung et al. 2007). That work focused on the genus *Cordyceps* Fr., which previously contained over 400 species attacking a range of insect and fungal hosts. Many species of *Cordyceps* sensu lato now reside in the families Cordycipitaceae, for which the genus *Cordyceps* has been retained owing to the placement of the type *C. militaris* (L.) Link, and the genera *Ophiocordyceps* Petch and *Elaphocordyceps* G.H. Sung, J.M. Sung & Spatafora in Ophiocordycipitaceae. However, there remains a significant number of *Cordyceps* s.l. species for which molecular or morphological data are insufficient or lacking, and are therefore considered incertae sedis with regards to family classification until new material is available for examination. Of the genera to emerge from the new taxonomy, *Metacordyceps* remains poorly understood owing to the relatively few species it contains and the convergent nature of morphological characters with other genera (Sung et al. 2007).

Sung et al. (2007) determined that morphological characters associated with color and texture of the stromata and ecological traits correlated with habitat were most consistent with the clades of *Cordyceps*, *Elaphocordyceps* and *Ophiocordyceps*. However, for *Metacordyceps*, morphological characters able to characterize the genus

remain elusive. The range of character states for all clavicipitaceous fungi are represented to a greater or lesser extent within the genus, although texture of the stipe and color were identified as useful. All seven known species of *Metacordyceps* produce stipitate fruiting structures that are typically fibrous in texture. The color may range from whitish to yellow-green to green to lilac or purple, turning deep purple or black upon bruising or drying. Some produce rhizomorphs or mycelial threads, connecting the aboveground structures to hosts that are buried in the soil (Liu et al. 2001; Zhang et al. 2004). Species of *Cordyceps* s.l. with stromata that are very bright and fleshy, e.g., *C. militaris*, or very dark and pliant or wiry, e.g., *O. unilateralis* (Tul.) Petch, are more likely to be found in the families Cordycipitaceae and Ophiocordycipitaceae, respectively, although exceptions are known. Characters of the perithecia, ascus and ascospore are the most variable and convergent throughout all clavicipitaceous fungi (Sung et al. 2007). Perithecia may be scattered or crowded and partially to completely immersed in the stroma with an ordinal to oblique angle. Asci are typical for the family, long and cylindrical with a well-defined apical cap, and ascospores, although filiform, may or may not form septations and may or may not disarticulate at maturity into partspores (Mains 1958; Kobayasi 1982; Rossman et al. 1999).

Host associations are also diverse for *Metacordyceps*. Soil dwelling species from several families of Coleoptera and Lepidoptera are the most commonly exploited hosts, however, *M. chlamydosporia* (H.C. Evans) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora attacks mollusc egg cases (Zare et al. 2001). Exact determination of host

species is often difficult owing to complications with identification of immature insects, and the extreme disfiguration of the cadaver that can accompany infection.

Although most morphological characteristics are inadequate for definitive placement in *Metacordyceps*, anamorph morphology can be useful. The genera *Metarhizium* and *Pochonia* were found to be associated only with the *Metacordyceps* clade (Sung et al. 2007). The number of species names associated with the anamorphic forms greatly exceeds those known for the teleomorphs. Currently, only seven species have been placed in *Metacordyceps*, while *Metarhizium* alone has 29 species with numerous forms and varieties. *Nomuraea* and *Paecilomyces*-like anamorphs have also been placed in the *Metacordyceps* clade (Sung et al. 2007; Sosa-Gómez et al. 2009). However, *Paecilomyces*-like anamorphs are found throughout Hypocreales (Luangsa-ard et al. 2004; Luangsa-ard et al. 2005), and *Nomuraea* is polyphyletic with green-spored species in Clavicipitaceae and lilac-spored species in Ophiocordycipitaceae (Sung et al. 2007). It is therefore difficult to assess the taxonomic properties of these anamorph genera. Anamorph-teleomorph connections have been established through observation of co-occurrence on the same host, cultures derived from single spores and molecular phylogenetic reconstructions (Liu et al. 2002; Liu et al. 2001). Linking sexual and asexual states of species in *Metacordyceps* remains a challenge, hampered by the inability to culture and induce formation of fertile stromata.

Biological control practitioners have been interested in the anamorphic forms of *Metacordyceps* for over a century. *Metarhizium anisopliae* (Metschn.) Sorokin was

one of the first organisms seriously investigated for use against agricultural pests. Metchnikoff undertook investigation of this fungus for use against the wheat cockchafer *Anisoplia austriaca* Herbst (Coleoptera: Scarabaeidae) in 1879 and although early successes were promising difficulties in large-scale production ultimately ended development. Since that time understanding of the basic biology and ecology has advanced and numerous other systems have been tested. Recent successes include applications against mosquito vectors of malaria (Scholte et al. 2005; Kanzok and Jacobs-Lorena 2006) and locusts (Lomer et al. 2001) throughout Africa. The host and environmental range for *Mr. anisopliae* has traditionally been considered broad, but a molecular evaluation of isolates in the *Mr. anisopliae* clade has revealed numerous phylogenetic species that may in fact show greater host specificity (Bischoff et al. 2009). *Nomuraea rileyi* (Farl.) Samson has a much more limited host range than *M. anisopliae* and is able to cause massive epizootic events against lepidopteran pests, especially species in the Noctuidae (Ignoffo 1981). *Pochonia chlamydosporia* (Goddard) Zare & W. Gams is able to control root nematodes (Kerry et al. 1982).

In this work, we examine teleomorphs currently considered incertae sedis or unexamined with molecular data obtained through extensive field collection efforts in China and Japan. By placing sequence data from five genes in a dataset comprised of taxa from the major clades of Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae identified by Sung et al. (2007) we conclude that *C. atrovirens* Kobayasi & Shimizu, *C. indigotica* Kobayasi & Shimizu, *C. khaoyaiensis* Hywel-

Jones, *C. kusanagiensis* Kobayasi & Shimizu, *C. martialis* Speg., *O. owariensis* Kobayasi & Shimizu and *C. pseudoatrovirens* Kobayasi & Shimizu should be transferred to the genus *Metacordyceps*. The addition of these taxa into the genus allows for a strengthening of the morphological concepts that define the genus and provides additional insight into evolution of the genus and species that should be targeted for future sampling as specimens are made available.

MATERIALS AND METHODS

Specimen collection

Specimens were collected from field sites in China and Japan during the months of Jun.-Aug. in the years 2007, 2008, 2009. Upon collection, specimens were cleaned of dirt and a piece of tissue was removed and placed in CTAB buffer for DNA extraction, with the remainder placed in a wax paper bag and later air dried for storage. Material of *C. owariensis* var. *viridescens* was obtained from ex-type culture deposited at the National Institute of Technology and Evaluation (NITE) Biotechnological Resource Center.

DNA extraction, PCR and sequencing

DNA extraction relied on a CTAB protocol consisting of an initial grinding of stromatal and perithecial tissue with a power drill affixed with an Eppendorf pestle in 50 μ l CTAB buffer. Following this initial mastication step, an additional 400 μ l of CTAB was combined with tissue in a FastDNA lysing matrix A tube (MP Biomedical, Salon, OH), and ground further with the fast prep machine for two cycles, 20 seconds each. A 20-minute water bath at 60°C following mastication was performed to aid cell lysis. Tissue was then separated from supernatant by centrifugation for 10 minutes at 14000rpm. Four hundred microliters of supernatant was then removed for further cleaning with centrifugation at 14000 rpm for 20 minutes in a gradient with 500 μ l chloroform:isoamyl alcohol (24:1). Purified DNA was obtained and concentrated by removing 300 μ l of top layer of liquid after chloroform:isoamyl centrifugation using the GeneCleanIII Kit, following the recommended protocol and eluting from glass milk with 30 μ l of water.

PCR methods were used to amplify a total of six nuclear loci for each specimen. As an initial quality control step, and to serve as a voucher for barcoding efforts, the complete span of the internal transcribed spacer region of ribosomal DNA (ITS1-5.8s-ITS2) was amplified and sequenced. A BLAST search of the GenBank database was used to determine that the closest hit was in Clavicipitaceae. After passing quality control measures, five nuclear loci were amplified and sequenced for phylogenetic analysis: fragments of small subunit and large subunits of nuclear ribosomal DNA (SSU and LSU, respectively), elongation factor 1 α (TEF), and the largest and second largest subunits of RNA polymerase II (RPB1 and RPB2,

respectively). Primer information is given in Table 1. PCR reactions were performed in either an iCycler or MyCycler thermocycler (BioRad, Hercules, CA) using MasterAmp 2X PCR premix E (Epicenter, Madison WI) and Novagen Taq polymerase. Reaction conditions were the same as those used in Johnson et al. (2009). PCR products were cleaned using the GeneClean III kit following the manufacturers instructions and sequenced using the Macrogen (Seoul, South Korea) sequencing service with the primers used for the initial amplifications.

Phylogenetic analyses

Raw sequence reads returned from Macrogen were edited using CodonCode Aligner, version 2.0.6 (Dedham, MA). Sequences generated in this study were combined with previously published data for species in Hypocreales. Genbank and specimen voucher information is provided in Table 2.2. Individual gene alignments were generated using MAFFT version 6 (Kato et al. 2002; Kato and Toh 2008), and improved by direct examination with the program BioEdit version 7.05 (Hall 1999). Ambiguously aligned regions were excluded from phylogenetic analyses and gaps were treated as missing data. The dataset consisted of 135 taxa and after exclusion of ambiguously aligned sites consisted of 1089 nucleotides for SSU, 884 for LSU, 998 for TEF, 698 for RPB1, and 1048 for RPB2. The total length of aligned sequences was 4717 nucleotides. For all specimens examined, at least three of the five genes sought were obtained (Table 1). Analysis of neighbor joining trees between individual

genes with *compat.py* (Kauff et al. 2002) did not reveal any significant levels of conflict among the sequences. Eleven data partitions were defined for the final combined dataset, one each for nu-rSSU and nu-rLSU plus nine for each of the three codon positions for the protein coding genes *TEF*, *RPB1*, and *RPB2*.

The phylogenetic relationships for *Metacordyceps* were estimated under two separate criteria: Maximum Likelihood and Bayesian. Maximum Likelihood (ML) estimation of phylogeny was performed with RAxML version 7.0.4 (Stamatakis 2006) with 500 rapid bootstrap replicates on a concatenated dataset containing all five genes. The CAT-GAMMA model of evolution was employed during the rapid bootstrapping phase and the GTR-GAMMA model of evolution was specified for the final likelihood tree as suggested by the program manual for large datasets. Bayesian estimation of phylogenetic relationships was conducted with the program Mr. Bayes v3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The partitions were applied in the same manner as for the ML analyses and the GTR+I+G nucleotide substitution model was used for all gene partitions. Two runs were conducted simultaneously, each with four chains for 10 million generations. Each chain was sampled every 500 generations and the tree saved with branch length information. Each run was examined with the program Tracer v1.5 (Drummond and Rambaut 2007) to determine burn-in and confirm both runs had converged. Summary of the model parameters was determined with the *sump* command. A strict consensus tree with branch lengths and posterior probabilities was then obtained with the *sumt* command. The same burnin value was used for both *sump* and *sumt* commands.

Analyses were conducted on the Genome High Performance Computing Cluster in the Center for Genome Research and Bioinformatics at Oregon State University.

RESULTS

The results of the ML analysis yielded a tree with a log likelihood of -103328.231253. For the Bayesian analysis, the burnin was determined to be 25000 after graphing the split likelihoods of both runs in Tracer. The topologies between the two programs were largely congruent with differences confined to nodes not strongly supported by bootstrap partitions in the RAxML analyses.

The addition of the new taxa did not affect the family level relationships observed in previous studies (Fig. 2.1). The family Clavicipitaceae was strongly supported in all analyses. The genus *Metacordyceps* was strongly supported in Clavicipitaceae as separate from other clades containing entomopathogenic fungi, e.g., *Hypocrella*. The placement of new taxa within *Metacordyceps* resulted in an emergent phylogenetic structure to the genus. The backbone consisted of a phylogenetic grade of teleomorphs associated with anamorphic forms of *Pochonia*, *Rotiferophthora* and *Paecilomyces*. This includes the species *M. chlamydosporia* and *M. yongmunensis* as determined previously, as well as *M. khaoyaiensis* and *M. kusanagiensis* placed in this work. Relationships among backbone taxa remain poorly supported with BP less than 70%. High support values were recovered for a clade containing the *M. taii* and anamorphic genera *Metarhizium* and *Nomuraea*. New

teleomorphs placed in this clade include *C. atrovirens*, *C. indigotica*, *C. martialis*, *C. owariensis* and *C. pseudoatrovirens*. The anamorph of *M. pseudoatrovirens* differs from other species and is morphologically similar to *Pochonia*. We also place what is apparently a new species (*Metacordyceps* sp. HMAS 199601) in the core clade. The anamorph of this species produces green conidia on a robust synemma. However only one specimen of the teleomorph was collected and we defer description of a new species until additional specimens can be collected to better understand the range of variability present for morphological characters.

The distribution of morphological characters reflects the structure suggested by phylogenetic reconstructions. Among taxa along the backbone, the stroma is a white or pallid color, with lilac colored perithecia in *C. khaoyaiensis*. The anamorphic forms are hyaline and otherwise lacking pigmentation. Within the core clade of *Metacordyceps*, stromata are usually strongly pigmented in some fashion. Bright yellow or green are the most common colors, but *C. martialis* is dark brick-red or orange. Similarly, *Metarhizium* and *Nomuraea* anamorphs found in this clade are pigmented green or brown. However, the anamorph of *C. pseudoatrovirens* differs from closely related anamorph types and is hyaline in color, lacking pigmentation. Based on these multi-gene phylogenetic results we propose the following taxonomic revisions and combinations.

TAXONOMY

The phylogenetic placement of the species studied in this analysis supports their inclusion in the genus *Metacordyceps*. We therefore make the following new combinations:

Metacordyceps atrovirens (Kobayasi & Shimizu) Kepler, G.-H. Sung, J.W.

Spatafora comb. nov.

≡ *Cordyceps atrovirens* Kobayasi & Shimizu, Bull. Natn. Sci. Mus. Tokyo, Ser. B, 4: 52. (1978).

Metacordyceps indigotica (Kobayasi & Shimizu) Kepler, G.-H. Sung, J.W. *Spatafora* comb. nov.

≡ *Cordyceps indigotica* Kobayasi & Shimizu, Bull. Natn. Sci. Mus. Tokyo, Ser. B, 4: 52. (1978).

Anamorph: *Metarhizium* like

Metacordyceps khaoyaiensis (Hywel-Jones) Kepler, G.-H. Sung, J.W. *Spatafora* comb. nov.

≡ *Cordyceps khaoyaiensis* Hywel-Jones, Mycol. Res. 98: 939 (1994).

Anamorph: *Pochonia* like

Metacordyceps kusanagiensis (Kobayasi & Shimizu) Kepler, G.-H. Sung, J.W.

Spatafora comb. nov.

≡ *Cordyceps kusanagiensis* Kobayasi & Shimizu, Bull. Natn. Sci. Mus. Tokyo, Ser B, 9:7 (1983).

Metacordyceps martialis (Speg.) Kepler, G.-H. Sung, J.W. *Spatafora* comb. nov.

≡*Cordyceps martialis* Speg., Boletín, Academia nacional de Ciencias, Córdoba 11: 535, (1889).

Metacordyceps owariensis (Kobayasi) Kepler, G.-H. Sung, J.W. Spatafora comb. nov.

≡*Ophiocordyceps owariensis* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones, Spatafora, Stud. Mycol. 57: 45 (2007)

≡*Cordyceps owariensis* Kobayasi, Bulletin of the biogeographical Society of Japan 9: 166 (1939).

Metacordyceps owariensis var. **viridescens** (Uchiy. & Udagawa) Kepler, G.-H. Sung, J.W. Spatafora comb. nov.

≡*Ophiocordyceps owariensis* var. *viridescens* (Uchiy. & Udagawa) G.H. Sung, J.M. Sung, Hywel-Jones, Spatafora, Stud. Mycol. 57: 45 (2007)

≡*Cordyceps owariensis* var. *viridescens* Uchiy. & Udagawa, Mycoscience 43: 136 (2002)

Anamorph: *Nomuraea owariensis* Uchiy. & Udagawa

Metacordyceps pseudoatrovirens (Kobayasi & Shimizu) Kepler, G.-H. Sung, J.W. Spatafora comb. nov.

≡*Cordyceps pseudoatrovirens* Kobayasi & Shimizu, Bull. Natn. Sci. Mus. Tokyo, Ser B, 8: 111 (1982).

DISCUSSION

Phylogenetic structure of *Metacordyceps*

The work of Sung et al. (2007) placed six teleomorphic species in the genus *Metacordyceps* and an additional species was added recently by Li et al. (2010). The data presented here more than doubles the number of associated taxa with the addition of seven species and one variety. The genus remains well supported as a distinct member of Clavicipitaceae, separate from both the plant symbionts and other insect pathogens. We identify a core clade of the genus *Metacordyceps* containing the species *M. atrovirens*, *M. indigotica*, *M. martialis*, *M. owariensis*, *M. pseudoatrovirens* and *Metacordyceps* sp. *HMAS 199601* from this study as well as *M. brittlebankisoides* (Zuo Y. Liu, Z.Q. Liang, Whalley, Y.J. Yao & A.Y. Liu) G.H. Sung, J.M. Sung, Hywel-Jones, Spatafora, *M. campsosterni* (W.M. Zhang & T.H. Li, *M. taii* Z.Q. Liang & A.Y. Liu) G.H. Sung, J.M. Sung, Hywel-Jones, Spatafora, and *M. guniujiangensis* (R. Li, B. Huang, M.Z. Fan & Z.Z. Li) G.H. Sung, J.M. Sung, Hywel-Jones, Spatafora. The core clade of *Metacordyceps* is subtended by a phylogenetic grade including the species *M. chlamydosporia*, *M. khaoyaiensis*, *M. kusanagiensis* and *M. yongmunensis* whose relationships to one another remain weakly supported by the data.

Morphology of the core *Metacordyceps*

Species in the core *Metacordyceps* clade produce one to several stromata that are pigmented in some manner, usually yellow or transitioning from yellow to green or dark green, especially in the region of the clava. Several species connect to the host below ground by mycelial threads or rhizomorphs that are usually bright yellow. In the case of *M. martialis* the stroma is not yellow, but a dark brick red-orange color. The tip of the stroma often forms a broad point lacking perithecia. The peridium of *Metacordyceps* sp. (HMAS 199601) is predominantly yellow in color, but the fertile area is a pink color due to the reddish perithecia under the yellow hyphae. The description of *M. owariensis* by Kobayasi (Kobayasi 1939) does not mention any yellow or green coloration, however, *M. owariensis* var. *viridescens* examined here, does have yellow-green pigmentation (Uchiyama and Udagawa 2002). Li et al. (2010) described *M. guniujiangensis* as a species similar to *M. owariensis* var. *viridescens*, and positioned in an ITS phylogeny with related anamorphic forms (see below), differing only in a more yellow stromatal tip. Examination of definitive material for the pale form of *M. owariensis* is necessary to determine the plasticity of color and other morphologies in this complex.

The perithecia of the core *Metacordyceps* are typically embedded in a relatively dense, fibrous stroma with only the ostiole protruding through the peridium. In the case of *M. brittlebankisoides* perithecia are erumpent (Liu et al. 2001). The orientation of perithecia is predominantly oblique, although this character is apparently variable with several species displaying ordinal orientation of perithecia (Table 2.3). Perithecia usually occupy a terminal or subterminal position on the stipe,

and maybe dispersed or aggregated, usually leaving bare spots on the stipe especially at the tip. The oblique orientation of perithecia was a defining characteristic of subgenus *Neocordyceps* in Kobayasi's classification scheme for the genus *Cordyceps* (Kobayasi 1941). Although this character is found in more than one clade of clavicipitaceous fungi, Sung et al. (2007) identify a distinct clade of “*Neocordyceps*” species in a subclade of Ophiocordycipitaceae exemplified by *O. sphecocephala* (Klotzsch ex Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora. Morphologies in this “*Neocordyceps*” clade differ significantly from species with oblique perithecia in *Metacordyceps*. The stipe is thin and often filiform with a wiry or pliant texture, and the perithecia are contained in a distinct capitate head, as opposed to the robust, fibrous stipe and variable perithecial distribution seen in *Metacordyceps*. The shape of the perithecia is also very different; species in the “*Neocordyceps*” clade possess perithecia with strongly curved necks, as opposed to the ampullaceous to ovular forms of *Metacordyceps*.

The characteristics of the ascus and ascospores in the core *Metacordyceps* are probably the most variable of characters within the clade. All species form septations when the ascospores are mature. However, the formation of septations does not necessarily result in ascospores that disarticulate into partspores, and at least four species produce whole ascospores (Table 2.3). *Metacordyceps owariensis* forms septations that do not result in partspores, but both *M. owariensis* var. *viridescens* and *M. guniujiangensis* have ascospores that disarticulate into partspores. The species *M. atrovirens* and *M. pseudoatrovirens* are sister taxa and highlight the plasticity and

convergent nature of ascospore characters between families of clavicipitaceous fungi. Both species form fusoid spores, developing relatively few septations and remaining whole at maturity, with attenuated tips. Petch (1931) used the fusoid ascospore morphology as a diagnostic feature in the original description of the genus *Ophiocordyeps*, and was previously considered limited to a subset of the taxa within the genus (Sung et al. 2007).

Morphology of species outside of the core *Metacordyceps*

The most striking feature separating the core *Metacordyceps* clade from species outside it is the lack of strong pigmentation. *Metacordyceps liangshanensis* is brown in color (Zang et al. 1982), while all other species are pale white or pallid. In the case of *M. khaoyaiensis*, the white stroma may develop a purplish or lilac hue, especially around the ostiole (Hywel-Jones 1994). Although the perithecia are embedded in *M. yongmunensis* and *M. khaoyaiensis*, they are superficial or erumpent in other species and may be ordinal or oblique. Ascospores are filiform and develop septations in all species, forming partspores in about half of the species (Table 2.3).

Anamorphic relationships of *Metacordyceps*

Characters of the anamorph continue to provide insight into systematic relationships not only between families but also within *Metacordyceps*. The

anamorphic genera *Pochonia* and *Rotiferophthora* are associated with species placed outside of the core *Metacordyceps*. The connection of *Pochonia* to teleomorphs in *Metacordyceps* was first noted with the placement of *P. chlamydosporia* with *M. chlamydosporia* (= *Cordyceps chlamydosporia*) growing on mollusc eggs (Zare et al. 2001). However, poor resolution among these species make determination of direct anamorph-teleomorph connections difficult. Among the taxa added in this study, *M. khaoyaiensis* is supported as a close relative of *P. marquandii*. There is a clade of *Pochonia* species basal to the rest of *Metacordyceps* that remains unassociated with any teleomorphs.

Within the core clade, anamorphs typically produce olive, green or yellowish conidia, never pink or purple, generally reflecting the spectrum of color seen in the teleomorph. The only teleomorph in this clade not producing a green-spored anamorph is *M. pseudoatrovirens*. Cultures obtained from single-ascospore isolates produce hyaline conidia and chlamydospores. The genus *Metarhizium* is known exclusively from the core clade of *Metacordyceps* and has known links to *M. brittlebankisoides*, *M. campsosterni* and *M. taii*. Also included are several green-spored species of *Nomuraea*, *N. cylindrospora* (Q.T. Chen & H.L. Guo) Tzean, L.S. Hsieh, J.L. Chen & W.J. Wu and *N. rileyi*. This contrasts sharply with the pink to purple spored *N. atypicola* (Yasuda) Samson, a pathogen of spiders and member of Ophiocordycipitaceae (Sung et al. 2007). *Metacordyceps* sp. HMAS 199601 produces green conidia similar to *Metarhizium* or *Nomuraea* on the surface of robust synnema that may co-occur with the teleomorph.

We find strong phylogenetic support that *M. indigotica* is a member of the *Mr. anisopliae* species complex (Figs. 1 & 2). Bischoff et al. (2009) identified nine species in the *Mr. anisopliae* complex, some of which were formerly recognized as varieties. Using the multigene dataset of Bischoff et al. we determine that *M. indigotica* is a member of the clade comprising *Mr. majus* (J.R. Johnst.) J.F. Bisch., Rehner & Humber and *Mr. guizhouensis* Q.T. Chen & H.L. Guo isolates, and possibly represents a unique clade in this complex *Metarhizium guizhouensis* is the anamorph of *M. taii*, a species morphologically similar to *M. indigotica*. This relationship should be investigated with more sensitive molecular markers and additional specimens of *M. indigotica* to determine the precise anamorph-teleomorph connection.

Surrounding the *Mr. anisopliae* clade are species of Hemipteran pathogens comprised of *Mr. album*, *N. cylindrospora*, and the teleomorph *M. owariensis* var. *viridescens*. The anamorph of *M. owariensis* var. *viridescens* was described as a *Nomuraea*, producing green, oblong spores in cultures derived from ascospores. Molecular data remains somewhat weak at resolving these phylogenetic links, however the habit of hemipteran pathogenesis appears to be suggestive. All species are pathogens of Hemiptera: *Mr. album* on leaf-hoppers (Cicadellidae), *M. owariensis* var. *viridescens* on cicada nymphs and *N. cylindrospora* on adults (Cicadidae). Li et al. (2010) described a *Metarhizium* anamorph for *M. guniujiangensis* and established a connection with ITS data to *N. cylindrospora* (referred to as *Mr. cylindrospora*). The apparent overlap in *Nomuraea* and *Metarhizium* is highlighted by the relationships within this clade and future work should examine more material adequately resolve

generic boundaries and anamorph-teleomorph connections of this complex group of fungi.

Host associations of *Metacordyceps*

The host range for *Metacordyceps* occupies a breadth of taxa that is remarkable considering the small number of species relative to *Cordyceps* and *Ophiocordyceps*. This includes the orders Coleoptera and Lepidoptera, hosts that are common throughout all *Cordyceps*-like fungi. One novel lifestyle is singularly represented by *M. chlamydosporia*, which attacks the eggs of molluscs. Work presented here and by Li et al. (2010) increases the host range to include species that are pathogens Cicadidae of the order Hemiptera. The current taxon sampling does not place any species attacking coleopteran hosts among the early diverging taxa, which attack only lepidopteran hosts and in the case of *M. chlamydosporia*, mollusc eggs. Within the core clade more diverse host utilization is seen. Coleopteran and lepidopteran hosts are utilized throughout the clade, and possibly a single origin of hemipteran pathogenesis. Theory on host associations will likely develop as more taxa are placed in this genus.

When anamorphic forms are considered, the diversity of hosts attacked expands dramatically. Among the early diverging taxa, anamorphic forms are known to attack nematodes and rotifers. Within the core clade, host ranges expand even further. The various isolates of *Mr. anisopliae* s.l. have been used to control a wide

range of pests, ranging from mosquitoes that vector malaria to garden thrips (Bischoff et al. 2009). Conversely, *Mr. acridium* is known from hosts in Orthoptera. The emerging picture is therefore complex, with sexual reproduction triggered by infection of specific hosts while infection of secondary hosts may allow species to persist in the environment as anamorphs.

Implications for biological control

The search for pest control options with minimal negative impacts on surrounding ecosystems has motivated research into the use of fungal pathogens as replacements for conventional chemistries. Species of *Metarhizium* have played an important role in this effort. Most fungal biocontrol agents are used in the anamorphic state due to the ease of artificial rearing and broader host ranges. However, the work conducted here and elsewhere indicates that a large portion of the host diversity may in fact be the result of cryptic species, e.g., Bischoff et al. (2009). Furthermore, the recognition of a much broader host range for *Metacordyceps* can aid biological control workers in searching for strains able to control specific pest species.

Population genetic work has revealed the evidence of sexual recombination in natural *Mr. anisopliae* populations with unobserved meiotic forms. Parasexual cycles and transfer of genetic material through anastomoses of hyphal walls are some of the mechanisms invoked to explain the genotypic diversity in the absence of observed sexual forms (Bidochka et al. 2001). The addition of *M. indigotica* to this study places

another teleomorph in the *Mr. anisopliae* clade, although its affinity to the cryptic species described by Bischoff et al. (2009) remains ambiguous. As the concept of *Metacordyceps* expands to include more species, sexual reproduction will likely emerge as the more significant driver of phylogenetic diversity through increased anamorph-teleomorph connections. Recognition of the sexually reproductive states sets the stage for possible development of strain improvement through breeding programs. However, it is possible that certain lineages of *Metarhizium* have escaped the need for sexual recombination (Bidochka and Koning 2001).

Conclusion

Molecular phylogenetic approaches to the taxonomy of clavicipitaceous fungi have enabled greater resolution of relationships at the family and genus level and aided understanding the range of morphological characters and lifestyle ecologies displayed by these fungi. The emerging picture is complex, with repeated convergent evolution of characters previously thought to be informative. Distinctions between the families Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae for species in *Cordyceps* s.l. can be delineated in part by the characters of color and texture of the stipe, ecological niche and the morphology of the anamorph (Sung et al. 2007). However, these characters are still convergent for some species and genus level designations should be based on at least two characters in the absence of molecular data. Certain characters, such as ascospore disarticulation and shape are best restricted

to use in identification between closely species. Species in the core *Metacordyceps* clade typically possess a robust, firm, fleshy stroma, with striking yellow to green pigmentation and ovoid perithecia usually set at an oblique angle. Although morphological distinction within the genus between the core clade and taxa along the backbone is possible based on color, delimitation from taxa in other genera remains difficult without knowledge of the anamorph owing to the convergent nature of other morphological characters.

The following species exhibit dark green or yellowish stipe coloration with a firm and fibrous texture, often coupled with the oblique orientation of perithecia. These characters are consistent with the species placed phylogenetically, however other morphological and ecological details are lacking in published descriptions to confidently support their placement in *Metacordyceps*. We suggest that these species receive priority for future phylogenetic examination of the genus. Their inclusion would help to further clarify concepts of morphological evolution, as well as the sources of high genetic diversity observed in anamorphic clades lacking teleomorphs:

Cordyceps brasiliensis Hennings, Hedwigia 36: 221 (1897).

Cordyceps joaquiensis Henn., Hedwigia 43: 248 (1904)

Cordyceps olivacea Rick, in Lloyd, Mycol. Writ. 7: 1118 (1922).

Cordyceps olivacens Mains, Mycologia 39: 537 (1947).

Cordyceps olivaceovirescens Henn., Hedwigia 39: 78 (1900).

Cordyceps pallidiolivacea, Kobayasi & Shimizu, Bull. Natn. Sci. Mus. Tokyo, Ser. B, 8: 83 (1982).

REFERENCES

- BIDOCHKA, M.J., AND J. DEKONING. 2001. Are Teleomorphs Really Necessary?: Modelling the Potential Effects of Muller's Ratchet on Deuteromycetous Entomopathogenic Fungi. *Mycological Research* 105: 1014-1019.
- BIDOCHKA, M.J., A.M. KAMP, T.M. LAVENDER, J. DEKONING, AND J.N.A. DE CROOS. 2001. Habitat Association in Two Genetic Groups of the Insect-Pathogenic Fungus *Metarhizium anisopliae*: Uncovering Cryptic Species? *Appl. Environ. Microbiol.* 67: 1335-1342.
- BISCHOFF, J.F., S.A. REHNER, AND R.A. HUMBER. 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* 101: 512-530.
- BISCHOFF, J.F., S.A. REHNER, AND R.A. HUMBER. 2006. *Metarhizium frigidum* sp. nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex. *Mycologia* 98: 737-745.
- CASTLEBURY, L.A., A.Y. ROSSMAN, G. SUNG, A.S. HYTEN, AND J.W. SPATAFORA. 2004. Multigene phylogeny reveals new lineage for *Stachybotrys chartarum*, the indoor air fungus. *Mycological Research* 108: 864-872.
- CHAVERRI, P., M. LIU, AND K. HODGE. 2008. A monograph of the entomopathogenic genera *Hypocrella*, *Moelleriella*, and *Samuelsia* gen. nov. (Ascomycota, Hypocreales, Clavicipitaceae), and their aschersonia-like anamorphs in the Neotropics. *Stud Mycol* 60: 1-66.
- CHAVERRI, P., J.F. BISCHOFF, H.C. EVANS, AND K.T. HODGE. 2005. *Regiocrella*, a new entomopathogenic genus with a pycnidial anamorph and its phylogenetic placement in the Clavicipitaceae. *Mycologia* 97: 1225-1237.
- DRUMMOND, A., AND A. RAMBAUT. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7: 214.
- HALL, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41: 95-98.
- HUELSENBECK, J.P., AND F. RONQUIST. 2001. Mrbayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.
- HYWEL-JONES, N.L. 1994. *Cordyceps khaoyaiensis* and *C. pseudomilitaris*, two new pathogens of lepidopteran larvae from Thailand. *Mycological Research* 98: 939-942.

- IGNOFFO, C. 1981. The fungus *Nomuraea rileyi* as a microbial insecticide. *In* Microbial Control of Pests and Plant Diseases: 1970-1980, 513-538. Academic Press, London.
- JOHNSON, D. ET AL. 2009. Systematics and evolution of the genus *Torrubiella* (Hypocreales, Ascomycota). *Mycological Research* 113: 279-289.
- KANZOK, S.M., AND M. JACOBS-LORENA. 2006. Entomopathogenic fungi as biological insecticides to control malaria. *Trends in Parasitology* 22: 49-51.
- KATOH, K., K. MISAWA, K. KUMA, AND T. MIYATA. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl. Acids Res.* 30: 3059-3066.
- KATOH, K., AND H. TOH. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* 9: 286-298.
- KAUFF, F., AND F. LUTZONI. 2002. Phylogeny of the Gyalectales and Ostropales (Ascomycota, Fungi): among and within order relationships based on nuclear ribosomal RNA small and large subunits. *Molecular Phylogenetics and Evolution* 25: 138-156.
- KERRY, B., D. CRUMP, AND L. MULLEN. 1982. Natural control of the cereal cyst nematode, *Heterodera avenae* Woll., by soil fungi at three sites. *Crop Protection* 1: 99-109.
- KOBAYASI, Y. 1939. On the genus *Cordyceps* and its allies on cicadae from Japan. *Bulletin of the Biogeographical Society of Japan* 9: 145-176.
- LI, C., B. HUANG, M. FAN, Y. LIN, AND Z. LI. 2010. *Metacordyceps guniujiangensis* and its *Metarhizium* anamorph: a new pathogen on cicada nymphs. *Mycotaxon* 111: 221-231.
- LIU, Y., S. WHELEN, AND B. HALL. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Mol Biol Evol* 16: 1799-1808.
- LIU, Z.Y., Z.Q. LIANG, A.Y. LIU, Y.J. YAO, K.D. HYDE, AND Z.N. YU. 2002. Molecular evidence for teleomorph-anamorph connections in *Cordyceps* based on ITS-5.8S rDNA sequences. *Mycological Research* 106: 1100-1108.

- LIU, Z.Y., Z.Q. LIANG, A.J.S. WHALLEY, Y.-. YAO, AND A.Y. LIU. 2001. Cordyceps brittlebankisoides, a New Pathogen of Grubs and Its Anamorph, Metarhizium anisopliae var. majus. *Journal of Invertebrate Pathology* 78: 178-182.
- LIU, Z., Y. YAO, Z.Q. LIANG, A. LIU, D.N. PEGLER, AND M.W. CHASE. 2001. Molecular evidence for the anamorph–teleomorph connection in Cordyceps sinensis. *Mycological Research* 105: 827-832.
- LOMER, C.J., R.P. BATEMAN, D.L. JOHNSON, J. LANGEWALD, AND M. THOMAS. 2001. Biological Control of Locusts and Grasshoppers. *Annual Review of Entomology* 46: 667-702.
- LUANGSA-ARD, J.J., N.L. HYWEL-JONES, L. MANOCH, AND R.A. SAMSON. 2005. On the relationships of Paecilomyces sect. Isarioidea species. *Mycological research* 109: 581-9.
- LUANGSA-ARD, J.J., N.L. HYWEL-JONES, AND R.A. SAMSON. 2004. The polyphyletic nature of Paecilomyces sensu lato based on 18S-generated rDNA phylogeny. *Mycologia* 96: 773-780.
- PETCH, T. 1931. Notes on entomogenous fungi. *Transactions of the British Mycological Society* 16: 55-75.
- ROGERSON, C.T. 1970. The hypocrealean fungi (Ascomycetes, Hypocreales). *Mycologia* 62: 865-910.
- RONQUIST, F., AND J.P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572 -1574.
- SCHOLTE, E. ET AL. 2005. An Entomopathogenic Fungus for Control of Adult African Malaria Mosquitoes. *Science* 308: 1641-1642.
- SOSA-GÓMEZ, D., R. HUMBER, K. HODGE, E. BINNECK, AND K. SILVA-BRANDÃO. 2009. Variability of the Mitochondrial SSU rDNA of Nomuraea Species and Other Entomopathogenic Fungi from Hypocreales. *Mycopathologia* 167: 145-154.
- SPATAFORA, J.W., G.-. SUNG, J.-. SUNG, N.L. HYWEL-JONES, AND J.R. WHITE. 2007. Phylogenetic evidence for an animal pathogen origin for ergot and the grass endophytes. *Molecular Ecology* 16: 1701-1711.
- STAMATAKIS, A. 2006. RAXML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688-2690.

- SUNG, G., N.L. HYWEL-JONES, J. SUNG, J.J. LUANGSA-ARD, B. SHRESTHA, AND J.W. SPATAFORA. 2007. Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. *Stud Mycol* 57: 5-59.
- SUNG, G., G.O. POINAR JR., AND J.W. SPATAFORA. 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. *Molecular Phylogenetics and Evolution* 49: 495-502.
- UCHIYAMA, S., AND S. UDAGAWA. 2002. Cordyceps owariensis f. viridescens and its new Nomuraea anamorph. *Mycoscience* 43: 0135-0141.
- VILGALYS, R., AND B.L. SUN. 1994. Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *PNAS* 91: 4599-4603.
- WHITE, T.J., T.D. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White [eds.], *PCR protocols, a guide to methods and applications.*, 315-322. Academic Press, San Diego.
- ZANG, M., L. DAOQING, AND H. RUOYING. 1982. Notes concerning the subdivisions of Cordyceps and a new species from China. *Acta Bot. Yunnanica* 4: 173-176.
- ZARE, R., W. GAMS, AND H. EVANS. 2001. A revision of *Verticillium* section Prostrata. V. The genus *Pochonia*, with notes on *Rotiferophthora*. *NOVA HEDWIGIA* 73: 51-86.
- ZARE, R., AND W. GAMS. 2008. A revision of the *Verticillium fungicola* species complex and its affinity with the genus *Lecanicillium*. *Mycological Research* 112: 811-824.
- ZHANG, W., H. TAI, Y. CHEN, AND H. LIANG. 2004. Cordyceps campsosterna, a new pathogen of Campsosternus auratus. *Fungal Diversity* 17: 239-242.

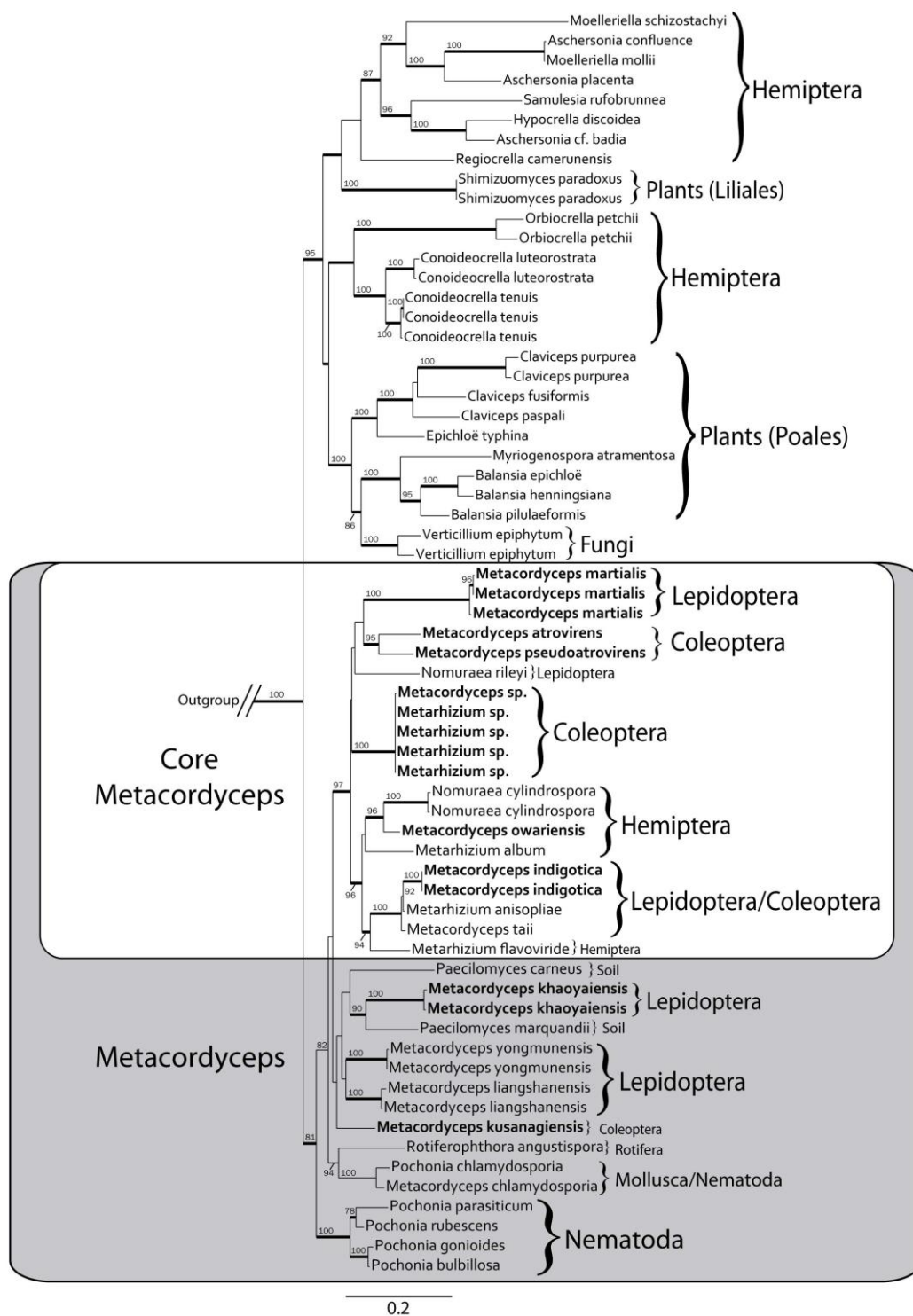


Figure 2.1

Figure 2.1 Clade for the family Clavicipitaceae extracted from a larger maximum likelihood tree (supplementary information) obtained from analysis of the 135 species concatenated, five-gene dataset. Numbers above branches indicate bootstrap support from 500 maximum likelihood replicates. Thickened branches indicate posterior probability support > 0.95 in Bayesian analyses.



Figure2.2

Figure 2.2 Examples of *Metacordyceps* from this study. A. *M. kusanagiensis* in the field fruiting from larvae of Coleoptera. B. Anamorph of undescribed *Metacordyceps* showing robust synnema fruiting from larvae of Coleoptera. C. *M. cylindrospora* on adult cicada (Hemiptera). D. Type specimen of *M. kusanagiensis*. E. Co-occurrence of anamorph and teleomorph for an undescribed *Metacordyceps*. F. Stromal tip of *M. owariensis* var. *viridescens*. G. *M. martialis* fruiting from larvae of Lepidoptera. H. *M. indigotica* fruiting from larvae of Lepidoptera. I. Rhizomorphs emerging from hemipteran host of *M. owariensis* var. *viridescens*. J. Fresh specimen of *M. pseudoatrovirens*. K. Ascus tip of *M. atrovirens*. L. Type specimen of *M. pseudoatrovirens*. M. Perithecium of *M. indigotica*. N. Fusoid ascospore of *M. atrovirens*. O. Asci of type specimen for *M. pseudoatrovirens*

Table 2.1 Information for primers used for PCR in this study.

Gene	Primer	5'-Sequences-3'	source
TEF	983F	GCYCCYGGHCAYCGTGAYTTYAT	Carbone & Kohn (1999)
TEF	2218R	ATGACACCRACRGCACRGTYTG	Steven Rehner, personal communication
LSU	LR5	ATCCTGAGGGAAACTTC	Vilgalys and Sun (1994)
LSU	LR0R	GTACCCGCTGAACTTAAGC	Vilgalys and Sun (1994)
SSU	SR7	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS4	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS3	GCAAGTCTGGTGCCAGCAGCC	White et al. (1990)
SSU	NS1	GTAGTCATATGCTTGTCTC	White et al. (1990)
RPB1	RPB1Cr	CCNGCDATNTCRTTRTCCATRTA	Castlebury et al. (2004)
RPB1	CRPB1A	CAYCCWGGYTTYATCAAGAA	Castlebury et al. (2004)
RPB2	fRPB2-7cR	CCCATRGCTTGTYRCCCAT	Liu et al. (1999)
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al. (1999)

Table 2.2 Specimen information for materials used in this study

Species	Voucher info.	Genbank Accession Number					
		ITS GB.	nSSU	nLSU	TEF	RPB1	RPB2
<i>Akanthomyces novoguineensis</i>	NHJ 11923		EU369095	EU369032	EU369013	EU369052	EU369072
<i>Aphysiostroma stercorarium</i>	ATCC 62321		AF543769	AF543792	AF543782	AY489633	EF469103
<i>Aschersonia cf. badia</i>	BCC 7016	Pending	DQ372091	DQ384941	DQ384969	DQ385009	DQ452460
<i>Aschersonia confluence</i>	BCC 7961	Pending	DQ372100	DQ384947	DQ384976	DQ384998	DQ452465
<i>Aschersonia placenta</i>	BCC 7869	Pending	EF469121	EF469074	EF469056	EF469085	EF469104
<i>Balansia epichloë</i>	AEG 96-15a	Pending	EF468949		EF468743	EF468851	EF468908
<i>Balansia henningsiana</i>	GAM 16112	Pending	AY545723	AY545727	AY489610	AY489643	DQ522413
<i>Balansia pilulaeformis</i>	AEG 94-2	Pending	AF543764	AF543788	DQ522319	DQ522365	DQ522414
<i>Bionectria ochroleuca</i>	CBS 114056		AY489684	AY489716	AY489611	DQ842031	DQ522415
<i>Claviceps fusiformis</i>	ATCC 26019	Pending	DQ522539	U17402	DQ522320	DQ522366	
<i>Claviceps paspali</i>	ATCC 13892	Pending	U32401	U47826	DQ522321	DQ522367	DQ522416
<i>Claviceps purpurea</i>	GAM 12885	Pending	AF543765	AF543789	AF543778	AY489648	DQ522417
<i>Claviceps purpurea</i>	SA cp11		EF469122	EF469075	EF469058	EF469087	EF469105
<i>Conoideocrella luteorostrata</i>	NHJ 12516	Pending	EF468994	EF468849	EF468800	EF468905	EF468946
<i>Conoideocrella luteorostrata</i>	NHJ 11343	Pending	EF468995	EF468850	EF468801	EF468906	
<i>Conoideocrella tenuis</i>	NHJ 6791	Pending	EU369113	EU369046	EU369028	EU369069	EU369089
<i>Conoideocrella tenuis</i>	NHJ 6293	Pending	EU369112	EU369044	EU369029	EU369068	EU369087
<i>Conoideocrella tenuis</i>	NHJ 345.01		EU369111	EU369045	EU369030		EU369088
<i>Cordyceps atrovirens</i>	TNM F10184	Pending	Pending	Pending		Pending	
<i>Cordyceps bifusispora</i>	EFCC 5690		EF468952	EF468806	EF468746	EF468854	EF468909
<i>Cordyceps brongniartii</i>	BCC 16585	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps cardinalis</i>	OSC 93610	Pending	AY184974	AY184963	EF469059	EF469088	EF469106
<i>Cordyceps coccidioperitheciata</i>	NHJ 6709	Pending	EU369110	EU369042	EU369025	EU369067	EU369086
<i>Cordyceps confragosa</i>	CBS 101247	Pending	AF339604	AF339555	DQ522359	DQ522407	DQ522466
<i>Cordyceps gunnii</i>	OSC 76404	Pending	AF339572	AF339522	AY489616	AY489650	DQ522426
<i>Cordyceps indigotica</i>	TNS F18553	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps indigotica</i>	TNS F18554	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps khaoyaiensis</i>	BCC 12687	Pending		Pending	Pending	Pending	
<i>Cordyceps khaoyaiensis</i>	BCC 14290	Pending		Pending	Pending	Pending	

Table 2.2 Continued

<i>Cordyceps kusanagiensis</i>	TNS F18494	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps kyusyuënsis</i>	EFCC 5886		EF468960	EF468813	EF468754	EF468863	EF468917
<i>Cordyceps martialis</i>	EFCC 6863			Pending	Pending		Pending
<i>Cordyceps martialis</i>	Pending	Pending	Pending	Pending		Pending	
<i>Cordyceps martialis</i>	HMAS 197472(S)	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps militaris</i>	OSC 93623	Pending	AY184977	AY184966	DQ522332	DQ522377	AY545732
<i>Cordyceps cf. ochraceostromata</i>	ARSEF 5691	Pending	EF468964	EF468819	EF468759	EF468867	EF468921
<i>Cordyceps owariensis</i>	NBRC 33258	Pending		Pending	Pending		Pending
<i>Cordyceps pseudoatrovirens</i>	TNSF 16380	Pending		Pending		Pending	Pending
<i>Cordyceps scarabaeicola</i>	ARSEF 5689	Pending	AF339574	AF339524	DQ522335	DQ522380	DQ522431
<i>Cordyceps sp.</i>	HMAS 199601	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps tuberculata</i>	OSC 111002	Pending	DQ522553	DQ518767	DQ522338	DQ522384	DQ522435
<i>Cosmospora coccinea</i>	CBS 114050	Pending	AY489702	AY489734	AY489629	AY489667	DQ522438
<i>Elaphocordyceps japonica</i>	OSC 110991	Pending	DQ522547	DQ518761	DQ522330	DQ522375	DQ522428
<i>Elaphocordyceps ophioglossoides</i>	OSC 106405		AY489691	AY489723	AY489618	AY489652	DQ522429
<i>Elaphocordyceps subsessilis</i>	OSC 71235	Pending	EF469124	EF469077	EF469061	EF469090	EF469108
<i>Engyodontium aranearum</i>	CBS 309.85	Pending	AF339576	AF339526	DQ522341	DQ522387	DQ522439
<i>Epichloë typhina</i>	ATCC 56429	Pending	U32405	U17396	AF543777	AY489653	DQ522440
<i>Gibellula sp.</i>	NHJ 13158	Pending	EU369100	EU369037	EU369020	EU369057	EU369077
<i>Glomerella cingulata</i>	CBS 114054		AF543762	AF543786	AF543773	AY489659	DQ522441
<i>Glomerella cingulata</i>	FAU 513		U48427	U48428	AF543772	DQ858454	DQ858455
<i>Haptocillium sinense</i>	CBS 567.95	AJ292417	AF339594	AF339545	DQ522343	DQ522389	DQ522443
<i>Hirsutella sp.</i>	NHJ 12525		EF469125	EF469078	EF469063	EF469092	EF469111
<i>Hirsutella sp.</i>	OSC 128575	Pending	EF469126	EF469079	EF469064	EF469093	EF469110
<i>Hydropisphaera peziza</i>	CBS 102038		AY489698	AY489730	AY489625	AY489661	DQ522444
<i>Hymenostilbe aurantiaca</i>	OSC 128578	Pending	DQ522556	DQ518770	DQ522345	DQ522391	DQ522445
<i>Hypocrea lutea</i>	ATCC 208838		AF543768	AF543791	AF543781	AY489662	DQ522446
<i>Hypocrea rufa</i>	CBS 114374		AY489694	AY489726	AY489621	AY489656	EF692510
<i>Hypocrella discoidea</i>	BCC 8237	Pending	-	DQ384937	DQ384977	DQ385000	DQ452461
<i>Isaria farinosa</i>	CBS 240.32	AY624178	Pending	Pending	Pending	Pending	Pending
<i>Isaria tenuipes</i>	ARSEF 5135	AY624196	Pending	Pending	Pending	Pending	Pending
<i>Lecanicillium attenuatum</i>	CBS 402.78	AJ292434	AF339614	AF339565	EF468782	EF468888	EF468935

Table 2.2 continued

<i>Lecanicillium psalliotae</i>	CBS 532.81	Pending	AF339609	AF339560	EF469067	EF469096	EF469112
<i>Mariannaea elegans</i> var. <i>punicea</i>	CBS 239.56	Pending	Pending	Pending	Pending	Pending	Pending
<i>Mariannaea pruinosa</i>	ARSEF 5413	Pending	AY184979	AY184968	DQ522351	DQ522397	DQ522451
<i>Metacordyceps chlamydosporia</i>	CBS 101244	Pending	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
<i>Metacordyceps liangshanensis</i>	EFCC 1452		EF468962	EF468815	EF468756		
<i>Metacordyceps liangshanensis</i>	EFCC 1523		EF468961	EF468814	EF468755		EF468918
<i>Metacordyceps taii</i>	ARSEF 5714	Pending	AF543763	AF543787	AF543775	DQ522383	DQ522434
<i>Metacordyceps yongmunensis</i>	EFCC 2131	Pending	EF468977	EF468833	EF468770	EF468876	
<i>Metacordyceps yongmunensis</i>	EFCC 2135		EF468979	EF468834	EF468769	EF468877	
<i>Metarhizium album</i>	ARSEF 2082	Pending	DQ522560	DQ518775	DQ522352	DQ522398	DQ522452
<i>Metarhizium anisopliae</i>	ARSEF 3145	Pending	AF339579	AF339530	AF543774	DQ522399	DQ522453
<i>Metarhizium flavoviride</i>	ARSEF 2037	Pending	AF339580	AF339531	DQ522353	DQ522400	DQ522454
<i>Metarhizium</i> sp.	HMAS 199596	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium</i> sp.	HMAS 199592	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium</i> sp.	HMAS 199590	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium</i> sp.	HMAS 199603	Pending	Pending	Pending	Pending	Pending	Pending
<i>Moelleriella mollii</i>	BCC 7963		DQ372087		DQ384964	DQ385004	DQ452466
<i>Moelleriella schizostachyi</i>	BCC 1985		DQ372105	DQ384939	DQ384959	DQ385012	DQ452471
<i>Myriogenospora atramentosa</i>	AEG 96-32	Pending	AY489701	AY489733	AY489628	AY489665	DQ522455
<i>Nectria cinnabarina</i>	CBS 114055		U32412	U00748	AF543785	AY489666	DQ522456
<i>Nectria</i> _cf_ sp	CBS 478.75		U47842	U17404	EF469068	EF469097	EF469115
<i>Nomuraea cylindrospora</i>	TNS 16371		Pending	Pending	Pending	Pending	
<i>Nomuraea cylindrospora</i>	RCEF 3632	Pending	Pending	Pending	Pending		
<i>Nomuraea rileyi</i>	CBS 806.71	AY624205	AY624205	AY624250	EF468787	EF468893	EF468937
<i>Ophiocordyceps acicularis</i>	OSC 128580	Pending	DQ522543	DQ518757	DQ522326	DQ522371	DQ522423
<i>Ophiocordyceps agriotidis</i>	ARSEF 5692	Pending	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
<i>Ophiocordyceps aphodii</i>	ARSEF 5498		DQ522541	DQ518755	DQ522323		DQ522419
<i>Ophiocordyceps brunneipunctata</i>	OSC 128576		DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
<i>Ophiocordyceps entomorrhiza</i>	KEW 53484	Pending	EF468954	EF468809	EF468749	EF468857	EF468911
<i>Ophiocordyceps gracilis</i>	EFCC 8572	Pending	EF468956	EF468811	EF468751	EF468859	EF468912
<i>Ophiocordyceps heteropoda</i>	EFCC 10125	Pending	EF468957	EF468812	EF468752	EF468860	EF468914
<i>Ophiocordyceps irangiensis</i>	OSC 128577	Pending	DQ522546	DQ518760	DQ522329	DQ522374	DQ522427

Table 2.2 continued

<i>Ophiocordyceps longissima</i>	EFCC 6814			EF468817	EF468757	EF468865	
<i>Ophiocordyceps nigrella</i>	EFCC 9247	Pending	EF468963	EF468818	EF468758	EF468866	EF468920
<i>Ophiocordyceps ravenelii</i>	OSC 110995		DQ522550	DQ518764	DQ522334	DQ522379	DQ522430
<i>Ophiocordyceps rhizoidea</i>	NHJ 12522	Pending	EF468970	EF468825	EF468764	EF468873	EF468923
<i>Ophiocordyceps sinensis</i>	EFCC 7287	Pending	EF468971	EF468827	EF468767	EF468874	EF468924
<i>Ophiocordyceps sobolifera</i>	KEW 78842	Pending	EF468972	EF468828		EF468875	EF468925
<i>Ophiocordyceps sphecocephala</i>	OSC 110998		DQ522551	DQ518765	DQ522336	DQ522381	DQ522432
<i>Ophiocordyceps stylophora</i>	OSC 111000	Pending	DQ522552	DQ518766	DQ522337	DQ522382	DQ522433
<i>Ophiocordyceps unilateralis</i>	OSC 128574		DQ522554	DQ518768	DQ522339	DQ522385	DQ522436
<i>Ophiocordyceps variabilis</i>	ARSEF 5365		DQ522555	DQ518769	DQ522340	DQ522386	DQ522437
<i>Ophionectria trichospora</i>	CBS 109876		AF543766	AF543790	AF543779	AY489669	DQ522457
<i>Orbiocrella patchii</i>	NHJ 5318		EU369105	EU369040	EU369021	EU369062	EU369080
<i>Orbiocrella patchii</i>	NHJ 6209	Pending	EU369104	EU369039	EU369023	EU369061	EU369081
<i>Paecilomyces carneus</i>	CBS 239.32	AY624171	EF468988	EF468843	EF468789	EF468894	EF468938
<i>Paecilomyces coleopterorum</i>	CBS 110.73	AY624177	Pending	Pending	Pending	Pending	Pending
<i>Paecilomyces lilacinus</i>	CBS 431.87	AY624188	AY624188	EF468844	EF468791	EF468897	EF468940
<i>Paecilomyces marquandii</i>	CBS 182.27	AY624193	EF468990	EF468845	EF468793	EF468899	EF468942
<i>Paecilomyces niphedodes</i>	CBS 364.76	AY624192	Pending	Pending	Pending	Pending	Pending
<i>Pochonia bulbillosa</i>	CBS 145.70		AF339591	AF339542	EF468796	EF468902	EF468943
<i>Pochonia chlamydosporia</i>	CBS 504.66	Pending	AF339593	AF339544	EF469069	EF469098	EF469120
<i>Pochonia gonioides</i>	CBS 891.72	AJ292409	AF339599	AF339550	DQ522354	DQ522401	DQ522458
<i>Pochonia parasiticum</i>	ARSEF 3436	Pending	EF468993	EF468848	EF468799	EF468904	EF468945
<i>Pochonia rubescens</i>	CBS 464.88		AF339615	AF339566	EF468797	EF468903	EF468944
<i>Pseudonectria rousseliana</i>	CBS 114049		AF543767	U17416	AF543780	AY489670	DQ522459
<i>Regiocrella camerunensis</i>	ARSEF 7682			DQ118735	DQ118743	DQ127234	
<i>Rotiferophthora angustispora</i>	CBS 101437		AF339584	AF339535	AF543776	DQ522402	DQ522460
<i>Roumegueriella rufula</i>	CBS 346.85		DQ522561	DQ518776	DQ522355	DQ522403	DQ522461
<i>Samuelsia rufobrunnea</i>	CUP 067858			AY986918	AY986944	DQ000345	-
<i>Septofusidium herbarum</i>	CBS 265.58	Pending	Pending	Pending	Pending	Pending	Pending
<i>Shimizuomyces paradoxus</i>	EFCC 6564		EF469130	EF469083	EF469072	EF469101	EF469118
<i>Shimizuomyces paradoxus</i>	EFCC 6279	Pending	EF469131	EF469084	EF469071	EF469100	EF469117
<i>Simplicillium lamellicola</i>	CBS 116.25	AJ292393	AF339601	AF339552	DQ522356	DQ522404	DQ522462

Table 2.2 continued

<i>Simplicillium lanosoniveum</i>	CBS 101267	AJ292395	AF339603	AF339554	DQ522357	DQ522405	DQ522463
<i>Sphaerostilbella berkeleyana</i>	CBS 102308		AF543770	U00756	AF543783	AY489671	DQ522465
<i>Torrubiella ratticaudata</i>	ARSEF 1915	Pending	DQ522562	DQ518777	DQ522360	DQ522408	DQ522467
<i>Torrubiella wallacei</i>	CBS 101237	Pending	AY184978	AY184967	EF469073	EF469102	EF469119
<i>Verticillium dahliae</i>	ATCC 16535		AY489705	AY489737	AY489632	AY489673	DQ522468
<i>Verticillium epiphytum</i>	CBS 154.61	AJ292404	AF339596	AF339547	EF468802		EF468947
<i>Verticillium epiphytum</i>	CBS 384.81		AF339596	AF339547	DQ522361	DQ522409	DQ522469
<i>Verticillium incurvum</i>	CBS 460.88		AF339600	AF339551	DQ522362	DQ522410	DQ522470
<i>Verticillium</i> sp.	CBS 102184	Pending	AF339613	AF339564	EF468803	EF468907	EF468948
<i>Viridispora diparietispora</i>	CBS 102797	Pending	AY489703	AY489735	AY489630	AY489668	DQ522471

Herbarium Codes: AEG, A. E. Glenn personal collection; ARSEF, USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; ATCC, American Type Culture Collection, Manassa, VA; BCC, BIOTEC Culture Collection, Klong Luang, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CUP, Cornell University Plant Pathology Herbarium; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; FAU, F. A. Uecker personal collection; GAM, Julian H. Miller Mycological Herbarium Athens, GA; HMAS, Chinese Academy of Sciences, Beijing, China; KEW, mycology collection of Royal Botanical Garden, KEW, Surrey, UK; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR; SA, S. Alderman personal collection; TNS, National Museum of Science and Nature, Tsukuba, Japan;

Table 2.3 Morphological characteristics of *Metacordyceps* species

Species	Host	Stipe Color	Anamorph	Perithecia		Ascospores		
				Stratification	Orientation	Shape	Septation	Disarticulation
<i>Metacordyceps atrovirens</i>	Coleoptera	yellow-green	Unknown	immersed	oblique	fusiform	Yes	No
<i>Metacordyceps brittlebankisoides</i>	Coleoptera	green	<i>Metarhizium anisopliae</i> var <i>majus</i>	errumpent	ordinal	filiform	Yes	Yes
<i>Metacordyceps campsosterni</i>	Coleoptera	green/yellow	<i>Metarhizium</i>	immersed	ordinal	filiform	Yes	Yes
<i>Metacordyceps chlamydosporia</i>	mollusc eggs	pallid	<i>Pochonia chlamydosporia</i>	errumpent	ordinal	filiform	Yes	No
<i>Metacordyceps guniujiangensis</i>	Hemiptera	green	<i>Metarhizium/Nomuraea</i>	immersed	oblique	filiform	Yes	No
<i>Metacordyceps indigotica</i>	Lepidoptera	green/yellow	<i>Metarhizium</i>	immersed	oblique	filiform	Yes	Yes
<i>Metacordyceps khaoyaiensis</i>	Lepidoptera	white-lilac	Unknown	immersed	ordinal	filiform	Yes	No
<i>Metacordyceps kusanagiensis</i>	Lepidoptera	pallid	Unknown	superficial	ordinal	filiform	Yes	Yes
<i>Metacordyceps liangshanensis</i>	Lepidoptera	brown	Unknown	superficial	oblique	filiform	Yes	Yes
<i>Metacordyceps martialis</i>	Lepidoptera	orange	<i>Cephalosporium</i>	immersed	oblique	filiform	Yes	Yes
<i>Metacordyceps owariensis</i>	Hemiptera	white	Unknown	immersed	oblique	filiform	Yes	Yes
<i>Metacordyceps owariensis</i> var <i>viridescens</i>	Hemiptera	green	<i>Nomuraea owariensis</i>	immersed	oblique	filiform	Yes	No
<i>Metacordyceps pseudoatrovirens</i>	Coleoptera	yellow-green	<i>Nomuraea</i> like	immersed	ordinal	fusiform	Yes	No
<i>Metacordyceps</i> sp	Coleoptera	Yellow	<i>Metarhizium/Nomuraea</i>	immersed	ordinal	filiform	No	N/A
<i>Metacordyceps taii</i>	Lepidoptera	yellow-red	<i>Metarhizium</i>	immersed	oblique	filiform	Yes	Yes
<i>Metacordyceps yongmunensis</i>	Lepidoptera	pallid	<i>Pochonia</i>	immersed	oblique	filiform	Yes	No

CHAPTER 3

***Veterocordyceps* gen. nov. represents a unique clade of diversity in Clavicipitaceae s.l.**

Ryan Kepler, Joseph W. Spatafora

Department of Botany and Plant Pathology
Oregon State University
Corvallis OR, 97331, USA

Sayaka Ban, Akira Nakagiri

Department of Biotechnology, National Institute of Technology and Evaluation
2-5-8 Katusakamatari, Kisarazu, Chiba 292-0818
Japan

Formatted for submission to *Fungal Biology*.

ABSTRACT

Molecular phylogenetic methods have revolutionized the way systematists and taxonomists approach understanding relationships between fungi. Application of these methods to Hypocreales has advanced understanding of the evolution of fungi with important roles in ecological settings and human enterprises. The clavicipitaceous fungi in particular have benefited from molecular phylogenetic investigation. Traditionally considered as belonging to the family Clavicipitaceae, they are now represented by three families, largely driven by reevaluation of the morphologically and ecologically diverse genus *Cordyceps*. Although morphological features of both sexual and asexual states were found to reflect the structure of phylogenies based on molecular data, many species remain of uncertain placement due to a lack of reliable data. A rigid, darkly pigmented stipe and the productions of a *Hirsutella*-like anamorph in culture was used to support transfer of the species *C. cuboidea*, *C. prolifica*, and *C. ryogamiensis* to the genus *Ophiocordyceps*, although molecular data adequate for placing these species remains lacking. In this paper we obtain molecular data for ribosomal and protein coding DNA from specimens of *O. cuboidea*, *O. ryogamiensis*, *O. paracuboidea*, *O. prolifica*, *C. ramosopulvinata*, *C. nipponica*, and the anamorphic species *Polycephalomyces formosus*. We find these species represent a new clade of diversity for the clavicipitaceous fungi consistent with generic level distinctions. We describe *Veterocordyceps* *gen. nov.* with two subgenera, *V. subg. Veterocordyceps* and *V. subg. Pulvinata* to accommodate the fungi present in this novel clade.

Keywords: Anamorph-teleomorph connection, *Cordyceps*, *Hirsutella*, Molecular phylogenetics, Morphological plasticity, *Polycephalomyces*

INTRODUCTION

The use of multigene phylogenetics has revolutionized fungal systematics. The effect has been felt across all levels of investigation; from kingdom wide, coordinated multi-researcher initiatives like the Assembling the Fungal Tree of Life project (AFTOL), to explorations of global species complexes, e.g., *Fusarium*. Because of their important roles as pathogens across a broad range of the tree of life, the order Hypocreales (Ascomycota: Sordariomycetes) has been the focus of several intensive molecular investigations. They can be important regulators of defoliating insect populations in forest ecosystems (Kamata et al. 1997), a feature that has been exploited in agricultural systems for biological control. However they may also be important pathogens of cultivated crops, causing serious economic harm. Others impact human health as disease agents (Castlebury et al. 2004) or sources of medicine (Powels et al. 1978, Hodge et al. 1996). Increased understanding of the relationships between these fungi will allow for more effective management of those that are problematic, and utilization of those that are beneficial.

Perhaps no group in Hypocreales represents the diverse breadth of ecological roles and niches better than the clavicipitaceous fungi. Originally considered a single family (Clavicipitaceae), molecular phylogenetics has revealed the family to be polyphyletic (Sung et al. 2007). This work resulted largely from investigation of the genus *Cordyceps*. Current concepts now recognize three families, with *Cordyceps* now divided among five genera (Sung et al. 2007, Chapter 3). Clavicipitaceae s.s. is

now considered to include all plant pathogenic species, e.g., the genera *Claviceps*, *Shimizuomyces* and *Ustilagoidea*) and animal pathogens, e.g., *Metacordyceps* and *Hypocrella*) infecting a range of animal hosts. Sister to Clavicipitaceae is the family Ophiocordycipitaceae, which also contains pathogens infecting a wide range of hosts. The genus *Elaphocordyceps* contains species previously placed in *Cordyceps* that attack false truffles in the genus *Elaphomyces*, as well as several pathogens of cicada nymphs. The genus *Ophiocordyceps* contains the majority of species transferred from *Cordyceps* s.l. These fungi are exclusively pathogens of animals, spanning at least seven orders of arthropods. Cordycipitaceae represents species associated with the type for the genus *Cordyceps*, *C. militaris* (L:Fr.) Link, as well as the genera *Torrubiella* which contains species attacking spiders and *Simplicillium*, a clade of asexual fungal pathogens. This family shares a sister relationship with the Hypocreaceae.

The reevaluation of *Cordyceps* revealed characters historically used to define genera and subgenera did not corroborate with results from molecular data. Traits such as orientation of the perithecia and morphology of the ascospores were found to be distributed across all three families. Morphological characters consistent with the resulting molecular phylogeny and correlated with generic boundaries include texture and color of the stroma as well as ecological niche (Sung et al. 2007). The genus *Cordyceps* s.s. contains species with a fleshy texture and brightly colored stroma occurring in leaf litter or shallow soil. The genus *Metacordyceps* contains species producing a stroma with a firm, fibrous texture and are predominantly green, pallid or

lilac colored and darken to a purple or black upon bruising or drying (Sung et al. 2007, Chapter 1). *Ophiocordyceps* is comprised of fungi producing a rigid, pliant or wiry stipe that is darkly colored and are typically found deeply buried in soil or in rotting wood. Although these characters correspond well to clades defined phylogenetically, exceptions do occur and a less ambiguous system of morphological classification is still lacking.

Host associations can be informative as well. For example, although sexually reproducing pathogens of other fungi can be found in Ophiocordycipitaceae and Clavicipitaceae, those attacking false truffles in the genus *Elaphomyces* are restricted to the genus *Elaphocordyceps* (Sung et al. 2007). Species infecting the sclerotia of *Claviceps* are restricted to the genus *Tyrannocordyceps* in Clavicipitaceae (Chapter 4). Pathogens of spiders are typically in the genus *Torrubiella*.

The form of the anamorph also conveys taxonomic information, although as with other morphological characters exceptions are known (Sung et al. 2007). For example species producing forms similar to *Verticillium* can be found throughout the Sordariomycetes (Zare et al. 2000). Species in the genus *Metacordyceps* of Clavicipitaceae produce anamorphs typified by the genera *Pochonia* and *Metarhizium*, as well as green spored forms of *Nomuraea* (Sung et al. 2007, Chapter 1). Pink or lilac forms of *Nomuraea* can be found in Ophiocordycipitaceae.

Ophiocordycipitaceae are also associated with *Hymenostilbe* and *Hirsutella* anamorphic forms. The genus *Simplicilium* lacks any known teleomorphic association and occupies a basal position in Cordycipitaceae. Other anamorphic forms restricted

to Cordycipitaceae include *Lecanicillium*, associated with *Cordyceps* teleomorphs and *Gibellula*, associated with *Torruiella* (Sung et al. 2007). Although these anamorphic forms are fairly indicative of family and genus level associations, successfully rearing cultures from fresh teleomorph material can be difficult, and is not possible for herbarium specimens.

Although the work of Sung et al. (2007) is the most expansive molecular investigation of this group of fungi to date, many species remained unsampled in the molecular dataset. Furthermore, the convergent nature of some characters across all three new families of clavicipitaceous fungi leaves a considerable number of taxa of uncertain placement. Ban et al. (2009) used the largest subunit of nuclear ribosomal DNA (LSU) and the complete span of the internal transcribed spacer region (ITS) to address the phylogenetic placement of incertae sedis taxa or those for which molecular data were lacking: *C. alboperithecata* Kobayasi & Shimizu, *O. cuboidea* (Kobayasi & Shimizu) S. Ban, Sakane & Nakagiri, *O. ryogamiensis* (Kobayasi & Shimizu) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, and *O. prolifica* (Kobayasi) S. Ban, Sakane & Nakagiri. They also successfully cultured the anamorphic forms from fresh material. The molecular data showed these species formed a well-supported clade sister to the species *C. ramosopulvinata* Kobayasi & Shimizu and *C. kansashiana* Kobayasi & Shimizu. Furthermore, a cryptic species was uncovered (*O. paracuboidea* S. Ban, Sakane & Nakagiri) and the name *C. alboperithecata* was found to be synonymous with *O. cuboidea*. However, deeper relationships for this group remained unsupported and a direct placement of these taxa

with molecular data in a genus consistent with the current understanding of diversity for clavicipitaceous fungi was not possible. The anamorphic forms were described as *Hirsutella*-like and this was used as justification to move these taxa into the genus *Ophiocordyceps*.

In this paper we obtain additional molecular data for small subunit ribosomal DNA (SSU) and three protein coding genes from specimens of *O. cuboidea*, *O. ryogamiensis*, *O. paracuboidea* and *O. prolifica* sampled in the Ban et al. (2009) paper. We also expand the sampling in the *C. ramosopulvinata* clade sister to these fungi to include *C. nipponica* Kobayasi, and the anamorphic species *Polycephalomyces formosus* Kobayasi. When incorporated into a multigene dataset including representatives from all the major clades of clavicipitaceous fungi, as well as the other hypocrealean families of Bionectriaceae, Hypocreaceae and Nectriaceae we find these taxa are not supported as members of *Ophiocordyceps* but rather occupy a unique position as sister to Clavicipitaceae s.s. Because these species were strongly supported as a distinct clade separate from the known genera of clavicipitaceous fungi we propose the name *Veterocordyceps* as a new genus with two subgenera reflecting morphological, host and phylogenetic divisions. We consider this genus to be a member of Clavicipitaceae sensu lato owing to its placement outside of other major clades of Clavicipitaceae. This work forces reevaluation of the utility of *Hirsutella* anamorph types as a character for phylogenetic placement of taxa lacking molecular data.

MATERIALS AND METHODS

Specimen collection

Tissue from cultures of *O. cuboidea*, *O. ryogamiensis*, *O. paracuboidea* and *O. prolifica* sampled in the Ban et al. (2009) paper were resampled from stocks maintained at the NITE Biological Resource Center. In addition fresh material was collected from field sites in Japan during the months of Jun.-Aug. in the years 2007 and 2008. Upon collection, specimens were cleaned of dirt and placed in a wax paper bag, and later air dried for herbarium storage and a piece of tissue was removed and placed in CTAB buffer for DNA extraction.

DNA extraction, PCR and sequencing

DNA extraction relied on a CTAB protocol. Tissue received an initial grinding by power drill with an Eppendorf pestle in 50 µl CTAB buffer. Four hundred microliters of CTAB was then added to the ground tissue in a FastDNA lysing matrix A tube (MP Biomedical, Salon, OH), and ground further with the fast prep machine for two cycles, 20 seconds each. Cell lysis was enhanced by soaking twenty minutes in a water bath at 60°C. Tissue was then separated from supernatant by centrifugation for 10 minutes at 14000rpm. Four hundred microliters of supernatant was then removed for further cleaning with centrifugation at 14000 rpm for 20 minutes in 500µl

chloroform:isoamyl alcohol (24:1). Cleaned and concentrated DNA was then obtained from 300 µl of the top layer of liquid after chloroform:isoamyl centrifugation with the GeneCleanIII Kit following the manufacturers protocol and eluting from glass milk in the final step with 30µl of water.

PCR methods were used to amplify a total of six nuclear loci for each specimen. As an initial quality control step, and to serve as a voucher for barcoding efforts, the complete span of the internal transcribed spacer region of ribosomal DNA (ITS1-5.8s-ITS2) was amplified and sequenced. A BLAST search of the GenBank database performed to ensure that DNAs obtained were not from contaminants outside of Hypocreales. After passing quality control measures, five nuclear loci were amplified and sequenced for phylogenetic analysis: SSU and LSU, elongation factor 1 α (TEF), and the largest and second largest subunits of RNA polymerase II (RPB1 and RPB2, respectively). Primer information is given in Table 3.1. PCR reactions were performed in either an iCycler or MyCycler thermocycler (BioRad, Hercules, CA) using MasterAmp 2X PCR premix E (Epicenter, Madison WI) and Novagen Taq polymerase. Reaction conditions were the same as those used in (Johnson et al. 2009). PCR products were cleaned using the GeneClean III kit following the manufactures instructions and sequenced using the Macrogen sequencing service (Seoul, South Korea) with the primers used for the initial amplifications.

Phylogenetic analyses

Processing and contig formation of raw sequence reads returned from MacroGen were edited using CodonCode Aligner, version 2.0.6 (Dedham, MA). These data were then added to alignments with previously published data for other clavicipitaceous fungi and species throughout Hypocreales in the families Hypocreaceae, Nectriaceae and Bionectriaceae in Hypocreales, as well as *Glomerella cingulata* and *Verticillium dahliae* from outside the order. Genbank and specimen voucher information is provided in Table 3.2. MAFFT version 6 (Katoh et al. 2002; Katoh and Toh 2008) was used to obtain an initial alignment that was then improved by hand with the program BioEdit version 7.05 (Hall 1999). Ambiguously aligned regions were excluded from phylogenetic analyses and gaps were treated as missing data. The final dataset contained 142 specimens and after exclusion of ambiguously aligned sites consisted of 1089 nucleotides for SSU, 884 for LSU, 998 for TEF, 698 for RPB1, and 1048 for RPB2. The total length of aligned sequences was 4717. For all specimens examined, at least three of the five genes sought were obtained (Table 3.2). Tests for conflict between loci were conducted with the program compat.py. Eleven data partitions were defined for the final combined dataset, one each for nu-rSSU and nu-rLSU plus nine for each of the three codon positions for the protein coding genes TEF, RPB1, and RPB2.

Maximum Likelihood (ML) estimation of phylogeny was performed with RAxML version 7.0.4 (Stamatakis 2006) with 500 rapid bootstrap replicates on a concatenated dataset containing all five genes. The CAT-GAMMA model of evolution was employed during the rapid bootstrapping phase and the GTR-GAMMA

model of evolution was specified for the final likelihood tree as suggested by the program manual for large datasets. Bayesian estimation of phylogenetic relationships was conducted with the program Mr. Bayes v3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The same eleven data partitions were applied with the GTR+I+G nucleotide substitution model was used for all gene partitions. Two runs were conducted simultaneously, each with four chains for 5 million generations. Each chain was sampled every 100 generations, and trees saved with branch length information every 500 generations. After the analysis finished, each run was examined with the program Tracer v1.5 (Drummond and Rambaut 2007) to determine burn-in and confirm that both runs had converged. Summary of the model parameters was determined with the sump command. A strict consensus tree with branch lengths and posterior probabilities was then obtained with the sumt command. The same burnin value was used for both sump and sumt commands.

RESULTS

Maximum likelihood and Bayesian analyses confirmed *C. ramosopulvinata*, *C. nipponica*, *O. cuboidea*, *O. ryogamiensis*, *O. paracuboidea*, *O. prolifica* and *Polycephalomyces formosus* form a well supported clade not associated with any previously identified genus of clavicipitaceous fungi. Maximum likelihood analyses recover high support for a relationship sister to all other taxa of Clavicipitaceae (log likelihood -105693.345561) (Figure 3.1). The same topology was recovered for

Bayesian analyses but support was not significant (less than 0.95 posterior probability). Although placement of this clade was found to be sensitive to taxon sampling (data not shown) at no time was a topology recovered showing an association with *Ophiocordyceps*. Support values for relationships between other families of Hypocreales were not affected by the addition of these taxa. The topology recovered here for relationships between families of Hypocreales is consistent with the results of previous analyses (Sung et al. 2007, Johnson et al. 2009, Chapter 1).

Structure was observed within this clade and found to correlate with both morphological and host differences. The species *O. cuboidea*, *O. ryogamiensis*, *O. paracuboidea* and *O. prolifica* form a subclade of species attacking a range of hosts which includes beetle larvae and cicada nymphs and forming clavate stroma with superficial perithecia. The second subclade consists of *C. ramosopulvinata*, *C. nipponica*, and *P. formosus*. Teleomorphs in this clade comprise species restricted to cicada nymphs (Hemiptera: Cicadidae) and form stroma with perithecia contained in a pulvinate pad that may be terminal or lateral.

Based on these results we describe the new genus *Veterocordyceps*. Furthermore, the subgeneric classifications *Veterocordyceps* and *Pulvinata* are erected to accommodate the phylogenetic, morphological and host association split observed between the *O. cuboidea*, *O. ryogamiensis*, *O. paracuboidea*, *O. prolifica* clade and the *C. ramosopulvinata*, *C. nipponica*, and *P. formosus* clade respectively. We consider this genus to be a member of Clavicipitaceae sensu lato, owing to its

placement outside of the currently recognized members of the family and the effects of taxon sampling depth on support values.

TAXONOMY

Veterocordyceps Kepler & J.W. Spatafora subgenus *Veterocordyceps* genus et subgenus novum

Stroma stiff, pliant, often multifurcating or with several stipes representing several cycles of growth, rhizomorphs present or absent; color ranging from white to pallid or yellow to brown. Perithecia superficial and scattered to gregarious, often concentrated on the upper portion of the stroma but leaving the tip bare. Asci long, ascospores forming many small partspores of nearly equal length on a side. Hosts include cicada nymphs (Hemiptera: Cicadidae) and beetle larvae (Coleoptera).

Type: *Cordyceps prolifica* Kobayasi

Anamorphic states: *Hirsutella* or *Acremonium*

Etymology: The genus name refers to the long-lived habit of some species, as well as the general *Cordyceps*-like morphology.

Veterocordyceps subg. *Veterocordyceps cuboidea* (Kobayasi & Shimizu) Kepler & J.W. Spatafora comb. nov.

≡ *Ophiocordyceps cuboidea* (Kobayasi & Shimizu) S. Ban, Sakane & Nakagiri.

Mycoscience 50(4): 268 (2009)

≡*Cordyceps cuboidea* Kobayasi & Shimizu. Bull. natn. Sci. Mus., Tokyo, Bot. 6(4): 131 (1980).

Host: Larvae of Coleoptera

Habitat: Rotten wood

Veterocordyceps subg. Veterocordyceps paracuboidea (S. Ban, Sakane & Nakagiri) Kepler & J.W. Spatafora comb. nov.

≡*Ophiocordyceps paracuboidea* S. Ban, Sakane & Nakagiri. Mycoscience 50(4): 268 (2009)

Host: Larvae of Coleoptera

Habitat: Rotten wood

Veterocordyceps subg. Veterocordyceps prolifica (Kobayasi) Kepler & J.W. Spatafora comb. nov.

≡*Ophiocordyceps prolifica* (Kobayasi & Shimizu) S. Ban, Sakane & Nakagiri. Mycoscience 50(4): 270 (2009)

≡*Cordyceps prolifica* Kobayasi in Kobayasi, Y.; Shimizu, D., Bulletin of the National Science Museum, Tokyo 6: 289 (1963)

Host: Nymph of Cicadidae (Hemiptera)

Habitat: Buried in soil

Veterocordyceps subg. Veterocordyceps ryogamiensis (Kobayasi & Shimizu) Kepler & J.W. Spatafora comb. nov.

≡*Ophiocordyceps ryogamiensis* (Kobayasi & Shimizu) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora in Sung, Hywel-Jones, Sung, Luangsa-ard, Shrestha & Spatafora, Stud. Mycol. 57: 45 (2007).

≡*Cordyceps ryogamiensis* Kobayasi & Shimizu. Bull. natn. Sci. Mus., Tokyo, Bot. 9(1): 4 (1983).

Host: Larvae of Coleoptera

Habitat: Rotten wood

Veterocordyceps Kepler & J.W. Spatafora subgenus *Pulvinata* subgenus novum

Stroma stiff, pliant, often multifurcating or with several stipes representing several cycles of growth, rhizomorphs present or absent; color ranging from white to pallid or yellow to brown. Perithecia immersed, contained in terminal or subterminal pads. Asci long, ascospores forming many rectangular partspores. Hosts restricted to cicada nymphs (Hemiptera: Cicadidae).

Type: *Cordyceps ramosopulvinata* Kobayasi & Shimizu

Anamorphic state: *Polycephalomyces*

Etymology: The subgenus name refers to the habit of forming perithecia in a pillow like pad.

Commentary: In addition to the species examined directly here, we include *C.*

kanzashiana because of the affinity of this species to *C. ramosopulvinata* in previous studies (Nikoh and Fukatsu 2000, Ban et al. 2009). *Cordyceps pleuricapitata* is included based on morphological examination of macro and microscopic features.

Veterocordyceps subg. Pulvinata kanzashiana (Kobayasi & Shimizu) Kepler & Spatafora comb. nov.

≡*Cordyceps kanzashiana* Kobayasi & Shimizu. Bulletin of the National Science Museum, Tokyo 8(3): 86 (1982).

Host: Nymph of Cicadidae (Hemiptera)

Habitat: Buried in soil

Veterocordyceps subg. Pulvinata nipponica (Kobayasi) Kepler & J.W. Spatafora comb. nov.

≡*Cordyceps nipponica* Kobayasi. Bulletin of the Biogeogr. Soc. Jap. 9: 151 (1939).

Host: Nymph of Cicadidae (Hemiptera)

Habitat: Buried in soil

Veterocordyceps subg. Pulvinata pleuricapitata (Kobayasi & Shimizu) Kepler & J.W. Spatafora comb. nov.

≡*Cordyceps pleuricapitata* Kobayasi, Y.; Shimizu, D., 1982, Bulletin of the National Science Museum, Tokyo 8(3): 87

Host: Nymph of Cicadidae (Hemiptera)

Habitat: Buried in soil

Veterocordyceps subg. Pulvinata ramosopulvinata (Kobayasi & Shimizu) Kepler & J.W. Spatafora comb. nov.

≡*Cordyceps ramosopulvinata* Kobayasi & Shimizu. Bull. natn. Sci. Mus., Tokyo, Bot. 9(1): 2 (1983).

Host: Nymph of Cicadidae (Hemiptera)

Habitat: Buried in soil

Discussion

Understanding of diversity in Hypocreales is progressing rapidly by the application of molecular phylogenetic methods. As modern concepts of taxonomy for the order have developed, morphological characters that once defined boundaries have shown multiple origins. The genus *Cordyceps* was found to be especially diverse, spanning five genera in three families (Sung et al. 2007, Chapter 3). Discrimination between genera with morphological characters was found to rely on color and texture of the stipe, habitat and in some cases host affiliation. The form of the anamorph was also found to be phylogenetically informative. *Veterocordyceps* gen. nov. described in this study represents the sixth genus to be described from species previously placed in *Cordyceps*. Associations amongst these taxa were first noted by Ban et al. (2009), who obtained strong support for a clade equating to *Veterocordyceps* with two subgenera. However, neither the depth of taxon sampling nor the nature of the molecular data was sufficient to support placement in relation to established genera. Ban et al. (2009) used a 504 base-pair fragment of large subunit nuclear ribosomal RNA (LSU), which fails to adequately resolve deep fungal nodes (Hofsetter et al. 2007). Previously, Chaverri et al. (2005) analyzed LSU, TEF and RBP1 data for *V. ramosopulvinata* and *P. formosus*, however the taxon sampling in this analysis was focused on scale insect pathogens in Clavicipitaceae s.s. and did not include members

of *V. subg. Veterocordyceps*. The dataset examined here includes additional ribosomal data, as well as protein coding genes which are shown to be better suited for addressing divergences of genera and families in Hypocreales (Zhang et al. 2006, Hofsetter et al. 2007, Sung et al. 2007). We also greatly expand the taxonomic scope, including species throughout the order. Increasing the number of taxa included in phylogenetic analyses reduces error and increases accuracy of the reconstruction (Zwickl and Hillis 2002). Strong support was obtained for placement of this genus as sister to Clavicipitaceae *sensu stricto*, which we consider here to contain the plant-associated species such as *Claviceps* and *Ustilaginoidea*, as well as animal pathogens such as *Hypocrella* and *Metacordyceps*. Although topologies for the relationship of *Veterocordyceps* to Clavicipitaceae are consistent across methods, support for this relationship was only significant in likelihood analyses. Therefore, we feel it is prudent to consider *Veterocordyceps* an incertae sedis member of Clavicipitaceae s.l. until modern taxonomic and phylogenetic concepts of clavicipitaceous fungi mature further.

Veterocordyceps is morphologically very different from the other animal pathogens of Clavicipitaceae. Both *V. subg. Veterocordyceps* and *V. subg. Pulvinata* are more similar to *Ophiocordyceps* in Ophiocordycipitaceae with regards to macromorphology (Figure 3.2). All species produce tough, wiry, long-lasting stromata, usually with a darkened color. Sung et al. (2007) included *V. ryogamiensis* in *Ophiocordyceps* based on morphological characters found to be consistent with the relationships inferred from molecular data. Ban et al. (2009) presented additional data

on the anamorph morphology for this species, as well as other closely related species in *V. subg. Veterocordyceps*. All species produce *Hirsutella*-like anamorphic forms, with *Acremonium* like morphologies developing near the edge of the colony.

Hirsutella was identified by Sung et al. (2007) as being associated only with the species of *Ophiocordyceps* and is distinguished from other anamorphs of clavicipitaceous fungi by having phialides swollen at the base then tapering to a tip where conidia are produced in a slimy mass. When naturally occurring on a host, *Hirsutella* produces grey or brown synnema. Phialides are typically solitary, although they may be whorled or verticillate. *Acremonium* is a diverse form genus primarily associated with Hypocreaceae. Several species associated with grass-endophytic species in Clavicipitaceae were moved from *Acremonium* to *Neotyphodium* (Glenn et al. 1996). Species of *Acremonium* also produce conidia in slimy heads, however the phialides are awl shaped, tapering at the tip without swollen bases. Both *Acremonium* and *Hirsutella* share a similarity to *Verticillium*, a highly polyphyletic form genus with multiple occurrences throughout Hypocreales (Zare et al. 2000, Gams and Zare 2001, Sung et al. 2001, Zare and Gams 2001, Zare et al. 2001). The range of anamorphic form genera reported by Ban et al. (2009) for the species of *V. subg. Veterocordyceps* is therefore polyphyletic. Historical uses of *Hirsutella* have been quite broad, with the name applied to species occurring outside of *Ophiocordyceps* that were later reclassified, e.g., *Simplicillium*. It is therefore possible that the *Hirsutella*-like anamorph types produced in culture by *Veterocordyceps* reflects

variation on a *Verticillium*-like anamorph commonly produced by other clavicipitaceous fungi in culture.

Veterocordyceps subg. *Pulvinata* was found to be associated with anamorphs of the genus *Polycephalomyces*, based on the placement of *P. formosus*. Members of the genus are similar to *Hirsutella* in that they produce conidia in slime at the tips of phialides, however phialides lack the swollen base and are concentrated at the tips of synnemata. The ecology of *Polycephalomyces* is unclear and specimens are reported as hyperparasites of other clavicipitaceous insect pathogens and myxomycetes, as well as insect cadavers (Seifert 1985). The specimen included here was collected from an unidentified insect pupa in a silk case. *Polycephalomyces* produces extremely tiny conidia (often 2µm or smaller in width) in a slimy mass at the tip of a prominent synnema. Seifert (1985) reported the teleomorphic state for *P. tomentosum* as *Byssostilbe stilbigera* (Berk. & Br.) Petch and was later found to be phylogenetically distinct from other species of *Polycephalomyces* (Bischoff et al. 2003). A connection between *P. formosus* and *V. ramosopulvinata* was recovered by Chaverri et al. (2005), however the taxon sampling was not sufficient for further inferences. Linking *P. formosus* to *V.* subg. *Pulvinata* closes the gap in understanding teleomorph associations of these fungi, however their nutritional mode is still unclear.

The host associations for *Veterocordyceps* subg. *Pulvinata* include only cicada pathogens, whereas *V.* subg. *Veterocordyceps* also contains pathogens of Coleoptera. Pathogens of cicada nymphs have evolved multiple times in Ophiocordycipitaceae, as well as in Clavicipitaceae (Sung et al. 2007, Ch 2 herein). These hosts occur buried

deeply in the soil, however the beetle pathogens in *V. subg. Veterocordyceps* were excavated from decaying wood. This pairing of habitats is possibly another convergent character between fungi of *Ophiocordyceps* and *Veterocordyceps* (Sung 2007).

Introduction of molecular phylogenetic methods to the study of clavicipitaceous fungi has enabled major advances in understanding the evolution of species relationships and key life-history traits and morphologies. However, such advances must be flexible in the face of new data. Although morphological characteristics such as stipe texture and especially color of teleomorphs are known to have exceptions, anamorphic forms were considered to be more robust and indicative of phylogenetic position (Sung et al. 2007). The work presented here calls into question the utility of *Hirsutella*-like anamorphs as informative characters for membership in *Ophiocordyceps* by showing that they also occur in *Veterocordyceps*. However, *Hirsutella*-like anamorphs are observed in other species outside of Ophiocordycipitaceae when growing in culture. To date no *Hirsutella* anamorph typical of the brown or grey synnemateous forms encountered in Ophiocordycipitaceae have been recovered for *Veterocordyceps*. We also make a teleomorph connection for *P. formosus*. It is possible *Polycephalomyces*-like anamorphs can be used as a diagnostic character, compensating somewhat for the convergent nature of *Hirsutella*. To fully understand the phylogenetic and taxonomic relations of *Veterocordyceps*, further sampling of clavicipitaceous fungi is needed, particularly those infecting cicada nymphs or ecologies not yet incorporated into the molecular framework.

REFERENCES

- BAN, S., T. SAKANE, K. TOYAMA, AND A. NAKAGIRI. 2009. Teleomorph-anamorph relationships and reclassification of *Cordyceps cuboidea* and its allied species. *Mycoscience* 50: 261-272.
- BISCHOFF, J.F., R. SULLIVAN, N.L. HYWEL-JONES, AND J. WHITE. 2003. Resurrection of *Blistum tomentosum* and its exclusion from *Polycephalomycetes* (Hyphomycetes, Deuteromycota) based on 28S rDNA sequence data. *Mycotaxon* 86: 433-444.
- CARBONE, I., AND L.M. KOHN. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91: 553-556.
- CASTLEBURY, L.A., A.Y. ROSSMAN, G. SUNG, A.S. HYTEN, AND J.W. SPATAFORA. 2004. Multigene phylogeny reveals new lineage for *Stachybotrys chartarum*, the indoor air fungus. *Mycological Research* 108: 864-872.
- CHAVERRI, P., J.F. BISCHOFF, H.C. EVANS, AND K.T. HODGE. 2005. *Regiocrella*, a new entomopathogenic genus with a pycnidial anamorph and its phylogenetic placement in the Clavicipitaceae. *Mycologia* 97: 1225-1237.
- DRUMMOND, A., AND A. RAMBAUT. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7: 214.
- GAMS, W., AND R. ZARE. 2001. A revision of *Verticillium* sect. *Prostrata*. III. Generic classification. *NOVA HEDWIGIA* 72: 329-337.
- GLENN, A.E., C.W. BACON, R. PRICE, AND R.T. HANLIN. 1996. Molecular Phylogeny of *Acremonium* and Its Taxonomic Implications. *Mycologia* 88: 369-383.
- HALL, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41: 95-98.
- HEDTKE, S.M., T.M. TOWNSEND, AND D.M. HILLIS. 2006. Resolution of Phylogenetic Conflict in Large Data Sets by Increased Taxon Sampling. *Systematic Biology* 55: 522-529.
- HODGE, K.T., S.B. KRASNOFF, AND R.A. HUMBER. 1996. *Tolypocladium inflatum* is the anamorph of *Cordyceps subsessilis*. *Mycologia* 88: 715-719.
- HOFSTETTER, V., J. MIADLIKOWSKA, F. KAUFF, AND F. LUTZONI. 2007. Phylogenetic comparison of protein-coding versus ribosomal RNA-coding sequence data: A

- case study of the Lecanoromycetes (Ascomycota). *Molecular Phylogenetics and Evolution* 44: 412-426.
- HUELSENBECK, J.P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.
- JOHNSON, D. ET AL. 2009. Systematics and evolution of the genus *Torrubiella* (Hypocreales, Ascomycota). *Mycological Research* 113: 279-289.
- KAMATA, N., H. SATO, AND M. SHIMAZU. 1997. Seasonal changes in the infection of pupae of the beech caterpillar, *Quadricalcarifera punctatella* (Motsch.) (Lep., Notodontidae), by *Cordyceps militaris* Link (Clavicipitales, Clavicipitaceae) in the soil of the Japanese beech forest. *Journal of Applied Entomology* 121: 17-21.
- KATOH, K., K. MISAWA, K. KUMA, AND T. MIYATA. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl. Acids Res.* 30: 3059-3066.
- KATOH, K., AND H. TOH. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* 9: 286-298.
- KOBAYASI, Y., AND D. SHIMIZU. 1980. *Cordyceps* species from Japan 3. *Bulletin of the National Science Museum, Tokyo*, Ser. B.6(4): 125-145.
- KOBAYASI, Y., AND D. SHIMIZU. 1983. *Cordyceps* species from Japan 6. *Bulletin of the National Science Museum, Tokyo*, Ser. B. 9: 1-21.
- KOBAYASI, Y., AND D. SHIMIZU. 1963. Monographic studies of *Cordyceps* 2. Group parasitic on Cicadae. *Bulletin of the National Science Museum, Tokyo*, 6: 286-314.
- POWLES, R. 1978. CYCLOSPORIN A FOR THE TREATMENT OF GRAFT-VERSUS-HOST DISEASE IN MAN. *The Lancet* 312: 1327-1331.
- RONQUIST, F., AND J.P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572 -1574.
- SEIFERT, K. 1985. A Monograph of *Stilbella* and Some Allied Hyphomycetes. *Studies in Mycology* 1-224.
- STAMATAKIS, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688-2690.

- SUNG, G., J. SPATAFORA, R. ZARE, K. HODGE, AND W. GAMS. 2001. A revision of *Verticillium* sect. *Prostrata*. II. Phylogenetic analyses of SSU and LSU nuclear rDNA sequences from anamorphs and teleomorphs of the Clavicipitaceae. *NOVA HEDWIGIA* 72: 311-328.
- SUNG, G., N.L. HYWEL-JONES, J. SUNG, J.J. LUANGSA-ARD, B. SHRESTHA, AND J.W. SPATAFORA. 2007. Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud Mycol* 57: 5-59.
- SUNG, G., J. SUNG, N.L. HYWEL-JONES, AND J.W. SPATAFORA. 2007. A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. *Molecular Phylogenetics and Evolution* 44: 1204-1223.
- VILGALYS, R., AND B.L. SUN. 1994. Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of ribosomal DNA sequences. *PNAS* 91: 4599-4603.
- WHITE, T.J., T.D. BRUNS, S. LEE, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White [eds.], *PCR protocols, a guide to methods and applications.*, 315-322. Academic Press, San Diego.
- ZARE, R., AND W. GAMS. 2001. A revision of *Verticillium* section *Prostrata*. IV. The genera *Lecanicillium* and *Simplicillium* gen. nov. *NOVA HEDWIGIA* 73: 1-50.
- ZARE, R., W. GAMS, AND A. CULHAM. 2000. A revision of *Verticillium* sect. *Prostrata* - I. Phylogenetic studies using ITS sequences. *NOVA HEDWIGIA* 71: 465-480.
- ZARE, R., W. GAMS, AND H. EVANS. 2001. A revision of *Verticillium* section *Prostrata*. V. The genus *Pochonia*, with notes on *Rotiferophthora*. *NOVA HEDWIGIA* 73: 51-86.
- ZHANG, N. ET AL. 2006. An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. *Mycologia* 98: 1076-1087.
- ZWICKL, D.J., AND D.M. HILLIS. 2002. Increased Taxon Sampling Greatly Reduces Phylogenetic Error. *Systematic Biology* 51: 588 -598.

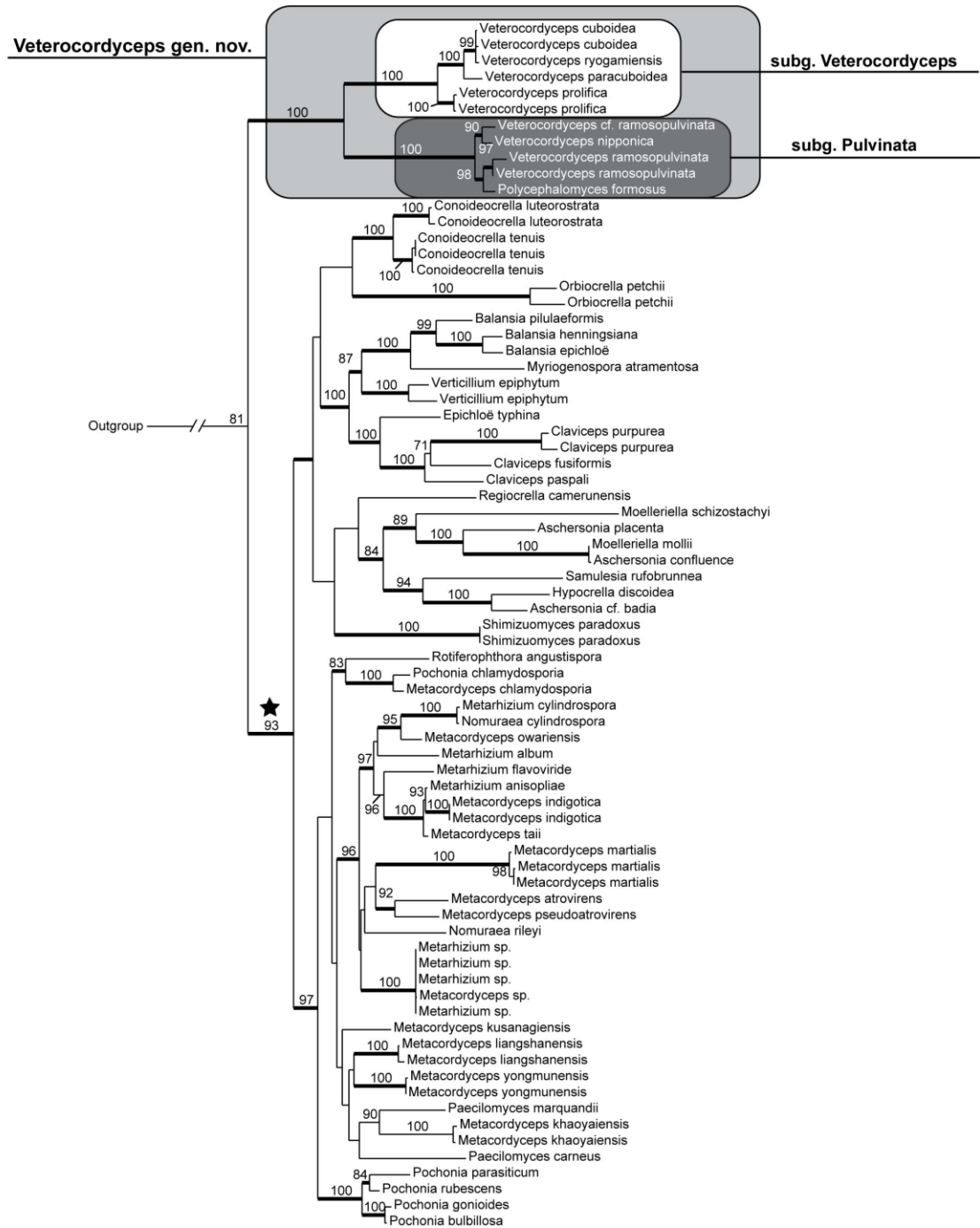


Figure 3.1

Figure 3.1 Maximum Likelihood tree obtained from analysis in RAxML of a concatenated 5 gene dataset (SSU, LSU, TEF, RPB1, RPB2) showing placement of *Veterocordyceps*. Values above branches represent bootstrap proportions from 500 replicates. Branches in bold denote strongly supported nodes in Bayesian analyses (≥ 0.95 posterior probability).



Figure 3.2. *Veterocordyceps prolifica* subg. *Veterocordyceps* and *Veterocordyceps pleuricapitata* subg. *Pulvinata*. A-C *Veterocordyceps prolifica*. A. Stroma emerging from soil. B. Ascus. C. Partspores. D-F *Veterocordyceps pleuricapitata*. D. Dried specimens. E. Ascus. F. Partspores.

Table 3.2 Information for primers used for PCR in this study.

Gene	Primer	5'-Sequences-3'	source
TEF	983F	GCYCCYGGHCAYCGTGAYTTYAT	Carbone & Kohn (1999)
TEF	2218R	ATGACACCRACRGCACRGTYTG	Steven Rehner, personal communication
LSU	LR5	ATCCTGAGGGAAACTTC	Vilgalys and Sun (1994)
LSU	LR0R	GTACCCGCTGAACTTAAGC	Vilgalys and Sun (1994)
SSU	SR7	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS4	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS3	GCAAGTCTGGTGCCAGCAGCC	White et al. (1990)
SSU	NS1	GTAGTCATATGCTTGTCTC	White et al. (1990)
RPB1	RPB1Cr	CCNGCDATNTCRTTRTCCATRTA	Castlebury et al. (2004)
RPB1	CRPB1A	CAYCCWGGYTTYATCAAGAA	Castlebury et al. (2004)
RPB2	fRPB2-7cR	CCCATRGCTTGTYRCCCAT	Liu et al. (1999)
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al. (1999)

Table 3.2 Taxon, specimen voucher and sequence information for specimens used in this study

Species	Voucher_info	ITS GB.	SSU	LSU	TEF	RPB1	RPB2
<i>Akanthomyces novoguineensis</i>	NHJ 11923		EU369095	EU369032	EU369013	EU369052	EU369072
<i>Aphysiostroma stercorarium</i>	ATCC 62321		AF543769	AF543792	AF543782	AY489633	EF469103
<i>Aschersonia badia_cf</i>	BCC 7016	Pending	DQ372091	DQ384941	DQ384969	DQ385009	DQ452460
<i>Aschersonia confluence</i>	BCC 7961	Pending	DQ372100	DQ384947	DQ384976	DQ384998	DQ452465
<i>Aschersonia placenta</i>	BCC 7869	Pending	EF469121	EF469074	EF469056	EF469085	EF469104
<i>Balansia epichloë</i>	AEG 96-15a	Pending	EF468949		EF468743	EF468851	EF468908
<i>Balansia henningsiana</i>	GAM 16112	Pending	AY545723	AY545727	AY489610	AY489643	DQ522413
<i>Balansia pilulaeformis</i>	AEG 94-2	Pending	AF543764	AF543788	DQ522319	DQ522365	DQ522414
<i>Bionectria ochroleuca</i>	CBS 114056		AY489684	AY489716	AY489611	DQ842031	DQ522415
<i>Claviceps fusiformis</i>	ATCC 26019	Pending	DQ522539	U17402	DQ522320	DQ522366	
<i>Claviceps paspali</i>	ATCC 13892	Pending	U32401	U47826	DQ522321	DQ522367	DQ522416
<i>Claviceps purpurea</i>	GAM 12885	Pending	AF543765	AF543789	AF543778	AY489648	DQ522417
<i>Claviceps purpurea</i>	SA cp11		EF469122	EF469075	EF469058	EF469087	EF469105
<i>Conoideocrella luteorostrata</i>	NHJ 12516	Pending	EF468994	EF468849	EF468800	EF468905	EF468946
<i>Conoideocrella luteorostrata</i>	NHJ 11343	Pending	EF468995	EF468850	EF468801	EF468906	
<i>Conoideocrella tenuis</i>	NHJ 6293	Pending	EU369112	EU369044	EU369029	EU369068	EU369087
<i>Conoideocrella tenuis</i>	NHJ 345.01		EU369111	EU369045	EU369030		EU369088
<i>Conoideocrella tenuis</i>	NHJ 6791	Pending	EU369113	EU369046	EU369028	EU369069	EU369089
<i>Cordyceps bifusispora</i>	EFCC 5690		EF468952	EF468806	EF468746	EF468854	EF468909
<i>Cordyceps brongniartii</i>	BCC 16585	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps cardinalis</i>	OSC 93610	Pending	AY184974	AY184963	EF469059	EF469088	EF469106
<i>Cordyceps cf. ochraceostromata</i>	ARSEF 5691	Pending	EF468964	EF468819	EF468759	EF468867	EF468921
<i>Cordyceps coccidioperitheciata</i>	NHJ 6709	Pending	EU369110	EU369042	EU369025	EU369067	EU369086
<i>Cordyceps confragosa</i>	CBS 101247	Pending	AF339604	AF339555	DQ522359	DQ522407	DQ522466
<i>Cordyceps gunnii</i>	OSC 76404	Pending	AF339572	AF339522	AY489616	AY489650	DQ522426
<i>Cordyceps kyusyuënsis</i>	EFCC 5886		EF468960	EF468813	EF468754	EF468863	EF468917
<i>Cordyceps militaris</i>	OSC 93623	Pending	AY184977	AY184966	DQ522332	DQ522377	AY545732
<i>Cordyceps nipponica</i>	BCC 18108	Pending	Pending	Pending	Pending	Pending	
<i>Cordyceps ramosopulvinata</i>	EFCC 5566	Pending		Pending	Pending	Pending	
<i>Cordyceps ramosopulvinata</i>	EFCC 9072		Pending	Pending	Pending	Pending	
<i>Cordyceps ramosopulvinata</i>	SU-65			DQ118742	DQ118753	DQ127244	

Table 3.2 continued

<i>Cordyceps scarabaeicola</i>	ARSEF 5689	Pending	AF339574	AF339524	DQ522335	DQ522380	DQ522431
<i>Cordyceps</i> sp.	HMAS 199601	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps tuberculata</i>	OSC 111002	Pending	DQ522553	DQ518767	DQ522338	DQ522384	DQ522435
<i>Cosmospora coccinea</i>	CBS 114050	Pending	AY489702	AY489734	AY489629	AY489667	DQ522438
<i>Elaphocordyceps japonica</i>	OSC 110991	Pending	DQ522547	DQ518761	DQ522330	DQ522375	DQ522428
<i>Elaphocordyceps ophioglossoides</i>	OSC 106405		AY489691	AY489723	AY489618	AY489652	DQ522429
<i>Elaphocordyceps subsessilis</i>	OSC 71235	Pending	EF469124	EF469077	EF469061	EF469090	EF469108
<i>Engyodontium aranearum</i>	CBS 309.85	Pending	AF339576	AF339526	DQ522341	DQ522387	DQ522439
<i>Epichloë typhina</i>	ATCC 56429	Pending	U32405	U17396	AF543777	AY489653	DQ522440
<i>Gibellula</i> sp.	NHJ 13158	Pending	EU369100	EU369037	EU369020	EU369057	EU369077
<i>Glomerella cingulata</i>	CBS 114054		AF543762	AF543786	AF543773	AY489659	DQ522441
<i>Glomerella cingulata</i>	FAU 513		U48427	U48428	AF543772	DQ858454	DQ858455
<i>Haptocillium sinense</i>	CBS 567.95	AJ292417	AF339594	AF339545	DQ522343	DQ522389	DQ522443
<i>Hirsutella</i> sp.	NHJ 12525		EF469125	EF469078	EF469063	EF469092	EF469111
<i>Hirsutella</i> sp.	OSC 128575	Pending	EF469126	EF469079	EF469064	EF469093	EF469110
<i>Hydropisphaera peziza</i>	CBS 102038		AY489698	AY489730	AY489625	AY489661	DQ522444
<i>Hypocrea lutea</i>	ATCC 208838		AF543768	AF543791	AF543781	AY489662	DQ522446
<i>Hypocrea rufa</i>	CBS 114374		AY489694	AY489726	AY489621	AY489656	EF692510
<i>Hypocrella discoidea</i>	BCC 8237	Pending	-	DQ384937	DQ384977	DQ385000	DQ452461
<i>Isaria farinosa</i>	CBS 240.32	AY624178	Pending	Pending	Pending	Pending	Pending
<i>Isaria tenuipes</i>	ARSEF 5135	AY624196	Pending	Pending	Pending	Pending	Pending
<i>Lecanicillium attenuatum</i>	CBS 402.78	AJ292434	AF339614	AF339565	EF468782	EF468888	EF468935
<i>Lecanicillium psalliotae</i>	CBS 532.81	Pending	AF339609	AF339560	EF469067	EF469096	EF469112
<i>Mariannaea elegans</i> var. <i>punicea</i>	CBS 239.56	Pending	Pending	Pending	Pending	Pending	Pending
<i>Mariannaea pruinosa</i>	ARSEF 5413	Pending	AY184979	AY184968	DQ522351	DQ522397	DQ522451
<i>Metacordyceps atrovirens</i>	TNM F10184	Pending	Pending	Pending		Pending	
<i>Metacordyceps chlamydosporia</i>	CBS 101244	Pending	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
<i>Metacordyceps indigotica</i>	TNS F18554	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metacordyceps indigotica</i>	TNS F18553	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metacordyceps khaoyaiensis</i>	BCC 14290	Pending		Pending	Pending	Pending	
<i>Metacordyceps khaoyaiensis</i>	BCC 12687	Pending		Pending	Pending	Pending	
<i>Metacordyceps kusanagiensis</i>	TNS F18494	Pending	Pending	Pending	Pending	Pending	
<i>Metacordyceps liangshanensis</i>	EFCC 1452		EF468962	EF468815	EF468756		

Table 3.2 continued

<i>Metacordyceps liangshanensis</i>	EFCC 1523		EF468961	EF468814	EF468755		EF468918
<i>Metacordyceps martialis</i>		Pending	Pending	Pending		Pending	
<i>Metacordyceps martialis</i>	HMAS 197472(S)	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metacordyceps martialis</i>	EFCC 6863			Pending	Pending		Pending
<i>Metacordyceps owariensis</i>	NBRC 33258	Pending		Pending	Pending		Pending
<i>Metacordyceps pseudoatrovirens</i>	TNSF 16380	Pending		Pending		Pending	Pending
<i>Metacordyceps taii</i>	ARSEF 5714	Pending	AF543763	AF543787	AF543775	DQ522383	DQ522434
<i>Metacordyceps yongmunensis</i>	EFCC 2131	Pending	EF468977	EF468833	EF468770	EF468876	
<i>Metacordyceps yongmunensis</i>	EFCC 2135		EF468979	EF468834	EF468769	EF468877	
<i>Metarhizium album</i>	ARSEF 2082	Pending	DQ522560	DQ518775	DQ522352	DQ522398	DQ522452
<i>Metarhizium anisopliae</i>	ARSEF 3145	Pending	AF339579	AF339530	AF543774	DQ522399	DQ522453
<i>Metarhizium flavoviride</i>	ARSEF 2037	Pending	AF339580	AF339531	DQ522353	DQ522400	DQ522454
<i>Metarhizium sp.</i>	HMAS 199592	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium sp.</i>	HMAS 199596	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium sp.</i>	HMAS 199603	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium sp.</i>	HMAS 199590	Pending	Pending	Pending	Pending	Pending	Pending
<i>Moelleriella mollii</i>	BCC 7963		DQ372087		DQ384964	DQ385004	DQ452466
<i>Moelleriella schizostachyi</i>	BCC 1985		DQ372105	DQ384939	DQ384959	DQ385012	DQ452471
<i>Myriogenospora atramentosa</i>	AEG 96-32	Pending	AY489701	AY489733	AY489628	AY489665	DQ522455
<i>Nectria cinnabarina</i>	CBS 114055		U32412	U00748	AF543785	AY489666	DQ522456
<i>Nectria_cf sp</i>	CBS 478.75		U47842	U17404	EF469068	EF469097	EF469115
<i>Nomuraea cylindrospora</i>	RCEF 3632	Pending	Pending	Pending	Pending		
<i>Nomuraea cylindrospora</i>	TNS 16371		Pending	Pending	Pending	Pending	
<i>Nomuraea rileyi</i>	CBS 806.71	AY624205	AY624205	AY624250	EF468787	EF468893	EF468937
<i>Ophiocordyceps acicularis</i>	OSC 128580	Pending	DQ522543	DQ518757	DQ522326	DQ522371	DQ522423
<i>Ophiocordyceps agriotidis</i>	ARSEF 5692	Pending	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
<i>Ophiocordyceps aphodii</i>	ARSEF 5498		DQ522541	DQ518755	DQ522323		DQ522419
<i>Ophiocordyceps brunneipunctata</i>	OSC 128576		DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
<i>Ophiocordyceps cuboidea</i>	NBRC 101740		Pending	Pending	Pending	Pending	
<i>Ophiocordyceps cuboidea</i>	TNS will be assigned		Pending	Pending	Pending		
<i>Ophiocordyceps entomorrhiza</i>	KEW 53484	Pending	EF468954	EF468809	EF468749	EF468857	EF468911
<i>Ophiocordyceps gracilis</i>	EFCC 8572	Pending	EF468956	EF468811	EF468751	EF468859	EF468912
<i>Ophiocordyceps heteropoda</i>	EFCC 10125	Pending	EF468957	EF468812	EF468752	EF468860	EF468914

Table 3.2 continued

<i>Ophiocordyceps longissima</i>	EFCC 6814			EF468817	EF468757	EF468865	
<i>Ophiocordyceps nigrella</i>	EFCC 9247	Pending	EF468963	EF468818	EF468758	EF468866	EF468920
<i>Ophiocordyceps paracuboidea</i>	NBRC 101742		Pending	Pending	Pending	Pending	Pending
<i>Ophiocordyceps prolifica</i>	TNS 18481	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps prolifica</i>	TNS 18547	Pending	Pending	Pending	Pending	Pending	Pending
<i>Ophiocordyceps ravenelii</i>	OSC 110995		DQ522550	DQ518764	DQ522334	DQ522379	DQ522430
<i>Ophiocordyceps rhizoidea</i>	NHJ 12522	Pending	EF468970	EF468825	EF468764	EF468873	EF468923
<i>Ophiocordyceps ryogamiensis</i>	NBRC 101751		Pending	Pending	Pending	Pending	
<i>Ophiocordyceps sinensis</i>	EFCC 7287	Pending	EF468971	EF468827	EF468767	EF468874	EF468924
<i>Ophiocordyceps sobolifera</i>	KEW 78842	Pending	EF468972	EF468828		EF468875	EF468925
<i>Ophiocordyceps stylophora</i>	OSC 111000	Pending	DQ522552	DQ518766	DQ522337	DQ522382	DQ522433
<i>Ophiocordyceps unilateralis</i>	OSC 128574		DQ522554	DQ518768	DQ522339	DQ522385	DQ522436
<i>Ophiocordyceps variabilis</i>	ARSEF 5365		DQ522555	DQ518769	DQ522340	DQ522386	DQ522437
<i>Ophionectria trichospora</i>	CBS 109876		AF543766	AF543790	AF543779	AY489669	DQ522457
<i>Orbiocrella petchii</i>	NHJ 6209	Pending	EU369104	EU369039	EU369023	EU369061	EU369081
<i>Orbiocrella petchii</i>	NHJ 5318		EU369105	EU369040	EU369021	EU369062	EU369080
<i>Paecilomyces carneus</i>	CBS 239.32	AY624171	EF468988	EF468843	EF468789	EF468894	EF468938
<i>Paecilomyces coleopterorum</i>	CBS 110.73	AY624177	Pending	Pending	Pending	Pending	Pending
<i>Paecilomyces lilacinus</i>	CBS 431.87	AY624188	AY624188	EF468844	EF468791	EF468897	EF468940
<i>Paecilomyces marquandii</i>	CBS 182.27	AY624193	EF468990	EF468845	EF468793	EF468899	EF468942
<i>Pochonia bulbillosa</i>	CBS 145.70		AF339591	AF339542	EF468796	EF468902	EF468943
<i>Pochonia chlamydosporia</i>	CBS 504.66	Pending	AF339593	AF339544	EF469069	EF469098	EF469120
<i>Pochonia gonioides</i>	CBS 891.72	AJ292409	AF339599	AF339550	DQ522354	DQ522401	DQ522458
<i>Pochonia parasiticum</i>	ARSEF 3436	Pending	EF468993	EF468848	EF468799	EF468904	EF468945
<i>Pochonia rubescens</i>	CBS 464.88		AF339615	AF339566	EF468797	EF468903	EF468944
<i>Polycephalomyces formosus</i>	ARSEF 1424			AY259544	DQ118754	DQ127245	
<i>Pseudonectria rousseliana</i>	CBS 114049		AF543767	U17416	AF543780	AY489670	DQ522459
<i>Regiocrella camerunensis</i>	ARSEF 7682			DQ118735	DQ118743	DQ127234	
<i>Rotiferophthora angustispora</i>	CBS 101437		AF339584	AF339535	AF543776	DQ522402	DQ522460
<i>Roumegueriella rufula</i>	CBS 346.85		DQ522561	DQ518776	DQ522355	DQ522403	DQ522461
<i>Samuelsia rufobrunnea</i>	P.C. 613			AY986918	AY986944	DQ000345	-
<i>Septofusidium herbarum</i>	CBS 265.58	Pending	Pending	Pending	Pending	Pending	Pending
<i>Shimizuomyces paradoxus</i>	EFCC 6564		EF469130	EF469083	EF469072	EF469101	EF469118

Table 3.2 cont

<i>Shimizuomyces paradoxus</i>	EFCC 6279	Pending	EF469131	EF469084	EF469071	EF469100	EF469117
<i>Simplicillium lamellicola</i>	CBS 116.25	AJ292393	AF339601	AF339552	DQ522356	DQ522404	DQ522462
<i>Simplicillium lanosoniveum</i>	CBS 101267	AJ292395	AF339603	AF339554	DQ522357	DQ522405	DQ522463
<i>Sphaerostilbella berkeleyana</i>	CBS 102308		AF543770	U00756	AF543783	AY489671	DQ522465
<i>Torrubiella ratticaudata</i>	ARSEF 1915	Pending	DQ522562	DQ518777	DQ522360	DQ522408	DQ522467
<i>Torrubiella wallacei</i>	CBS 101237	Pending	AY184978	AY184967	EF469073	EF469102	EF469119
<i>Verticillium dahliae</i>	ATCC 16535		AY489705	AY489737	AY489632	AY489673	DQ522468
<i>Verticillium epiphytium</i>	CBS 384.81		AF339596	AF339547	DQ522361	DQ522409	DQ522469
<i>Verticillium epiphytium</i>	CBS 154.61	AJ292404	AF339596	AF339547	EF468802		EF468947
<i>Verticillium incurvum</i>	CBS 460.88		AF339600	AF339551	DQ522362	DQ522410	DQ522470
<i>Verticillium sp.</i>	CBS 102184	Pending	AF339613	AF339564	EF468803	EF468907	EF468948
<i>Viridispora diparietispora</i>	CBS 102797	Pending	AY489703	AY489735	AY489630	AY489668	DQ522471

Herbarium Codes: AEG, A. E. Glenn personal collection; ARSEF, USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; ATCC, American Type Culture Collection, Manassa, VA; BCC, BIOTEC Culture Collection, Klong Luang, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CUP, Cornell University Plant Pathology Herbarium; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; FAU, F. A. Uecker personal collection; GAM, Julian H. Miller Mycological Herbarium Athens, GA; HMAS, Chinese Academy of Sciences, Beijing, China; KEW, mycology collection of Royal Botanical Garden, KEW, Surrey, UK; NBRC, National Institute of Technology and Evaluation, Chiba, Japan; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR; SA, S. Alderman personal collection; TNS, National Museum of Science and Nature, Tsukuba, Japan.

Chapter 4

Host jumping onto close relatives and across kingdoms by *Tyrannocordyceps fratricida* gen. nov. and *Ustilaginoidea*

Ryan M. Kepler, Joseph W. Spatafora,

Department of Botany and Plant Pathology
Oregon State University
Corvallis, OR 97331
USA

Gi-Ho Sung,

Mushroom Research Division, Dept of Herbal Crop Research
National Institute of Horticultural and Herbal Science
Rural Development Administration
150, Suin-Ro, Gwonseon-Gu, Suwon Gyunggi-Do, 404-707, Republic of Korea

Eiji Tanaka

Ishikawa Prefectural University
1-308, Suematsu, Nonoichi-machi, Ishikawa 921-8836
Japan

Tsuyoshi Hosoya

Department of Botany, National Museum of Nature and Science
4-1-1 Amakubo, Tsukuba, Ibaraki 305-005
Japan

To be submitted to *The American Journal of Botany*

ABSTRACT

Understanding the conditions that allow for pathogen movement between hosts is important for ecological and human concerns alike. The family Clavicipitaceae contains fungal pathogens exploiting hosts across three kingdoms of life in a pattern that features multiple interkingdom host shifts. Although molecular systematic studies have advanced understanding of phylogenetic relationships and hypotheses on host associations, many species with unique host associations remain unsampled. In particular, phylogenetic placement of grass pathogens in the tribe Ustilaginoidea as well as the mycoparasites (fungi that parasitize other fungi) of ergot sclerotia remains elusive. Incorporation of these groups could have a major impact on the understanding of host shifts in Clavicipitaceae. Material from the grass pathogen *Ustilaginoidea virens* and the ergot parasite *C. fraticida* were collected in Japan and subjected to multigene phylogenetic and ancestral character state reconstructions of host association. Microscopic morphological observations of sexual and asexual states were also performed. Placement of *U. virens* represents a third acquisition of the plant pathogenic lifestyle among clavicipitaceous fungi. *Cordyceps fraticida* was placed in a clade with *Verticillium epiphytum*, and represents a sexually reproducing stage for a clade of mycoparasites that was known previously as being exclusively asexual. The new genus *Tyrannocordyceps* is proposed for the mycoparasites of ergot. Ancestral character-state reconstructions indicate changes to a plant pathogenic lifestyle are facilitated by first infecting hemipteran insect hosts. The placement of *T. fraticida*

and *U. virens* strengthen hypotheses of host evolution and suggest that co-occurrence in the same habitat facilitates the observed interkingdom host shifts in Clavicipitaceae, and that pathogenicity of hemipteran hosts predisposed these fungi for host jumps onto plants.

Key words: anamorph-teleomorph connection; Clavicipitaceae; evolution; host association; pathogen

INTRODUCTION

Fungal pathogens of Hypocreales (Ascomycota: Sordariomycetes) play important roles in systems under anthropogenic influence, e.g., agriculture, and in those operating in natural ecological settings by infecting a diverse range of hosts (Kamata et al., 1997; Haarmann et al., 2009). Clavicipitaceous fungi (sensu Rogerson 1970) represent a significant portion of the diversity of Hypocreales and are now recognized as polyphyletic across three families: Clavicipitaceae s.s., Cordycipitaceae and Ophiocordycipitaceae (Sung et al., 2007). Although polyphyletic, common ecological and morphological characters are spread throughout these fungi and they are still studied as a group by mycologists. The known diversity of host affiliations currently spans the plant, animal and fungal branches of the tree of life (Spatafora et al., 2007; Sung et al., 2008). A given species of fungus typically forms sexually reproductive (teleomorph) structures on one host, or perhaps several closely related species. However, closely related fungi may attack hosts of considerable phylogenetic distance spanning orders or even kingdoms. The asexually reproductive (anamorphic) forms are frequently less specific, and often show considerable variability in substrates suitable for growth. It is important to note that separate naming conventions for different stages of a fungal lifecycle are allowed under article 59 of the International Code of Botanical Nomenclature. For example, *Cordyceps bassiana* Li, Z.-Z.; Li, C.-R.; Huang, B.; Fan, M.-Z. is only known to attack species of the family Cossidae (Lepidoptera) (Li et al., 2001), whereas the asexual form *Beauveria bassiana* (Bals.-

Criv.) Vuill. is used as a biological control agent against a wide range of insects, and has shown growth competence when injected endophytically in a variety of plants (Rehner and Buckley, 2005; Posada and Vega, 2005; Akello et al., 2007).

Advances in the use of molecular systematics have allowed formation of hypotheses on the polarity of interkingdom host jumps (Spatafora et al., 2007; Sung et al., 2008). Pathogens of plants are unique to Clavicipitaceae s.s. and are hypothesized to have evolved from animal pathogenic ancestors. The major clade of plant pathogens evolved to exploit hosts in the Poaceae and is commonly referred to as the “grass endophytes”, e.g., *Balansia*, *Claviceps*, *Neotyphodium* etc. A separate evolution of the plant pathogenic lifestyle in Clavicipitaceae is the genus *Shimizuomyces*, which infects seeds of the genus *Smilax*. Importantly, both of these origins share a close relationship with pathogens of scale insects in Hemiptera, but explicit investigations as to whether a particular group of animal hosts may serve disproportionately as ancestral host association for the jump to plants have not been performed.

The tribe Ustilaginoidea represents a third possible origin of plant pathogenesis among clavicipitaceous fungi (Diehl, 1950; Bischoff et al., 2004). This tribe includes the genera *Ustilaginoidea*, *Munkia* and *Neomunkia* and these fungi are mostly known from their anamorphic forms. The teleomorph of *U. virens* (Cooke) Takah. is rarely observed in nature and was classified as *Claviceps virens* Sakurai ex Nakata. Subsequent work cast doubt on this association and it was determined to be morphologically distinct from *Claviceps* and described as a new genus, *Villosiclava* (Tanaka and Tanaka 2008). *Ustilaginoidea virens* is the causal agent of rice false

smut disease, an agriculturally important pathogen resulting in reduced rice yields. *Ustilaginoidea virens* produces thick-walled conidia that are dark-green in color, similar to smut spores, e.g., *Ustilago*, from which the genus name is derived. The conidia are produced holoblastically and pleurogenously on conidiophores in the outer layer of a mycelial ball (= pseudosclerotia) on the spikelet of rice. This type of anamorph is atypical for grass-associated genera of Ascomycota, e.g., *Balansia*, *Claviceps*, *Epichloë* (Diehl 1950).

Bischoff et al. (2005) provided phylogenetic evidence on the monophyly of the tribe Ustilaginodeae, but its phylogenetic placement among the clavicipitaceous fungi or its relationship to other grass-associated genera (e.g., *Balansia*, *Claviceps*, *Epichloë*) using data from the large subunit of rDNA was not resolved. In an analysis of acetaldehyde dehydrogenase sequence data (Tanaka and Tanaka 2008), *U. virens* was resolved as monophyletic with the other grass-associated genera of Clavicipitaceae and Balansiae, but the taxon sampling was insufficient to test alternative hypotheses of the phylogenetic placement among the clavicipitaceous fungi of Hypocreales. Due to the differences in anamorph morphology, a lack of extensive knowledge regarding the teleomorphs of *Ustilaginoidea*, and inconclusive molecular data, its relationship within Clavicipitaceae remains tenuous (Tanaka and Tanaka 2008).

Mycoparasitism is present throughout Hypocreales including all three families of clavicipitaceous fungi: *Simplicillium* of Cordycipitaceae, *Elaphocordyceps* of Ophiocordycipitaceae, *Verticillium* of Clavicipitaceae (Spatafora et al., 2007; Sung et al., 2008). An unusual example of mycoparasitism, which has not been incorporated

into current phylogenetic hypotheses, however, are the species of *Cordyceps* s. l. that infect the sclerotia of *Claviceps* spp. Five species have been described, with a significant representation of diversity in Japan. Young specimens are whitish, developing pigmentation from yellow to brown to vermillion at maturity. Presentation of the perithecia is variable among species and they may be pseudoimmersed (surrounded by loosely woven hyphal threads) or superficial and scattered on the stipe or clustered at the tip. Ascospores are long and filiform, forming septations and partspores in most species and similar to other clavicipitaceous fungi (Örtegren, 1916; Imai, 1936; Kobayasi, 1980; Tanda and Kobayasi, 1984). These morphological characteristics are convergent throughout the clavicipitaceous fungi, prohibiting systematic inference based on morphology alone (Sung et al. 2007).

Ergot parasitism is striking in that it involves a species of *Cordyceps* s. l. attacking another clavicipitaceous fungus. While such hyperparasitic interactions involving mitotic forms (or anamorphs) of clavicipitaceous fungi have been inferred for other species (e.g., *Polycephalomyces*), *C. fraticida* Tanda & Kobayasi and relatives are the only known meiotic forms (or teleomorphs) known to behave in this way. However because mycoparasitism is known from all three families of clavicipitaceous fungi, it remains to be determined how closely related the host and parasite are. Thus, resolving the placement of ergot pathogens will further our understanding of host switching in these diverse fungi. As a pathogen of *Claviceps* they are also potentially valuable as a biological control agent in their own right, since

ergotism of wheat, barley and sorghum continue to pose threats to human and animal food supplies (Haarman et al. 2009).

In this paper, we have collected new material for *C. fraticida* and the tribe Ustilaginoidea and incorporated them into a multigene phylogeny of Clavicipitaceae s.s. We find significant support for placement of *C. fraticida* in Clavicipitaceae where it forms a monophyletic group with *V. epiphytum*, a hyperparasite of the coffee rust fungus. Incorporation of material from *U. virens* provides evidence for a third independent evolution of the plant pathogenic lifestyle in Clavicipitaceae s.s. Ancestral character state reconstructions were performed with insect hosts coded to order to investigate the repeated pattern of host jumping between insects and plants. Plant pathogens are found to have arisen from hemipteran hosts in the clades leading to *Shimizuomyces* and the grass endophytes. The host-state giving rise to *U. virens* is less certain owing to its early diverging placement and inconsistent support across maximum likelihood analyses. However, the emerging hypothesis to explain interkingdom host jumping suggests a hemipteran pathogenic ancestor is the most common. To resolve the taxonomic conflict posed by species of *Cordyceps* placed outside of Cordycipitaceae we propose the genus *Tyrannocordyceps* for *C. fraticida* and other mycoparasites of *Claviceps* sclerotia.

MATERIAL AND METHODS

Specimen Collection

Ustilaginoidea virens was isolated from rice false smut balls that were collected at two different rice paddy fields: IPU010 from Higashiomi, Shiga, Japan on Sep.; IPU009 from Kanazawa, Ishikawa, Japan on Sep. 5, 2007. The isolated cultures were deposited in MAFF (Ministry of Agriculture, Forestry and Fisheries) (MAFF240420 from IPU009 and MAFF240421 from IPU010). Specimens of *C. fraticida* were collected from Mt. Iwakisan, Hirosaki-shi, Aomori Pref. on *Claviceps* sp. sclerotia infecting *Phragmites* sp. in 2007. Sclerotia were taken back to the lab and incubated over winter. Stroma emerged the following spring. Cultures were obtained from single ascospore isolates.

DNA extraction, PCR and sequencing

Genomic DNA of *U. virens* was extracted using QuickGene DNA extraction kit (Fujifilm, Japan). Samples of *C. fraticida* were prepared for downstream molecular work by grinding stromatal and perithecial tissue with a plastic Eppendorf pestle attached to a power drill in 50 µl CTAB buffer. Following drill mastication, tissue was transferred to a FastDNA lysing matrix A tube (MP Biomedical, Salon, OH), combined with an additional 400µl CTAB buffer and further masticated with the fast prep machine for two rounds, 20 seconds each. Tubes were then placed in a water bath at 60°C for 20 minutes to further facilitate cell lysis. Coarse fungal tissue was then separated from the supernatant by centrifugation for 10 minutes at 14,000rpm.

The supernatant was cleaned further by transferring 400µl of supernatant to 1.5 ml tubes eppendorf tubes with 500µl chloroform:isoamyl alcohol (24:1) and another round of centrifugation at 14,000 rpm for 20 minutes. 300 µl of supernatant was removed and DNA in the supernatant was then concentrated using the GeneCleanIII Kit, following the recommended protocol and eluting with 30µl of water.

The complete span of the internal transcribed spacer region of ribosomal DNA (ITS1-5.8s-ITS2) was sequenced as a quality control measure and to serve as a template for later barcoding efforts. Attempts were made to amplify five nuclear loci for phylogenetic analysis: nu-rSSU and nu-rLSU, elongation factor 1 α (TEF), and the largest and second largest subunits of RNA polymerase II (RPB1 and RPB2 respectively) with a total read length nearing 5000bp. Primer information is given in Table 4.1. PCR reactions were performed in either an iCycler or MyCycler thermocycler (BioRad, Hercules, CA) using MasterAmp 2X PCR premix E (Epicenter, Madison WI) and Novagen Taq polymerase. Reaction conditions were the same as those used in Johnson et al. (2009). PCR products were cleaned using the GeneClean III kit following the manufactures instructions and sequenced using the MacroGen (Seoul, South Korea) sequencing service with the primers used for the initial amplifications.

Phylogenetic analyses

Raw sequence reads returned from Macrogen were edited using CodonCode Aligner, version 2.0.6 (Dedham, MA). Sequences generated in this study were combined with previously published data for species in Clavicipitaceae, as well as outgroup taxa from Ophiocordycipitaceae. Genbank and specimen voucher information is provided in Table 4.2. Individual gene alignments were generated using MAFFT version 6 (Katoh et al. 2002, Katoh and Toh 2008), and improved by hand with the program BioEdit version 7.05 (Hall 1995). Ambiguously aligned regions were excluded from phylogenetic analyses and gaps were treated as missing data. Generation of Maximum Likelihood trees was performed with RAxML version 7.0.4 with 500 rapid bootstraps (Stamatakis 2006). The CAT-GAMMA model of evolution was employed during the bootstrapping phase and the GTR-GAMMA model of evolution was specified for the final likelihood tree. The dataset consisted of 11 data partitions, one each for SSU and LSU plus nine for each of the three codon positions for the protein coding genes TEF, RPB1, and RPB2. Bayesian analyses were conducted with the program Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Two runs were executed simultaneously, each with one hot and three cold chains for three million generations. The dataset was partitioned as in the ML run with the GTR+G+I model specified for each partition. After completion of the analysis both runs were inspected with Tracer v1.4 to determine if they had reached stationarity and configure the burnin. The burnin value was then used to construct a strict consensus tree in Mr.Bayes. All analyses were

conducted on the Genome High Performance Computing Cluster located at the Center for Genome Research and Bioinformatics at Oregon State University.

Ancestral Character-State Reconstruction

Species with insect hosts were coded to order. It is often difficult to diagnose insect cadavers to finer taxonomic levels due to the extreme morphological disfiguration that accompanies infection and the relatively character poor morphologies of some of the life stages attacked. Species attacking the following orders were represented in the taxon sampling: Coleoptera, Lepidoptera, and Hemiptera. Categories for hosts outside of the class Insecta (e.g., Molluscs or nematodes) and specimens obtained from soil samples were also included. Species attacking plants or fungi were scored as “plant” or “fungi”, respectively.

Results of the Bayesian analysis were used to reconstruct ancestral character states. The final two hundred trees saved during one run of the Bayesian analysis were sampled for use in Bayesian posterior mapping of ancestral characters (Huelsenbeck et al., 2003) with the program SIMMAP (Bollback, 2006). An empirical prior was used for the bias parameter and a fixed prior of 1 was used for the rate parameter. Maximum likelihood reconstructions were performed on the Bayesian consensus tree in the program Mesquite 2.7 (Maddison and Maddison, 2010). The Mk1 model of state changes was used for the reconstructions.

Morphological Examination

To observe conidial mass in *Ustilaginoidea virens*, cross sections were processed using a paraffin embedding technique. Briefly, they were fixed in FAA solution [4% formaldehyde, 5% glacial acetic acid, and 50% ethanol (v/v)], dehydrated, embedded in paraffin and sectioned at a 10 μm thickness. To observe the conidiogenesis of *U. virens* in the cultures, it was incubated in 50 ml of a simple liquid medium [0.1% yeast extract, 0.1% tryptone, 1% glucose (w/v)] at 25°C on rotary shaker (180 rpm) in 300-ml conical flask for several days and then mounted on a slide covering with a coverslip. The conidiogenesis of the fungus was observed after incubating the mounted slide for a few hours in a humid chamber.

Culturing of *C. fraticida* was achieved by shooting ascospores onto PDA plates. Plates were incubated at 18°C for several days, after which conidia were mounted in sterile water and observed under bright field and DIC light microscopy.

RESULTS

Gene sampling and phylogenetic analyses

Amplification and sequencing efforts for *U. virens* were successful for LSU, TEF, and RPB2. Repeated efforts failed to obtain quality sequence reads for SSU and RPB1. For *C. fraticida* amplification and sequencing was successful for SSU, LSU,

RPB1 and RPB2. After removing ambiguously aligned positions the combined alignment contained 4,717 bp.

Cordyceps fratricida was placed phylogenetically within Clavicipitaceae as a sister taxa to the *V. epiphytum* clade of asexual fungi, a finding supported by both ML and Bayesian analyses. The *C. fratricida/V. epiphytum* clade comprises mycoparasites and is nested within the clade of plant-associated species. The taxa most closely related to *C. fratricida* in our phylogeny are species of the genera *Balansia* and *Myriogenospora*. *Ustilaginoidea virens* occupies a basal position to the major clade of scale insect pathogens in Clavicipitaceae. Placement of *U. virens* in this position is supported in the Bayesian analyses, however this topology was recovered but did not receive significant bp support with ML analysis.

Ancestral character-state reconstructions

Ancestral character state reconstruction was performed on the final 200 trees, as well as the consensus tree, inferred from the Bayesian analyses and from the most likely RAxML tree. The values for the highest scoring state obtained from Bayesian and ML reconstruction analyses are given in Table 3 for the relevant nodes of Clavicipitaceae Clade 1 (Fig. 4.1). The ancestral host character states for grass endophyte clade and the *Shimizuomyces* clade were confidently resolved as hemipteran under both reconstruction criteria (Table 4.3). The hemipteran pathogenic lifestyle also received the most support for the base of the plant/hemipteran clade in

the SIMMAP analysis, although this was considerably weaker than support for the other plant associated species. Maximum likelihood reconstructions for this node were ambiguous, and the likelihood was distributed across several host associations. The stability of this node in Bayesian analyses and character reconstructions should be viewed with caution since ML phylogenetic analyses fail to corroborate these results.

Morphological examination

Ustilaginoidea virens is characterized by producing a mycelial ball (= pseudosclerotia) on the spikelet of rice (Fig. 4.2 C,G). Its conidia are produced in the outer layer of the mycelial ball that is comprised of “spore-bearing hyphae” which produces conidia pleurogenously and holoblastically on short sterigmata (Bischoff et al. 2004; Takahashi 1896) (Fig. 4.2 A). Its conidia are verrucose, thick-walled, and dark-green colored and can germinate to produce secondary conidia, which are hyaline and globose to subglobose (Fig. 4.3B,F). Conidiogenesis of secondary conidia involves conidiogenous cells that are typically simple and hyaline and conidia are produced holoblastically and sympodially at the apex of each conidiophore (Fig. 4.3 D). In its sympodial conidiogenesis, these holoblastic conidia appeared to be produced in whorls at the apex of conidiogeneous cells (Fig. 4.3 E).

Cordyceps fraticida produces a stipe that emerges from the ergot sclerotia after overwintering (Fig. 4.3 H). The stipe is pale to ochraceous with perithecia clustered apically in a loosely defined clava, and partially pseudoimmersed. The ascus

is cylindrical with a prominent apical cap and foot, typical of Clavicipitaceae (Fig. 4.3 I,K). Ascospores are filiform and form partspores at maturity (Fig. 4.3 J).. Cultures obtained from germinated ascospores (Fig. 4.3 l) produced a *Verticillium*-like anamorph. Hyphae and conidia are hyaline (Fig. 4.3 M,N). Conidia were produced in a droplet at the tips of the phialides (Fig. 4.3 N). The ITS locus was amplified from DNA extracted from stromata and culture as a quality control step and found to be 100% identical for both sources.

TAXONOMY

Tyrannocordyceps R.M. Kepler & J.W. Spatafora genus novum

Stroma, pallid, yellow or sometimes vermilion, arising from sclerotia of species in the genus *Claviceps*. Perithecia scattered over stipe, or clustered at the apex. Asci cylindrical with apical cap. Ascospores filiform, forming septations that may disarticulate into part-spores.

The morphology of species attacking the sclerotia of *Claviceps* is fairly similar across taxa, with the most variation seen in the color of the stroma, although this is always within a fairly narrow range of white to dark yellow or red. At the microscopic level species descriptions are relatively similar as well, all producing ascospores with septations that usually disarticulate into partspores.

Tyrannocordyceps clavicipiticola (Tokun. & S. Imai) R.M. Kepler & J.W. Spatafora
comb. nov.

Basionym: *Cordyceps clavicipiticola* Tokun. & S. Imai, Transactions of the Sapporo
Natural History Society 14: 104 (1935).

Tyrannocordyceps clavicipitis (Örtegren) R.M. Kepler & J.W. Spatafora comb. nov.

Basionym: *Cordyceps clavicipitis* Örtegren, Svensk botanisk Tidskrift 10: 57 (1916).

Tyrannocordyceps ergoticola (Tanda & Kawat.) R.M. Kepler & J.W. Spatafora comb.
nov.

Basionym: *Cordyceps ergoticola* Tanda & Kawat. J. Jap. Bot. 52:19 (1977).

Tyrannocordyceps fratricida (Tanda & Kobayasi) R.M. Kepler & J.W. Spatafora
comb. nov.

Basionym: *Cordyceps fratricida* Tanda & Kobayasi, J. Agric. Sci., Tokyo Univ.
Agric., Volume 29, Number 1, p.36-39 (1984).

Tyrannocordyceps sclerotium (Kobayasi) R.M. Kepler & J.W. Spatafora comb. nov.

Basionym: *Cordyceps sclerotium* Kobayasi, Journ. Jap. Bot., Volume 55, p.86-92
(1980).

Etymology: The genus name is in reference to the life-history characteristic of attacking closely related species, thereby acting like a tyrant, and to the *Cordyceps*-like macromorphology.

DISCUSSION

The array of host-pathogen relationships found in Clavicipitaceae are the most diverse among clavicipitaceous fungi and are marked by repeated interkingdom host jumps, a fact highlighted by the inclusion of *U. virens* and *T. fratricida*. The evolution of plant associated fungi, such as *Claviceps*, *Balansia* and *Shimizuomyces* took place against a background of animal pathogens (Spatafora et al., 2007; Sung et al., 2008). The placement of *U. virens* represents a third independent evolution of this lifestyle. With the placement of *T. fratricida* we see that the plant pathogenic lifestyle in turn gave way to one utilizing other fungi for nutrition. This change in nutritional mode is all the more remarkable because the host for *T. fratricida* is a closely related species in the genus *Claviceps*. Ancestral character-state reconstructions reveal an initial association with scale insect hosts in Hemiptera, facilitates host shifts onto plants and supports the insight of previous authors (Hywel-Jones and Samuels, 1998; Bischoff et al., 2004, 2005; Korocho et al., 2004).

Although the plant/Hemiptera clade of Clavicipitaceae are well resolved in Bayesian analyses, maximum likelihood reconstructions fail to adequately resolve the earliest diverging nodes of this clade (Fig. 4.1). Addition of *U. virens* appears to exert

a destabilizing effect on this clade, because analyses without *U. virens* result in a well supported early diverging node in both ML and Bayesian reconstruction methods (Chapter 2). The plant/Hemipteran clade of Clavicipitaceae appears to represent a source of diversity previously unappreciated, as several unique lineages of fungi have recently been described or recognized here (Chaverri et al. 2005a; Chaverri et al., 2005b; Chaverri et al. 2008; Johnson et al. 2009). This suggests further sampling may reveal additional taxa that help resolve these relationships. Additional sampling of species in this clade may reveal that the host switch onto Hemiptera represents a key innovation that has resulted in an expansion of species and multiple interkingdom host jumps.

Previous studies have suggested interkingdom host shifts onto plants are facilitated by scale insects (Hywel-Jones and Samuels, 1998; Bischoff et al., 2004, 2005; Korocho et al., 2004). Scale insects have a sessile lifestyle where their stylet is inserted directly into plant tissue. Pathogens infecting these insects are therefore in close proximity to a consistent source of nutrition greatly exceeding the original biomass of the host. Several species of *Hypocrella* were assumed to obtain plant nutrients indirectly through the scale insect host producing stroma considerably larger than their hosts, and were not at first realized to be entomopathogenic (Hywel-Jones and Samuels 1998). Species of *Dussiella*, whose taxonomic affinities within Clavicipitaceae remain ambiguous, also attains sizes much larger than the size of their host (Korocho et al. 2004). The same mechanisms are operating outside of Clavicipitaceae s.s. The genera *Ascopolyporous* and *Hyperdermium* also produce

large fruit bodies originating from a scale insect host, and molecular evidence places these genera within Cordycipitaceae (Sullivan et al., 2000; Bischoff et al., 2005). Further development of hypotheses on the interkingdom host jumps from scale insect to plant amongst clavicipitaceous fungi will rely on documentation of the host affiliation of unexamined plant-associated species (e.g., *Munkia* and *Neomunkia*) and increased taxon sampling in a multi-gene phylogenetic framework across associated families.

Mycoparasitism has evolved in all three families of clavicipitaceous fungi, although several clades are represented by anamorphic forms only. The placement of sexually reproducing *T. fraticida* in a clade with asexually reproducing *V. epiphytum*, a pathogen of the coffee rust *Hemileia vastatrix*, is consistent with their shared nutritional mode. Establishing anamorph-teleomorph connections is vital to gaining a comprehensive understanding of not only the autecologies and life histories of individual fungi with pleomorphic lifecycles, but the reproductive biology of fungi in general. Asexual lineages are thought to be evolutionarily short lived, occupying short branches in phylogenetic reconstructions due to the inability to purge deleterious alleles, although other mechanisms such as clonal turnover may be involved (Janko et al., 2008). In the handful of asexually reproducing fungal systems that have been examined, evidence of genetic recombination is commonly found in spite of no observed sexual states (Taylor et al., 1999; Paoletti et al., 2005). However, many anamorphic fungi are able to persist and sporulate on a more diverse array of substrates than those observed for teleomorphic fruiting. Increased observation and

sampling have recovered sexual states for many clades of fungi considered strictly anamorphic. For example *Neosartorya fumigata* was described for the sexual state of the widespread and widely studied human pathogenic fungus *Aspergillus fumigatus* from Irish isolates (O'Gorman et al., 2009). The phylogenetic distance between *V. epiphytum* and *T. fraticida* does not suggest a direct or conspecific anamorph-teleomorph connection. However, the anamorph produced from germinated ascospores is *Verticillium*-like, and corroborates the phylogenetic placement. It is likely that other unobserved anamorphic and teleomorphic members of this clade are present on the landscape including other species of *Tyrannocordyceps* not directly studied as part of this work.

There are some striking similarities in the jump between insects and plants or plant and fungal hosts observed here, and the host jumping that occurred in the genus *Elaphocordyceps*. In their examination of *Elaphocordyceps* spp. (as *Cordyceps* s.l.), infecting false truffles in the genus *Elaphomyces*, Nikoh and Fukatsu (2000) invoked the “host habitat hypothesis” as an explanation for the apparent jump from cicada nymphs to truffles. This hypothesis posits that host jumping by a pathogen between two distantly related hosts might be facilitated by co-occurrence and contact in the same habitat in which both truffles and cicada nymphs both occur below ground and obtain nutrients from tree roots by a physical connection. The polarity of this host jump was more recently interpreted as arthropod-truffle-cicada (Sung et al. 2007) with the “host habitat hypothesis” extended more broadly to the diversification of clavicipitaceous fungi (Sung et al. 2007, 2008). The results presented here show that

this phenomenon is operating widely throughout clavicipitaceous fungi facilitating interkingdom host jumps within Clavicipitaceae. Ancestral state reconstructions of Clavicipitiaceae show that the clade containing *V. epiphytum* and *T. fratricida* was derived from a plant pathogenic lifestyle. We hypothesize the switch from plant-based nutrition to mycoparasitism was facilitated by the repeated co-infection of the same host plant by the ancestor of both *T. fratricida* and a species of *Claviceps*, placing them in close physical proximity and setting the stage for resource competition. Likewise, the close association of scale insects with plants has repeatedly created conditions that allow for the movement of their pathogens onto plants, ultimately bypassing the scale insect entirely.

Understanding the adoption of new hosts by pathogens and parasites is important for protecting biodiversity, human health, agricultural systems and biological control of pest organisms. This need is pressing as the global economy expands and the rate of species introductions outside of their native ranges increases. The pathogens of Clavicipitaceae in particular, and the broader clavicipitaceous fungi in general, exhibit a remarkable expanse of host associations that provides a tractable system in which to study the phenomenon of host jumping. Establishing the placement of *T. fratricida* within the grass endophyte clade of Clavicipitaceae with *V. epiphytum* fills in important gaps in understanding the evolution of host affiliation in clavicipitaceous fungi by linking both reproductive and nutritional habits. This is made all the more remarkable in that the host switch for *Tyrannocordyceps* spp. involves movement onto closely related species, possibly as a result of competition

after co-infection of the same host plant. These findings are also significant for the placement of sexually reproducing fungal pathogens with a clade previously known only from asexual types. Future work with these fungi should focus on the potential of *Tyrranocordyceps* species for endophytic growth in living plants, production of ergot alkaloids and other secondary metabolites, and biological control properties.

REFERENCES

- AKELLO, J., T. DUBOIS, C.S. GOLD, D. COYNE, J. NAKAVUMA, AND P. PAPARU. 2007. *Beauveria bassiana* (Balsamo) Vuillemin as an endophyte in tissue culture banana (*Musa* spp.). *Journal of Invertebrate Pathology* 96: 34-42.
- BISCHOFF, J.F., P. CHAVERRI, AND J.F. WHITE. 2005. Clarification of the host substrate of *Ascopolyporus* and description of *Ascopolyporus philodendrus* sp. nov. *Mycologia* 97: 710-717.
- BISCHOFF, J., R. SULLIVAN, K. KJER, AND J. WHITE. 2004. Phylogenetic placement of the anamorphic tribe Ustilaginoideae (Hypocreales, Ascomycota). *Mycologia* 96: 1088-1094.
- BOLLBACK, J. 2006. SIMMAP: Stochastic character mapping of discrete traits on phylogenies. *BMC Bioinformatics* 7: 88.
- CHAVERRI, P., M. LIU, AND K. HODGE. 2008. A monograph of the entomopathogenic genera *Hypocrella*, *Moelleriella*, and *Samuelsia* gen. nov. (Ascomycota, Hypocreales, Clavicipitaceae), and their aschersonia-like anamorphs in the Neotropics. *Stud Mycol* 60: 1-66.
- CHAVERRI, P., J.F. BISCHOFF, H.C. EVANS, AND K.T. HODGE. 2005. *Regiocrella*, a new entomopathogenic genus with a pycnidial anamorph and its phylogenetic placement in the Clavicipitaceae. *Mycologia* 97: 1225-1237.
- CHAVERRI, P., J.F. BISCHOFF, M. LIU, AND K.T. HODGE. 2005. A new species of *Hypocrella*, *H. macrostroma*, and its phylogenetic relationships to other species with large stromata. *Mycological Research* 109: 1268-1275.
- DIEHL, W.W. 1950. *Balansia* and the *Balansiae* in America. Agricultural monograph No. 4. U. S. D. A., Washington.
- HAARMANN, T., Y. ROLKE, S. GIESBERT, AND P. TUDZYNSKI. 2009. Ergot: from witchcraft to biotechnology. *Molecular Plant Pathology* 10: 563-577.
- HODGE, K.T., S.B. KRASNOFF, AND R.A. HUMBER. 1996. *Tolypocladium inflatum* is the anamorph of *Cordyceps subsessilis*. *Mycologia* 88: 715-719.
- HUELSENBECK, J.P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.

- HYWEL-JONES, N.L., AND G.J. SAMUELS. 1998. Three species of *Hypocrella* with large stromata pathogenic on scale insects. *Mycologia* 90: 36-46.
- IMAI, S. 1936. Studies on the Hypocreaceae of Japan II. *Transaction of the Sapporo Natural History Society* 14: 101-106.
- JANKO, K., P. DROZD, J. FLEGR, AND J.R. PANNELL. 2008. CLONAL TURNOVER VERSUS CLONAL DECAY: A NULL MODEL FOR OBSERVED PATTERNS OF ASEXUAL LONGEVITY, DIVERSITY AND DISTRIBUTION. *Evolution* 62: 1264-1270.
- JOHNSON, D. ET AL. 2009. Systematics and evolution of the genus *Torrubiella* (Hypocreales, Ascomycota). *Mycological Research* 113: 279-289.
- JW TAYLOR, DJ JACOBSON, AND MC FISHER. 2003a. THE EVOLUTION OF ASEXUAL FUNGI: Reproduction, Speciation and Classification.
- JW TAYLOR, DJ JACOBSON, AND MC FISHER. 2003b. THE EVOLUTION OF ASEXUAL FUNGI: Reproduction, Speciation and Classification.
- KAMATA, N., H. SATO, AND M. SHIMAZU. 1997. Seasonal changes in the infection of pupae of the beech caterpillar, *Quadricalcarifera punctatella* (Motsch.) (Lep., Notodontidae), by *Cordyceps militaris* Link (Clavicipitales, Clavicipitaceae) in the soil of the Japanese beech forest. *Journal of Applied Entomology* 121: 17-21.
- KEIGHTLEY, P.D., AND A. EYRE-WALKER. 2000. Deleterious Mutations and the Evolution of Sex. *Science* 290: 331-333.
- KOBAYASI, Y. 1980. Miscellaneous notes on the genus *Cordyceps* and its allies. *Journ. Jap. Bot.* 55: 86-92.
- KOROCH, A. ET AL. 2004. Examination of plant biotrophy in the scale insect parasitizing fungus *Dussiella tuberiformis*. *Symbiosis* 37: 267-280.
- LI, Z.-., C.-. LI, B. HUANG, AND M.-. FAN. 2001. Discovery and demonstration of the teleomorph of *Beauveria bassiana* (Bals.) Vuill., an important entomopathogenic fungus. *Chinese Science Bulletin* 9: 751-753.
- MADDISON, W.P., AND D.R. MADDISON. 2004. Mesquite: a modular system for evolutionary analysis. Version 1.05.
- MADDISON, W., AND D. MADDISON. Mesquite: a modular system for evolutionary analysis. Version 2.74 <http://mesquiteproject.org>.

- MCLEISH, M.J., S. VAN NOORT, AND K.A. TOLLEY. 2010. Parasitoid fig-wasp evolutionary diversification and variation in ecological opportunity. *Molecular Ecology* 19: 1483-1496.
- NIKOH, N., AND T. FUKATSU. 2000. Interkingdom Host Jumping Underground: Phylogenetic Analysis of Entomoparasitic Fungi of the Genus *Cordyceps*. *Molecular Biology and Evolution* 17: 629-638.
- O'GORMAN, C.M., H.T. FULLER, AND P.S. DYER. 2009. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* 457: 471-4.
- ÖRTEGREN, R. 1916. *Cordyceps clavicipitis* N. SP.,. Parasit Pa *Claviceps purpurea*. *Svensk Botanisk Tidskrift* 10: 53-58.
- PAOLETTI, M. ET AL. 2005. Evidence for Sexuality in the Opportunistic Fungal Pathogen *Aspergillus fumigatus*. *Current Biology* 15: 1242-1248.
- POSADA, F., AND F.E. VEGA. 2005. Establishment of the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales) as an endophyte in cocoa seedlings (*Theobroma cacao*). *Mycologia* 97: 1195-1200.
- REHNER, S.A., AND E. BUCKLEY. 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1- α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97: 84-98.
- ROGERSON, C.T. 1970. The hypocrealean fungi (Ascomycetes, Hypocreales). *Mycologia* 62: 865-910.
- RONQUIST, F., AND J.P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572 -1574.
- RYKARD, D.M., C.W. BACON, AND E.S. LUTTREL. 1985. Host relations of *Myriogenospora atramentosa* and *Balansia epichloe* (Clavicipitaceae). *Phytopathology* 75: 950-956.
- SAMUELS, G.J., AND N.L. HYWEL-JONES. 1998. Three species of *Hypocrella* with large stromata pathogenic on scale insects. *Mycologia* 90: 36-46.
- SPATAFORA, J.W., G.-. SUNG, J.-. SUNG, N.L. HYWEL-JONES, AND J.R. WHITE. 2007. Phylogenetic evidence for an animal pathogen origin for ergot and the grass endophytes. *Molecular Ecology* 16: 1701-1711.

- STAMATAKIS, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688-2690.
- SULLIVAN, R.F., G.F. BILLS, N.L. HYWEL-JONES, AND J.F. WHITE. 2000. Hyperdermium: a new clavicipitalean genus for some tropical epibionts of dicotyledonous plants. *Mycologia* 92: 908-918.
- SUNG, G., N.L. HYWEL-JONES, J. SUNG, J.J. LUANGSA-ARD, B. SHRESTHA, AND J.W. SPATAFORA. 2007. Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. *Stud Mycol* 57: 5-59.
- SUNG, G., G.O. POINAR JR., AND J.W. SPATAFORA. 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. *Molecular Phylogenetics and Evolution* 49: 495-502.
- TANAKA, E., T. ASHIZAWA, R. SONODA, AND C. TANAKA. 2008. Villosiclava gen. nov., com. nov., teleomorph of Ustilaginoidea virens, the causal agent of rice false smut. *Mycotaxon* 106: 491-501.
- TANAKA, E., AND C. TANAKA. 2008. Phylogenetic study of clavicipitaceous fungi using acetaldehyde dehydrogenase gene sequences. *Mycoscience* 49: 115-125.
- TANDA, S., AND Y. KOBAYASI. 1984. A New Fratricidal Cordyceps Found on the Sclerotia of Claviceps microcephala. *J. Agric. Sci., Tokyo Univ. Agric.* 29: 36-39.
- TAYLOR, J., D. JACOBSON, AND M. FISHER. 1999. THE EVOLUTION OF ASEXUAL FUNGI: Reproduction, Speciation and Classification. *Annual Review of Phytopathology* 37: 197.
- WHITE, J.F., AND A.E. GLENN. 1994. A Study of Two Fungal Epibionts of Grasses: Structural Features, Host Relationships, and Classification in the Genus Myriogenospora (Clavicipitales). *American Journal of Botany* 81: 216-223.

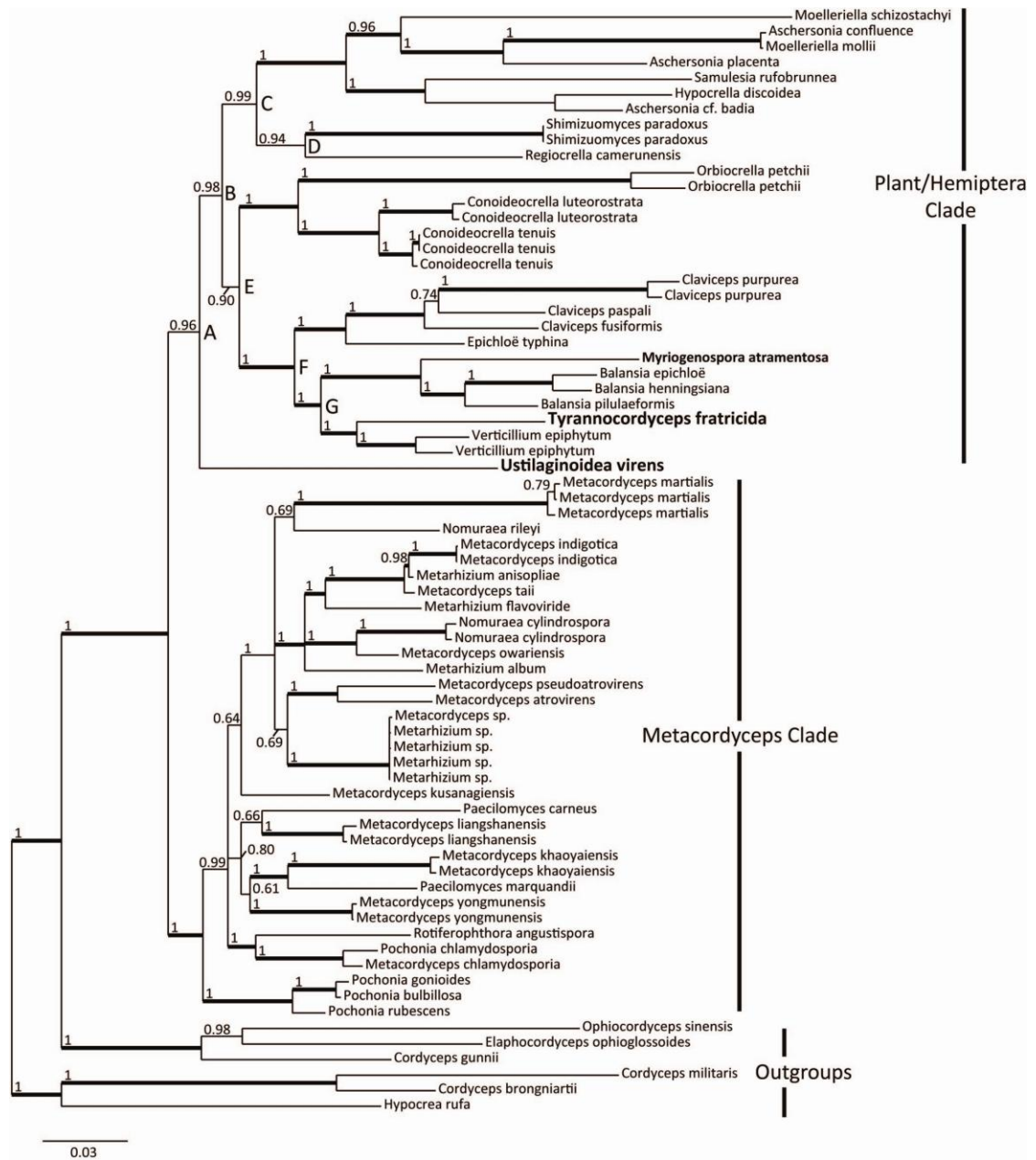


Figure 4.1 Strict consensus tree produced by Bayesian analysis of a concatenated dataset of 5 genes (SSU, LSU, TEF, RPB1, RPB2) showing the placement of *T. fraticida* and *U. virens*. Numbers above branches represent bayesian posterior probabilities. Branches in bold were well supported in maximum likelihood reconstructions (≥ 70 bootstrap proportions). Letters indicate nodes of interest in ancestral character-state reconstructions.

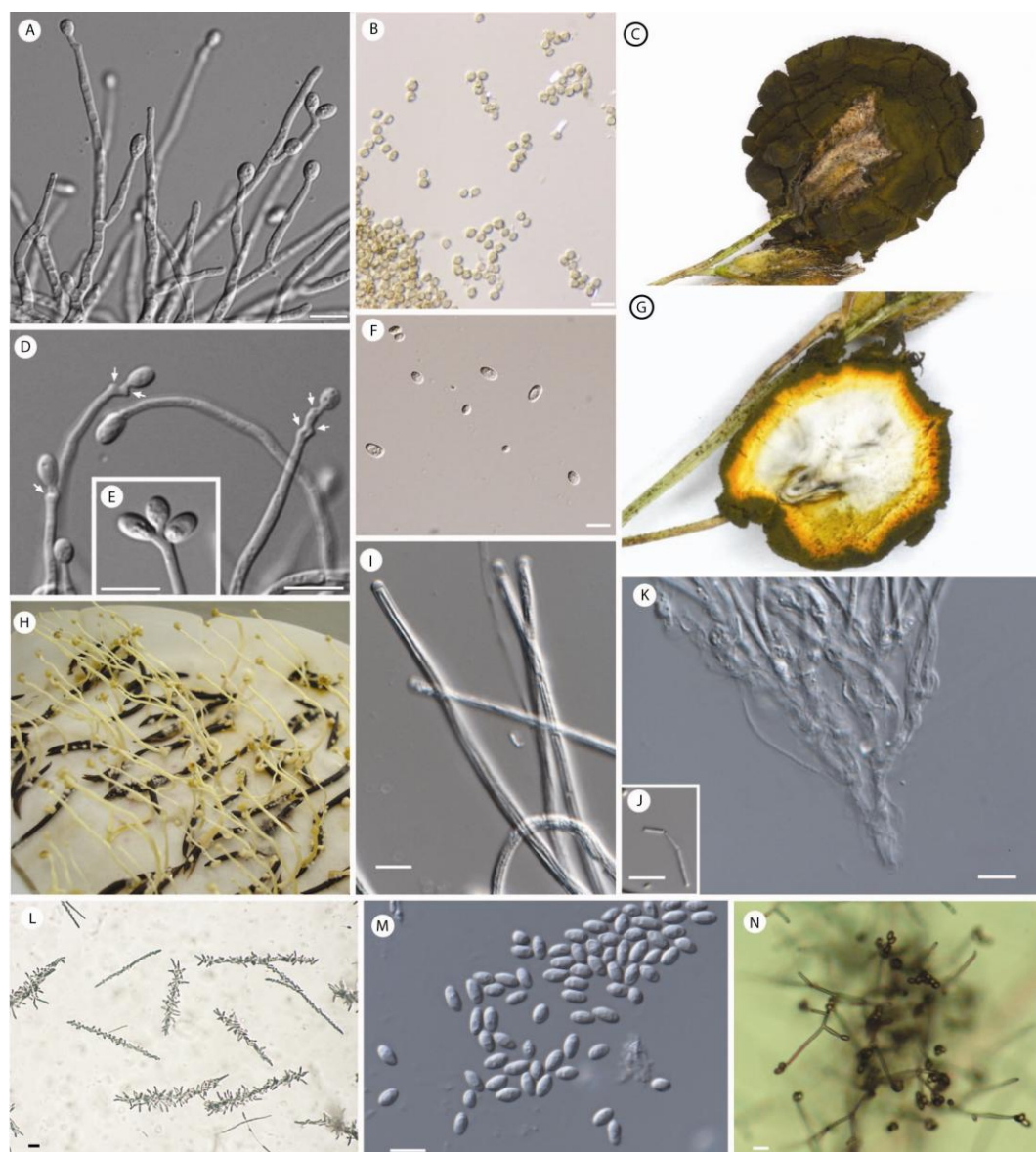


Figure 4.2

Figure 4.2 Macroscopic and microscopic features of *U. virens* and *T. fraticida*. A-G. *U. virens*. A. Conidia developing as swelling of the entire tip of hyphal conidiogenous cells. Scale bar 5µm. B. Dark-colored, verrucose thick-walled conidia. Scale bar 10µm. C. Rice false smut ball with remnants of the glumes. D. Sympodial branching of conidiophore in succession of short lateral proliferation. Detached points (arrows) of conidia. Scale bar 5µm. E. Conidia appear to occur in whorls at the apex of conidiogenous cell. F. Secondarily produced spherical conidia. G. Cross section of false smut ball which contain remnants of the ovule. H-N. *T. fraticida*. H. *T. fraticida* emerging from sclerotia of ergot after overwintering. I. Ascus. Scale bar 10µm. J. Partspores. Scale bar 10µm. K. Foot of asci. Scale bar 10µm. L. Germinating ascospores. Scale bar 50µm. M. Conidia produced by culture from germinated ascospores. Scale bar 10µm. N. Phialides of *Verticillium*-like anamorph, showing conidia in droplet at tips. Scale bar 10µm.

Table 4.1 Information for primers used to amplify sequences used in this study.

Gene	Primer	5'-Sequences-3'	source
TEF	983F	GCYCCYGGHCAYCGTGAYTTYAT	Carbone & Kohn (1999)
TEF	2218R	ATGACACCRACRGCRACRGTYTG	Steven Rehner, personal communication
LSU	LR5	ATCCTGAGGGAAACTTC	Vilgalys and Sun (1994)
LSU	LR0R	GTACCCGCTGAACTTAAGC	Vilgalys and Sun (1994)
SSU	SR7	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS4	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS3	GCAAGTCTGGTGCCAGCAGCC	White et al. (1990)
SSU	NS1	GTAGTCATATGCTTGTCTC	White et al. (1990)
RPB1	RPB1Cr	CCNGCDATNTCRTTRTCCATRTA	Castlebury et al. (2004)
RPB1	CRPB1A	CAYCCWGGYTTYATCAAGAA	Castlebury et al. (2004)
RPB2	fRPB2-7cR	CCCATRGCTTGTYRCCCAT	Liu et al. (1999)
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al. (1999)

Table 4.2 Taxon, specimen voucher and sequence information for specimens used in this study.

Species	Voucher_info	MSA_host	Genbank Accession Numbers					
			ITS GB.	SSU	LSU	TEF	RPB1	RPB2
<i>Aschersonia badia_cf</i>	BCC 7016	Hemiptera	Pending	DQ372091	DQ384941	DQ384969	DQ385009	DQ452460
<i>Aschersonia confluence</i>	BCC 7961	Hemiptera	Pending	DQ372100	DQ384947	DQ384976	DQ384998	DQ452465
<i>Aschersonia placenta</i>	BCC 7869	Hemiptera	Pending	EF469121	EF469074	EF469056	EF469085	EF469104
<i>Balansia epichloë</i>	AEG 96-15a	Plant	Pending	EF468949		EF468743	EF468851	EF468908
<i>Balansia henningiana</i>	GAM 16112	Plant	Pending	AY545723	AY545727	AY489610	AY489643	DQ522413
<i>Balansia pilulaeformis</i>	AEG 94-2	Plant	Pending	AF543764	AF543788	DQ522319	DQ522365	DQ522414
<i>Claviceps fusiformis</i>	ATCC 26019	Plant	Pending	DQ522539	U17402	DQ522320	DQ522366	
<i>Claviceps paspali</i>	ATCC 13892	Plant	Pending	U32401	U47826	DQ522321	DQ522367	DQ522416
<i>Claviceps purpurea</i>	SA cp11	Plant		EF469122	EF469075	EF469058	EF469087	EF469105
<i>Claviceps purpurea</i>	GAM 12885	Plant	Pending	AF543765	AF543789	AF543778	AY489648	DQ522417
<i>Conoideocrella luteorostrata</i>	NHJ 12516	Hemiptera	Pending	EF468994	EF468849	EF468800	EF468905	EF468946
<i>Conoideocrella luteorostrata</i>	NHJ 11343	Hemiptera	Pending	EF468995	EF468850	EF468801	EF468906	
<i>Conoideocrella tenuis</i>	NHJ 345.01	Hemiptera		EU369111	EU369045	EU369030		EU369088
<i>Conoideocrella tenuis</i>	NHJ 6293	Hemiptera	Pending	EU369112	EU369044	EU369029	EU369068	EU369087
<i>Conoideocrella tenuis</i>	NHJ 6791	Hemiptera	Pending	EU369113	EU369046	EU369028	EU369069	EU369089
<i>Cordyceps atrovirens</i>	TNM F10184	Coleoptera	Pending	Pending	Pending		Pending	
<i>Cordyceps brongniartii</i>	BCC 16585	Lepidoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps fraticida</i>	TNS 19011	Fungi	Pending	Pending	Pending		Pending	Pending
<i>Cordyceps gunnii</i>	OSC 76404	Lepidoptera	Pending	AF339572	AF339522	AY489616	AY489650	DQ522426
<i>Cordyceps militaris</i>	OSC 93623	Lepidoptera	Pending	AY184977	AY184966	DQ522332	DQ522377	AY545732
<i>Cordyceps sp.</i>	HMAS 199601	Coleoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metacordyceps indigotica</i>	TNS F18553	Lepidoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metacordyceps indigotica</i>	TNS F18554	Lepidoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metacordyceps khaoyaiensis</i>	BCC 14290	Lepidoptera	Pending		Pending	Pending	Pending	
<i>Metacordyceps khaoyaiensis</i>	BCC 12687	Lepidoptera	Pending		Pending	Pending	Pending	
<i>Metacordyceps kusanagiensis</i>	TNS F18494	Coleoptera	Pending	Pending	Pending	Pending	Pending	
<i>Metacordyceps martialis</i>	EFCC 6863	Lepidoptera			Pending	Pending		Pending
<i>Metacordyceps martialis</i>	Pending	Lepidoptera	Pending	Pending	Pending		Pending	
<i>Metacordyceps martialis</i>	HMAS 197472(S)	Lepidoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metacordyceps owariensis</i>	NBRC 33258	Hemiptera	Pending		Pending	Pending		Pending
<i>Metacordyceps pseudoatrovirens</i>	TNSF 16380	Coleoptera	Pending		Pending		Pending	Pending

Table 4.2 cont.

<i>Elaphocordyceps ophioglossoides</i>	OSC 106405	Fungi		AY489691	AY489723	AY489618	AY489652	DQ522429
<i>Epichloë typhina</i>	ATCC 56429	Plant	Pending	U32405	U17396	AF543777	AY489653	DQ522440
<i>Hypocrea rufa</i>	CBS 114374	Hemiptera		AY489694	AY489726	AY489621	AY489656	EF692510
<i>Hypocrella discoidea</i>	BCC 8237	Hemiptera	Pending	-	DQ384937	DQ384977	DQ385000	DQ452461
<i>Metacordyceps chlamydosporia</i>	CBS 101244	Nematode/Rotifer	Pending	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
<i>Metacordyceps liangshanensis</i>	EFCC 1523	Lepidoptera		EF468961	EF468814	EF468755		EF468918
<i>Metacordyceps liangshanensis</i>	EFCC 1452	Lepidoptera		EF468962	EF468815	EF468756		
<i>Metacordyceps taii</i>	ARSEF 5714	Lepidoptera	Pending	AF543763	AF543787	AF543775	DQ522383	DQ522434
<i>Metacordyceps yongmunensis</i>	EFCC 2135	Lepidoptera		EF468979	EF468834	EF468769	EF468877	
<i>Metacordyceps yongmunensis</i>	EFCC 2131	Lepidoptera	Pending	EF468977	EF468833	EF468770	EF468876	
<i>Metarhizium album</i>	ARSEF 2082	Hemiptera	Pending	DQ522560	DQ518775	DQ522352	DQ522398	DQ522452
<i>Metarhizium anisopliae</i>	ARSEF 3145		Pending	AF339579	AF339530	AF543774	DQ522399	DQ522453
<i>Metarhizium flavoviride</i>	ARSEF 2037		Pending	AF339580	AF339531	DQ522353	DQ522400	DQ522454
<i>Metarhizium sp.</i>	HMAS 199590	Coleoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium sp.</i>	HMAS 199592	Coleoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium sp.</i>	HMAS 199596	Coleoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Metarhizium sp.</i>	HMAS 199603	Coleoptera	Pending	Pending	Pending	Pending	Pending	Pending
<i>Moelleriella mollii</i>	BCC 7963	Hemiptera		DQ372087		DQ384964	DQ385004	DQ452466
<i>Moelleriella schizostachyi</i>	BCC 1985	Hemiptera		DQ372105	DQ384939	DQ384959	DQ385012	DQ452471
<i>Myriogenospora atramentosa</i>	AEG 96-32	Plant	Pending	AY489701	AY489733	AY489628	AY489665	DQ522455
<i>Nomuraea cylindrospora</i>	TNS 16371	Hemiptera		Pending	Pending	Pending	Pending	
<i>Nomuraea cylindrospora</i>	RCEF 3632	Hemiptera	Pending	Pending	Pending	Pending		
<i>Nomuraea rileyi</i>	CBS 806.71	Lepidoptera	AY624205	AY624205	AY624250	EF468787	EF468893	EF468937
<i>Ophiocordyceps sinensis</i>	EFCC 7287	Lepidoptera	Pending	EF468971	EF468827	EF468767	EF468874	EF468924
<i>Orbiocrella petchii</i>	NHJ 6209	Hemiptera	Pending	EU369104	EU369039	EU369023	EU369061	EU369081
<i>Orbiocrella petchii</i>	NHJ 5318	Hemiptera		EU369105	EU369040	EU369021	EU369062	EU369080
<i>Paecilomyces carneus</i>	CBS 239.32		AY624171	EF468988	EF468843	EF468789	EF468894	EF468938
<i>Paecilomyces marquandii</i>	CBS 182.27		AY624193	EF468990	EF468845	EF468793	EF468899	EF468942
<i>Pochonia bulbillosa</i>	CBS 145.70	Nematode/Rotifer		AF339591	AF339542	EF468796	EF468902	EF468943
<i>Pochonia chlamydosporia</i>	CBS 504.66	Nematode/Rotifer	Pending	AF339593	AF339544	EF469069	EF469098	EF469120
<i>Pochonia gonioides</i>	CBS 891.72	Nematode/Rotifer	AJ292409	AF339599	AF339550	DQ522354	DQ522401	DQ522458
<i>Pochonia rubescens</i>	CBS 464.88	Nematode/Rotifer		AF339615	AF339566	EF468797	EF468903	EF468944
<i>Regiocrella camerunensis</i>	ARSEF 7682	Hemiptera			DQ118735	DQ118743	DQ127234	

Table 4.2 cont.

<i>Rotiferophthora angustispora</i>	CBS 101437	Nematode/Rotifer		AF339584	AF339535	AF543776	DQ522402	DQ522460
<i>Samuelsia rufobrunnea</i>	P.C. 613	Hemiptera			AY986918	AY986944	DQ000345	-
<i>Shimizuomyces paradoxus</i>	EFCC 6279	Plant	Pending	EF469131	EF469084	EF469071	EF469100	EF469117
<i>Shimizuomyces paradoxus</i>	EFCC 6564	Plant		EF469130	EF469083	EF469072	EF469101	EF469118
<i>Ustilagoidea virens</i>	MAFF 240421	Plant			Pending	Pending		Pending
<i>Verticillium epiphytum</i>	CBS 154.61	Fungi	AJ292404	AF339596	AF339547	EF468802		EF468947
<i>Verticillium epiphytum</i>	CBS 384.81	Fungi		AF339596	AF339547	DQ522361	DQ522409	DQ522469

Herbarium Codes: AEG, A. E. Glenn personal collection; ARSEF, USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; ATCC, American Type Culture Collection, Manassa, VA; BCC, BIOTEC Culture Collection, Klong Luang, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CUP, Cornell University Plant Pathology Herbarium; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; FAU, F. A. Uecker personal collection; GAM, Julian H. Miller Mycological Herbarium Athens, GA; HMAS, Chinese Academy of Sciences, Beijing, China; KEW, mycology collection of Royal Botanical Garden, KEW, Surrey, UK; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR; SA, S. Alderman personal collection; TNS, National Museum of Science and Nature, Tsukuba, Japan;

Table 4.3. Likelihood scores and posterior probabilities from Maximum Likelihood and Bayesian ancestral character-state reconstructions

Node	MESQUITE Likelihood	
	scores	SIMMAP Posterior Probabilities
A	Hemiptera 0.462968	Hemiptera 0.582192
B	Hemiptera 0.780803	Hemiptera 0.983905
C	Hemiptera 0.853140	Hemiptera 0.998637
D	Hemiptera 0.838046	Hemiptera 0.992661
E	Hemiptera 0.779418	Hemiptera 0.978298
F	Plant 0.909214	Plant 0.999818
G	Plant 0.893017	Plant 0.974649

CHAPTER 5

The Evolution of Stipe Morphology in Hypocreales

Ryan Kepler, Joseph W. Spatafora

Department of Botany and Plant Pathology
Oregon State University
Corvallis, OR 97331
USA

ABSTRACT

- The evolution of stipe morphology in Hypocreales is marked by multiple, independent gains and losses, as determined by ancestral character-state reconstruction.
- The independent evolution of stipitate morphologies in Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae explains differences in the characters of stipe texture and color used in part to distinguish between genera.
- Strong correlation was found between the stipe character (absence or presence) and habitat (terrestrial or arboreal) in evolution. Astipitate forms are hypothesized to occur primarily in arboreal habitats, in most cases originating from a terrestrial, stipitate ancestor.
- The loss of stipe in arboreal species is hypothesized to be an adaptation to removal of selection pressure to elevate reproductive tissues above substrates encountered in terrestrial habitats.

INTRODUCTION

Molecular phylogenetics has been used to test and refine character state homologies across all branches of the fungal tree of life with a particular emphasis on the evolution of multicellular sporocarps or fruiting bodies. While all sporocarp-forming fungi are members of the Dikarya, which comprises Ascomycota and Basidiomycota, current phylogenetic hypotheses support independent origins of multicellular sporocarps within the two phyla (Schoch et al., 2009; Stajich et al., 2010). Furthermore, numerous investigations consistently demonstrate that many sporocarp morphologies are products of convergent evolution. For example, the mushroom, which is characterized by the elevation of the spore-producing tissue on a stipe above the substrate in which the fungal thallus resides, has been gained and lost multiple times during the evolution of the Basidiomycota (Hibbett 2007, 2006). These gains and losses of the stipe have been interpreted as correlating with differences in mechanisms for spore dispersal. Similarly, the sporocarp morphologies of the Ascomycota are of limited phylogenetic informativeness with major sporocarp morphologies intermixed and distributed across major clades of Ascomycota (Schoch et al. 2009). As in Basidiomycota, the elevation of spore-producing tissue on a stipe is found in several disparately related lineages of Ascomycota. However, no phylogenetic analyses have been conducted within Ascomycota to discern patterns of gains and losses of stipitate sporocarps and whether there are other life history traits that correlate with stipe evolution.

Hypocreales (Ascomycota: Sordariomycetes) contains species that display a staggering range of morphologies, as well as life-history traits (Rossman et al. 1999, Sung et al. 2007). The evolution of nutritional ecology for Hypocreales has been well characterized. A plant-associated ancestor, either pathogenic or saprobic, is hypothesized to be the ancestral state for the order (Spatafora et al. 2007, Sung et al. 2008). From this background, a complex pattern of nutritional shifts evolved, best exemplified by the expansive host range of the pathogenic clavicipitaceous fungi (Spatafora 2006, Sung 2008, Chapter 3). Co-occurrence in the same habitat has facilitated numerous inter-kingdom host shifts, resulting in associations with animals, fungi and plants (Chapter 3).

Sung et al. (2007) used seven genes to investigate relationships of clavicipitaceous fungi within Hypocreales. This work was largely based on evaluation of insect pathogenic fungi and the genus *Cordyceps*, whose members are now distributed throughout the families Clavicipitaceae s.s., Cordycipitaceae and Ophiocordycipitaceae. *Cordyceps* sensu lato was recognized based upon the production of stipitate, cylindrical to clavate stromata that emerge from an insect cadaver. The recent reclassification of *Cordyceps* s.l. (Sung et al. 2007) did not follow any subgeneric classification previously proposed based upon historical interpretations of morphological character states (e.g., Kobayasi 1941, Mains 1958), and new genera were subsequently named and character state homologies reassessed. Species that placed closely with the type for the genus *C. militaris* (L.: Fr.) Link remained in the genus and now comprise the majority of Cordycipitaceae. In Ophiocordycipitaceae,

the genus *Ophiocordyceps* sensu Petch (1931) was resurrected owing to the placement of *O. unilateralis* (Tul. and C. Tul.) Petch, and the genus *Elaphocordyceps* was created for species that are parasites of deer truffles in *Elaphomyces*, as well as several closely related cicada and beetle pathogens. For those insect pathogen species remaining in Clavicipitaceae s.s. the genus *Metacordyceps* was created, but Clavicipitaceae also contains ergot pathogens recently accommodated in the genus *Tryannocordyceps* (Chapter 3). Finally, another clade of *Cordyceps*-like fungi was found to be separate from all other genera, and basal to the Clavicipitiaceae s.s. and classified in the new genus *Veterocordyceps* (Chapter 2). Morphological and ecological characters found to correlate with the new phylogenetic genera inferred from molecular data include the color and texture of the stipe, ecological niche (i.e. occurrence in soil or rotten wood versus in leaf litter), as well as anamorph morphology. *Cordyceps* s.s. tend to produce brightly colored stromata with a soft and fleshy texture; *Elaphocordyceps*, *Ophiocordyceps* and *Veterocordyceps* often form darkly pigmented stromata with fibrous to wirey stipes; and *Metacordyceps* produces a variety of pigments (white to lilac to green) and the texture of the stroma is typically fibrous. However, species are known that violate all of these stromatal characteristics, with the obvious example of astipitate morphologies present in numerous clades, and definitive morphological synapomorphies to define taxa are still lacking.

Related to *Cordyceps*, and essential to understanding sporocarp evolution within Hypocreales, are the astipitate insect pathogenic genera *Torrubiella* and *Hypocrella*. *Torrubiella* is mainly distinguished from *Cordyceps* by the production of

astipitate fruiting structures with superficial perithecia produced on a subiculum, a morphology referred to here as torrubielloid. The monophyly of *Torrubiella* was rejected with species of the genus distributed across all three families of clavicipitaceous fungi and interspersed among species of *Cordyceps* s.l. (Johnson et al. 2009). A core *Torrubiella* clade composed of spider pathogens was recovered in Cordycipitaceae, which also contains separate astipitate torrubielloid species attacking mites and scale insects. Two new genera (*Orbiocrella*, *Conoidiocrella*) were proposed in Clavicipitiaceae for torrubielloid species attacking scale insects as part of a larger clade including astipitate scale pathogens of the genus *Hypocrella*, and a handful of torrubielloid species attacking leaf hoppers were reclassified in *Ophiocordyceps* of Ophiocordycipitaceae. Also, Kepler et al. (2010) described a new astipitate pathogen of ants, *O. pulvinata* Kepler, Kaitsu and Spatafora, unrelated to other torrubielloid species and closely related to the stipitate ant pathogen *O. unilateralis*.

Stipe morphology therefore presents itself as a contradicting set of characters. On the one hand stipe morphology is evolutionarily labile, with astipitate and stipitate forms repeatedly intertwined throughout all families of clavicipitaceous fungi, as well as Hypocreaceae. Stipe morphology also appears to be independent of host association, with astipitate forms found on scale insect, ant, and plant hosts or as saprobes. On the other hand, the families and genera of clavicipitaceous fungi can be defined in part by stipe texture and color when present. However, in spite of the importance stipe morphology continues to play in the taxonomy of this group, explicit hypotheses concerning the evolutionary of this feature have not been tested.

Within Hypocreales, the astipitate form is interpreted as ancestral. No species in the early diverging Bionectriaceae or Nectriaceae produce a stipe and stipe production is rare in the Hypocreaceae, sister-group to Cordycipitaceae.

Understanding when stipitate morphologies were derived would inform the utility of these characters for taxonomic purposes. It is possible that the distribution of astipitate morphologies represents the retention of the ancestral morphology for Hypocreales, with widespread repeated stipe gains across four families. However, although astipitate forms are not united phylogenetically or by a common host, a significant number occur in elevated or arboreal habitats. For example, members of the core clade of *Torrubiella* occur on spiders affixed to leaf surfaces or other elevated substrates. Species such as *Hypocrella* attack scale insects that are permanently affixed to living leaf surfaces still attached to the plant. In *O. pulvinata* and *O. unilateralis*, ants exhibit summit disease, which results in infected individuals seeking out an elevated position before death, after which they are affixed to the substrate by biting into the midvein of the leaf and by hyphal threads that emerge from the insect cadaver. Therefore, an alternative hypothesis for the phylogenetic distribution of astipitate morphologies would be loss of the stipe in response to an elevated, arboreal habitat. In these environments, the elevation of fertile tissues above a substrate is not necessary for spore dispersal. Using the results of a multigene phylogeny of hypocrealean fungi, we perform ancestral character-state reconstruction of stipe morphology and habitat, and correlation analysis these characters to test hypotheses for the distribution of astipitate species.s

MATERIALS AND METHODS

Specimen Collection

Specimens new to this study were collected in Japan from June to August in 2007 and 2008, as well as in China from June –September 2009. Collection details are given in Table 1. Specimens were taken from the field and attempts were made to obtain cultures from viable ascospores on PDA plates. A small piece of tissue was also placed in CTAB buffer for DNA extraction.

DNA extraction, PCR and sequencing

Genomic DNA was extracted by grinding tissues from cultures or field collected specimens with a plastic Eppendorf pestle attached to a power drill in 50 µl CTAB buffer. Following drill mastication, tissue was transferred to a FastDNA lysing matrix A tube (MP Biomedical, Salon, OH), combined with an additional 400µl CTAB buffer and further masticated with the fast prep machine for two rounds, 20 seconds each. Tubes were then placed in a water bath at 60°C for 20 minutes to promote cell lysis. Fungal tissue was then separated by centrifugation for 10 minutes at 14,000 rpm. The supernatant was retained and cleaned further by transferring 400µl of supernatant to 1.5 ml tubes eppendorf tubes with 500µl chloroform:isoamyl alcohol

(24:1) and another round of centrifugation at 14,000 rpm for 20 minutes. Three hundred μ l of supernatant was removed and DNA in the supernatant was then concentrated using the GeneCleanIII Kit, following the recommended protocol and eluting with 30 μ l of water.

The complete span of the internal transcribed spacer region of ribosomal DNA (ITS1-5.8s-ITS2) was sequenced as a quality control measure and to serve as a template for later barcoding efforts. Attempts were made to amplify five nuclear loci for phylogenetic analyses: SSU and LSU, elongation factor 1 α (TEF), and the largest and second largest subunits of RNA polymerase II (RPB1 and RPB2 respectively) with a total read length nearing 5000bp. Primer information is given in Table 5.1. PCR reactions were performed in either an iCycler or MyCycler thermocycler (BioRad, Hercules, CA) using MasterAmp 2X PCR premix E (Epicenter, Madison WI) and Novagen Taq polymerase. Reaction conditions were the same as those used in Johnson et al. (2009). PCR products were cleaned using the GeneClean III kit following the manufactures instructions and sequenced using Macrogen (Seoul, South Korea) sequencing service with the primers used for the initial amplifications.

Phylogenetic analyses

Raw sequence reads returned from Macrogen were edited using CodonCode Aligner, version 2.0.6 (Dedham, MA). Sequences generated in this study were combined with previously published data representative of the diversity in

Hypocreales. Because the purpose of this study is to investigate the evolution of stipe morphology, only teleomorphic taxa were included. Genbank and specimen voucher information is provided in Table 5.2. Individual gene alignments were generated using MAFFT version 6 (Katoh et al. 2002, Katoh and Toh 2008), and improved by hand with the program BioEdit version 7.05 (Hall 1999). Ambiguously aligned regions were excluded from phylogenetic analyses and gaps were treated as missing data. Maximum Likelihood analyses were performed with RAxML version 7.2.6 (Stamatakis 2006) with 500 rapid bootstrap replicates on the Genome Bioinformatics Computing Cluster at the Center for Genome Research and Biocomputing (CGRB), Oregon State University. The CAT-GAMMA model of evolution was employed during the bootstrapping phase and the GTR-GAMMA model of evolution was specified for the final likelihood tree. The dataset consisted of 11 data partitions, one each for nu-rSSU and nu-rLSU plus nine for each of the three codon positions for the protein coding genes TEF, RPB1, and RPB2.

Ancestral Character-State Reconstruction and correlation analyses

All taxa were scored for two characters: habitat where stroma are produced (terrestrial or arboreal) and for stipe morphology (present or absent) (Table 1). Terrestrial habitats include leaf litter, buried in soil and rotten wood or exposed but lying on the ground. Arboreal habitats include the undersides of living plant leaves or stems and above ground rock surfaces or twigs. A species was considered to have a

stipe if perithecia are presented on stromatal tissue elevated above the substrate. Astipitate species produce perithecia directly from the mycelia covering the substrate, usually in the form of a subiculum or pulvinate mass. Ancestral traits were reconstructed and tested for correlation with the program Mesquite 2.74 (Maddison and Maddison 2010). Character histories were estimated for each node using the Trace Character History function. Nodes were scored for a state if the raw likelihood was 2 log units or greater between states. Proportional likelihoods are reported in table three for nodes of interest. If no best character state was found the node is considered ambiguous (Table 5.3). In order to understand the influence of phylogenetic uncertainty in character reconstructions, the Trace Over Trees function with the 500 bootstrap trees generated during RAxML analyses. For each node of interest, the number of bootstrap trees containing the node is reported, as well as the number of trees assigned to a given state or marked as ambiguous (Table 5.3). Estimations of the numbers of state changes were obtained using the Summarize State Changes Over Trees function. All reconstructions were conducted using the Asymmetrical Markov two state model for binary characters (AsymmMK). This model allows different rates for gain and loss of a character.

Correlation analyses between stipe morphology and habitat were conducted over the consensus tree from RAxML with the Pagel94 method (Pagel 1994) using the Correl module in Mesquite. In this test the rates of character change are estimated from a model with four parameters representing independent evolution, and another with eight parameters representing rates of change when the characters are dependent.

The ratio for the likelihood values of the dependent and independent models was compared with a chi-squared test. A p-value to test for significance of the test was obtained from 1000 simulations with 5 replicates each.

RESULTS AND DISCUSSION

Phylogenetic analyses

After exclusion of ambiguously aligned regions, the concatenated dataset contained 4717 sites. Maximum likelihood phylogenetic reconstructions recovered relationships within Hypocreales consistent with previous studies (final tree log likelihood -115974.587842) (Figure 5.1). The exclusion of anamorphic forms did not significantly alter support values for relationships between genera or families, and the overall topology recovered here does not conflict with previous studies of the order (Sung et al. 2007, Johnson et al. 2009). The negligible effects of excluding anamorphs were confirmed by RAxML analyses that included anamorphic taxa (data not shown). The only instances of topologies differing between datasets including asexual species and the data analyzed here involve relations to clades for which no sexually reproducing forms have been observed. Relationships for taxa not included in previous studies were recovered for the families Cordycipitaceae and Ophiocordycipitaceae, however, taxonomic treatments for these taxa will be covered in subsequent works.

Relationships and ancestral character-state reconstructions of Hypocreales

Ancestral character-state reconstructions indicate Hypocreales arose from an astipitate, terrestrial ancestor (node 1, Table 5.3). Bionectriaceae contains no arboreal or stipitate species (Table 5.1). In Nectriaceae all species are astipitate and only two are found in arboreal habitats (Table 5.1). The stipitate morphology was estimated to have arisen from two to seven times (average 4.6), and subsequently lost again one to seven times (average 2). Reconstructions for habitat indicate zero to eight changes from terrestrial to arboreal (average 2.7), as well as zero to eight changes back to terrestrial (average 2.4). The common ancestor at node 3 joining Hypocreaceae and Cordycipitaceae retains the ancestral astipitate morphology with a terrestrial lifestyle. Node 12 joining Ophiocordycipitaceae and Clavicipitaceae reconstructed as terrestrial. Reconstructions of stipe morphology are largely ambiguous with less than half the bootstrap trees resolving this node assigned a state. However, those that do assign a state to this node are reconstructed as stipitate.

Hypocreaceae

The base of this clade (node 4, Fig. 5.2) is strongly supported as having retained the astipitate, terrestrial lifestyle. The stipitate forms in Hypocreaceae therefore represent a novel evolution of this character state. Historically, stipitate

forms were placed in either *Podostroma* P. Karst or *Podocrea* (Sacc.) Lindau, however these genera were later synonymized with *Hypocrea* (Chamberlain et al. 2004). Stipitate species were later shown to have a polyphyletic distribution in *Hypocrea*, with *H. alutacea* placed outside of the other taxa (Jaklitsch et al. 2008). *Hypocrea leucopus*, included here, represents the major clade of stipitate, indicating at least one if not two origins of stipitate morphology in the family.

Cordycipitaceae

Although the base of Cordycipitaceae (node 5, Fig. 5.2) is ambiguously resolved for both characters, the majority bootstrap trees are reconstructed as stipitate. However, the origin of when stipitate forms emerged remains ambiguous. Early diverging lineages in this family display a complex mix of stipitate and astipitate species, as well as poor phylogenetic resolution. The astipitate condition for *T. wallacei* appears to be from the retention of the ancestral state for the order. However, for the core *Torrubiella* clade (node 8, Fig. 5.2), it is unclear whether the astipitate morphology is ancestral or derived. The weakly supported branch subtending *Torrubiella*, *C. nelumboides* Kobayasi and Shimizu, *C. cardinalis* G.H. Sung and Spatafora and *C. pseudomilitaris* Hywel-Jones and Sivichai is not recovered when members of anamorphic genera *Simplicillium*, *Lecanicillium* and *Engyodontium* are included. *Cordyceps cardinalis* and *C. pseudomilitaris* have macromorphologies typical for most species of *Cordyceps*. Both species produce brightly colored stipitate

stromata with perithecia pseudoimmersed, but differ in possessing non-septate ascospores. Inclusion of anamorphic forms resolves a well-supported clade of *Torrubiella* plus stipitate *C. nelumboides*. This is the first inclusion of a stipitate species in *Torrubiella*, a genus of spider pathogens producing pseudoimmersed or superficial perithecia on a subiculum. *Cordyceps piperis* is a species with a torrubielloid morphology, however this species is placed outside of the *Torrubiella* clade and is a pathogen of scale insects (Bishchoff and White 2004, Johnson et al. 2009). Although node 9 subtending *C. piperis* is well supported in phylogenetic analyses, ancestral character-state reconstructions are unable to assign states either habitat or stipe morphology (Fig. 5.2).

Other novel placements in Cordycipitaceae include an unidentified species morphologically similar to *C. cardinalis* but with ascospores forming septations sister to *C. confragosa* (Mains) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora, *C. coccidioperitheciata* Kobayasi and Shimizu and *C. tuberculata* (Lebert) Maire, which produces superficial perithecia on a reduced stipe. This clade is strongly reconstructed as being derived from a stipitate ancestor, however habitat reconstructions are equivocal. *Cordyceps tuberculata* and *C. confragosa* both occur in arboreal habitats, however macromorphologically they are very different. *Cordyceps tuberculata* is stipitate and similar to *C. coccidioperitheciata* with superficial perithecia, whereas the astipitate morphology of *C. confragosa* is a derived condition (node 10, Fig. 5.2) previously used to include the species *Torrubiella*.

From node 11 forward, all species are reconstructed as stipitate (Fig. 5.2).

Cordyceps albocitrinus Koval was placed in a clade containing other species with a white or cream colored stroma. This species is placed between two clades of *C. bifusispora* O.E. Erikss., which has bola-ascospores. The spores of *C. albocitrinus* are filiform and disarticulate at maturity. Bola-ascospores are also found in the brightly colored *C. pruinosa* Petch clade which expanded sampling shows to be a phylogenetically diverse complex well supported in analyses with anamorphic taxa (data not shown), although poorly supported in here. The distribution of species possessing bola-ascospores raises questions on the evolution of spore morphology beyond the scope of this paper.

Ophiocordycipitaceae

Relationships at the base of Ophiocordycipitaceae were slightly affected by the exclusion of anamorphic taxa (Fig. 5.3). *Cordyceps cylindrica* Petch was resolved separate from *C. gunnii* Berk., but these species form a well-supported clade when *Haptocillium* and *Paecilomyces* anamorphs are added. This clade represents species that are currently considered incertae sedis following the work of Sung et al. 2007. We place a new unidentified stipitate species, which fruits from Coleoptera larvae in wood, in this clade sister to *C. gunnii*. *Cordyceps gunnii* is also terrestrial but fruits from Lepidoptera larvae in soil.

We find support for the placement of *C. formosana* Kobayasi and Shimizu in *Ophiocordyceps*. Sung et al. (2007) considered this species to be a member of *Cordyceps* s.s. due to its brightly colored stipe, but the ecological niche of fruiting from rotten wood is more common to *Ophiocordyceps*. *Ophiocordyceps purpureostromata* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora is placed in a clade including other species producing perithecia in a lateral pad. *Ophiocordyceps neovolkiana* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora is placed sister to *O. melolonthae* (Tul. and C. Tul.) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora, both pathogens of scarabs. Sampling of species originally considered belonging to *Cordyceps* subg. *Neocordyceps* was greatly expanded and supports the recognition of a “*C. sphecocephala*” clade identified by Sung et al. (2007). The species added to this clade are: *O. dipterigena* (Berk. and Broome) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora, *O. formicarum* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora, *O. lloydii* (H.S. Fawc.) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora, and *O. myrmecophila* (Ces.) G.H. Sung, J.M. Sung, Hywel-Jones and Spatafora.

We also find that the genus *Podonectria* of the Tubeufiaceae (Dothidiomycetes; Pleosporales) is polyphyletic due to the placement of *P. citrina* Kobayasi and Shimizu in *Ophiocordyceps*. *Podonectria citrina* is a pathogen of spittlebugs (Hemiptera; Cercopidae) producing an astipitate stroma that attaches the host to the underside of plant leaves. This species is placed close to *O. pruinosa* (Petch) D. Johnson, G.H. Sung, Hywel-Jones and Spatafora, another astipitate species

previously considered to belong to *Torrubiella* and also occurring in arboreal habitats. Both *O. pruinosa* and *P. citrina* also produce ascospores that are septate, but remain whole at maturity. These species are both considered to be derived from a stipitate ancestor.

In spite of the diversity of morphologies in Ophiocordycipitaceae, nearly all of them are stipitate. The exclusion of anamorphic species introduced some uncertainty into the phylogeny, but this did not affect ancestral character-state reconstructions. The basal node 13 of Ophiocordycipitaceae is reconstructed strongly as terrestrial and stipitate (Fig. 5.3). The astipitate morphology of *E. subessilis* (Petch) G.H. Sung, J.M. Sung and Spatafora is regarded as a loss (node 14, Fig. 5.3), as are other astipitate species in Ophiocordycipitaceae. *Elaphocordyceps subessilis* is the only astipitate species from the family occurring in a terrestrial setting, however it does produce well-developed rhizomorphic strands. These rhizomorphs essentially function as a stipe in that they elevate the spore producing tissue to the surface of substrate (e.g., wood, soil, dung) in which its hosts (primarily coleopteran larvae) are buried. In *Ophiocordyceps*, all astipitate species occur in arboreal habitats. However, there are several species attacking hosts in arboreal habitats that possess a stipe: *O. dipterigena*, *O. lloydii* (node 15, Fig. 5.3) and *O. unilateralis* (node 17, Fig. 5.3). Species like *P. citrina* and *O. pruinosa* (node 16, Fig. 5.3) attack hosts already occurring in arboreal habitats. However, *O. unilateralis* modifies the behavior of its ant host, causing it to seek out an elevated site on the underside of leaves to die. Although *O. unilateralis* is

stipitate, its sister species *O. pulvinata* produces perithecia in a collar-like pad around the neck of the ant.

Clavicipitaceae

Following the work of Kepler et al. (chapter 3) we consider Clavicipitaceae sensu stricto to contain the genus *Metacordyceps*, as well as the plant associated species and scale insect pathogens in *Regiocrella*, *Conoideocrella*, *Orbiocrella*, *Hypocrella*, *Samuelsia* and *Molleriella*. The genus *Veterocordyceps* is considered to be a member of Clavicipitaceae sensu lato. Phylogenetic relationships for Clavicipitaceae have been studied in detail (Chapters 1,2,3), and relationships for this family obtained here are in agreement with previous work. Support values for the clade comprised of scale insect pathogens and plant associated species has been shown to be sensitive to taxon sampling, and several relationships supported in other studies are not recovered with comparable support here.

It is clear that Clavicipitaceae arose from a terrestrial and likely stipitate ancestor (node 12 Fig. 5.1). However, the base of Clavicipitaceae s.l. (node 18, Fig. 5.4), although strongly supported, is ambiguously reconstructed in approximately half of the bootstrap trees containing this node for both habitat and stipe morphology. Those trees for which a state is scored are overwhelmingly terrestrial and stipitate. All species in *Veterocordyceps* are both terrestrial and stipitate. The base of Clavicipitaceae s.s. (node 19, Fig. 5.4), is split nearly equally between terrestrial,

arboreal and ambiguous reconstructions. However, the common ancestor of *Metacordyceps* is strongly reconstructed as being terrestrial and stipitate (node 20, Fig. 5.4).

The poorly supported and complex relationships between scale insect and plant pathogenic fungi confound inferences into the character state changes, but several trends are clear within this group of taxa. All astipitate species occur in arboreal habitats, and inferred to have been derived from a stipitate, terrestrial ancestor (node 21, Fig. 5.4), however bootstrap support for this node is weak. This shift in morphology and habitat coincides with the shift to a scale insect pathogenic lifestyle, which then facilitated movement onto plants, and finally another host jump onto other fungi by the genus *Tyrannocordyceps*. Both *T. fraticida* and hosts in *Claviceps* are stipitate and have evolved to exploit a terrestrial niche from an arboreal astipitate ancestor.

Character correlations

A high degree of correlation was found for the stipe morphology and habitat (p-value approximately 0.0). The vast majority of species occurring in arboreal habitats are astipitate, however the path to that combination of traits appears to vary between families. Arboreal species in Nectriaceae, as well as *T. wallacei* in Cordycipitaceae, moved into this habitat while retaining the ancestral, astipitate morphology. The core *Torrubiella* clade may also represent the retention of the

astipitate morphology, with a gain of the stipe occurring in *C. nelumboides*. However, reconstructions for the nodes subtending the core *Torrubiella*, as well as *C. piperis*, are ambiguous with regards to stipe morphology and the understanding trajectory of morphological changes must await more data. It is clear that stipitate species in Cordycipitaceae have evolved a stipitate morphology separate from all other hypocrealean fungi, possibly multiple times. All other astipitate species occurring in arboreal habitats were inferred as derived from a stipitate, terrestrial ancestor. This is not to say that all arboreal species have lost a stipe. Several arboreal species in Ophiocordycipitaceae possess a stipe, but this morphology is inferred to be maintenance of the morphology present as a terrestrial species. We therefore consider the evolution of an astipitate arboreal lifestyle from astipitate terrestrial ancestors to be a rare case for Hypocreales. The data presented here suggest that it is more common for species to adopt the arboreal lifestyle from a stipitate, terrestrial ancestor later followed by loss of the stipe. The stipitate morphology is necessary for terrestrial species because hosts are often buried or otherwise obscured. The stipe serves to elevate fertile tissues above this substrate to facilitate spore dispersal. The move to an arboreal habitat would make the utility of the stipe redundant, relieving the evolutionary pressure to expend energy incurred with stipe production. With the possible exception of *C. nelumboides*, there are currently no species definitely known to have acquired a stipitate morphology after the move to arboreal habitats.

The multiple gains and losses of a stipitate morphology indicate this character is highly malleable, able to adapt to evolution of new ecologies. The range of

morphologies and life-history characteristics in Clavicipitaceae illustrate this point. Kepler et al. (chapter 3) showed that the acquisition of scale insect hosts allowed for the host shift onto plants. This shift is also coincident with stipe loss, albeit with weak support. However, species of *Claviceps* infect the ovules of developing grass seeds and fall to the ground in autumn as a sclerotium, or resistant hyphal mass, that fruits the following spring. Character state reconstructions suggest that the stipitate morphology of *Claviceps* has been regained from an astipitate ancestor. The same evolutionary trajectory was followed by the *Claviceps* pathogens in *Tyrannocordyceps*. These species are hypothesized to have evolved the fungipathogenic lifestyle through competition of plant pathogens co-occurring in the same host. The ancestor of *Tyrannocordyceps* evolved to infect its *Claviceps* competition, and fruits from sclerotia after they have fallen to the ground. As a consequence of this shift in lifestyle, *Tyrannocordyceps* has also regained a stipitate morphology from an astipitate ancestor.

Conclusions

The changes in fungal systematics brought about by the introduction of molecular phylogenetic tools have often been at the expense of classifications based on morphology. Morphological diversity in Hypocreales varies in the extreme (Fig. 5.5), making consensus between morphological and molecular phylogenies difficult. Throughout Hypocreales presence or absence of a stipe has often served as an

important character to separate genera. Molecular phylogenetics repeatedly refutes this concept, however evolutionary hypotheses to explain this phenomenon have been lacking. Understanding of morphological evolution in Hypocreales is further confounded by the work of Sung et al. (2007) who proposed that texture and color of the stipe provided a means to distinguish between the genera *Cordyceps*, *Elaphocordyceps*, *Metacordyceps* and *Ophiocordyceps*. Through the use of ancestral character-state reconstructions and character correlation analyses we reconcile the contradictions posed for the utility of stipe morphologies as taxonomic characters. In the majority of cases, the astipitate morphology is an evolutionary response to changes in habitat use, namely the move from a terrestrial to an arboreal niche. Although the exact number of gains is currently unknown, it is clear that stipitate species in Cordycipitaceae are unrelated to those in other families. The acquisition of stipitate forms in Ophiocordycipitaceae and Clavicipitaceae is still an open question. However a shared stipitate ancestor would explain why some species originally placed in *Ophiocordyceps* by Sung et al. (2007) have later been found to be members of *Metacordyceps* or *Veterocordyceps*.

There are still a large number of species for Hypocreales not yet incorporated into molecular phylogenies. Resolution of lingering questions on the evolution of morphology, as well as host and habitat use, will rely on expanded taxon sampling. However, this work advances understanding of morphological plasticity throughout kingdom fungi by providing one of the first deeply sampled investigations within an order. These results should be of interest not only to systematists, but also

developmental biologists seeking to understand links between evolution and development. The field evolutionary-development in mycology has lagged far behind other biological disciplines (Taylor 2010). However, the increasing rate at which Hypocrealean genomes are published sets the stage for investigations into the cellular and genetic mechanisms that underlie the morphological complexity described here.

REFERENCES

- BERBEE, M.L., AND J.W. TAYLOR. 1992. Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. *Molecular Biology and Evolution* 9: 278 -284.
- BISCHOFF, J.F., AND J.F. WHITE. 2003. The plant-infecting clavicipitaleans. In J. F. White, M. Backlund, N. L. Hywel-Jones, and J. W. Spatafora [eds.], *Clavicipitalean fungi: Evolutionary biology, chemistry, biocontrol, and cultural impacts.*, 125-150. Marcel Decker, Inc., New York.
- CHAMBERLAIN, H., A. ROSSMAN, E. STEWART, T. ULVINEN, AND G. SAMUELS. 2004. The stipitate species of *Hypocrea* (Hypocreales, Hypocreaceae) including *Podostroma*. *Karstenia* 44: 1-24.
- HALL, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41: 95-98.
- HIBBETT, D.S. 2006. A phylogenetic overview of the Agaricomycotina. *Mycologia* 98: 917-925.
- HIBBETT, D.S. 2007. After the gold rush, or before the flood? Evolutionary morphology of mushroom-forming fungi (Agaricomycetes) in the early 21st century. *Mycological Research* 111: 1001-1018.
- HUELSENBECK, J.P., AND F. RONQUIST. 2001. Mrbayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.
- JAKLITSCH, W.M., S. GRUBER, AND H. VOGLMAYR. 2008. *Hypocrea seppoi*, a new stipitate species from Finland. *Karstenia* 48: 1-11.
- JAMES, T.Y. ET AL. 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* 443: 818-822.
- JOHNSON, D. ET AL. 2009. Systematics and evolution of the genus *Torrubiella* (Hypocreales, Ascomycota). *Mycological Research* 113: 279-289.
- KATOH, K., K. MISAWA, K. KUMA, AND T. MIYATA. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl. Acids Res.* 30: 3059-3066.

- KATOH, K., AND H. TOH. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* 9: 286-298.
- KEPLER, R.M., Y. KAITSU, E. TANAKA, S. SHIMANO, AND J.W. SPATAFORA. 2010. *Ophiocordyceps pulvinata* sp. nov., a pathogen of ants with a reduced stroma. *Mycoscience*.
- KOBAYASI, Y. 1941. The genus *Cordyceps* and its allies. *Science reports of the Tokyo Bunrika Daigaku* 84: 53-260.
- LIU, Y.J., AND B.D. HALL. 2004. Body plan evolution of ascomycetes, as inferred from an RNA polymerase II phylogeny. *Proceedings of the National Academy of Sciences of the United States of America* 101: 4507 -4512.
- MADDISON, W., AND D. MADDISON. Mesquite: a modular system for evolutionary analysis. Version 2.74 <http://mesquiteproject.org>.
- MADDISON, W.P. 2006. Confounding Asymmetries in Evolutionary Diversification and Character Change. *Evolution* 60: 1743-1746.
- MAINS, E.B. 1958. North American entomogenous species of *Cordyceps*. *Mycologia* 50: 169-222.
- MILLER, A.N., AND S.M. HUHNDORF. 2005. Multi-gene phylogenies indicate ascomal wall morphology is a better predictor of phylogenetic relationships than ascospore morphology in the Sordariales (Ascomycota, Fungi). *Molecular Phylogenetics and Evolution* 35: 60-75.
- PAGEL, M. 1994. Detecting Correlated Evolution on Phylogenies: A General Method for the Comparative Analysis of Discrete Characters. *Proceedings: Biological Sciences* 255: 37-45.
- ROGERS, D.P. 1954. The genus *Cordyceps* and Fries's Observations. *Mycologia* 46: 248-253.
- ROGERSON, C.T. 1970. The hypocrealean fungi (Ascomycetes, Hypocreales). *Mycologia* 62: 865-910.
- ROSSMAN, A.Y., G.J. SAMUELS, C.T. ROGERSON, AND R. LOWEN. 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). *STUDIES IN MYCOLOGY* 1-248.
- SCHMITT, I., R.D. PRADO, M. GRUBE, AND H.T. LUMBSCH. 2009. Repeated evolution of closed fruiting bodies is linked to ascoma development in the largest group of

- lichenized fungi (Lecanoromycetes, Ascomycota). *Molecular Phylogenetics and Evolution* 52: 34-44.
- SCHOCH, C.L. ET AL. 2009. The Ascomycota Tree of Life: A Phylum-wide Phylogeny Clarifies the Origin and Evolution of Fundamental Reproductive and Ecological Traits. *Syst Biol* 58: 216-231.
- SHIMIZU, D. 1997. Illustrated Vegetable Wasps and Plant Worms in Colour. Ie-No-Hikari Association, Tokyo.
- SPATAFORA, J.W., G.-. SUNG, J.-. SUNG, N.L. HYWEL-JONES, AND J.R. WHITE. 2007. Phylogenetic evidence for an animal pathogen origin for ergot and the grass endophytes. *Molecular Ecology* 16: 1701-1711.
- SPATAFORA, J.W. ET AL. 2006. A five-gene phylogeny of Pezizomycotina. *Mycologia* 98: 1018-1028.
- SPATAFORA, J., T. MITCHELL, AND R. VILGALYS. 1995. Analysis of genes coding for small-subunit rRNA sequences in studying phylogenetics of dematiaceous fungal pathogens. *J. Clin. Microbiol.* 33: 1322-1326.
- STAJICH, J.E. ET AL. 2010. Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). *Proceedings of the National Academy of Sciences* 107: 11889 - 11894.
- STAMATAKIS, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688-2690.
- SUNG, G., N.L. HYWEL-JONES, J. SUNG, J.J. LUANGSA-ARD, B. SHRESTHA, AND J.W. SPATAFORA. 2007. Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. *Stud Mycol* 57: 5-59.
- SUNG, G., G.O. POINAR JR., AND J.W. SPATAFORA. 2008. The oldest fossil evidence of animal parasitism by fungi supports a Cretaceous diversification of fungal-arthropod symbioses. *Molecular Phylogenetics and Evolution* 49: 495-502.
- TAYLOR, J.W., AND C.E. ELLISON. 2010. Mushrooms: Morphological complexity in the fungi. *Proceedings of the National Academy of Sciences* 107: 11655 - 11656.

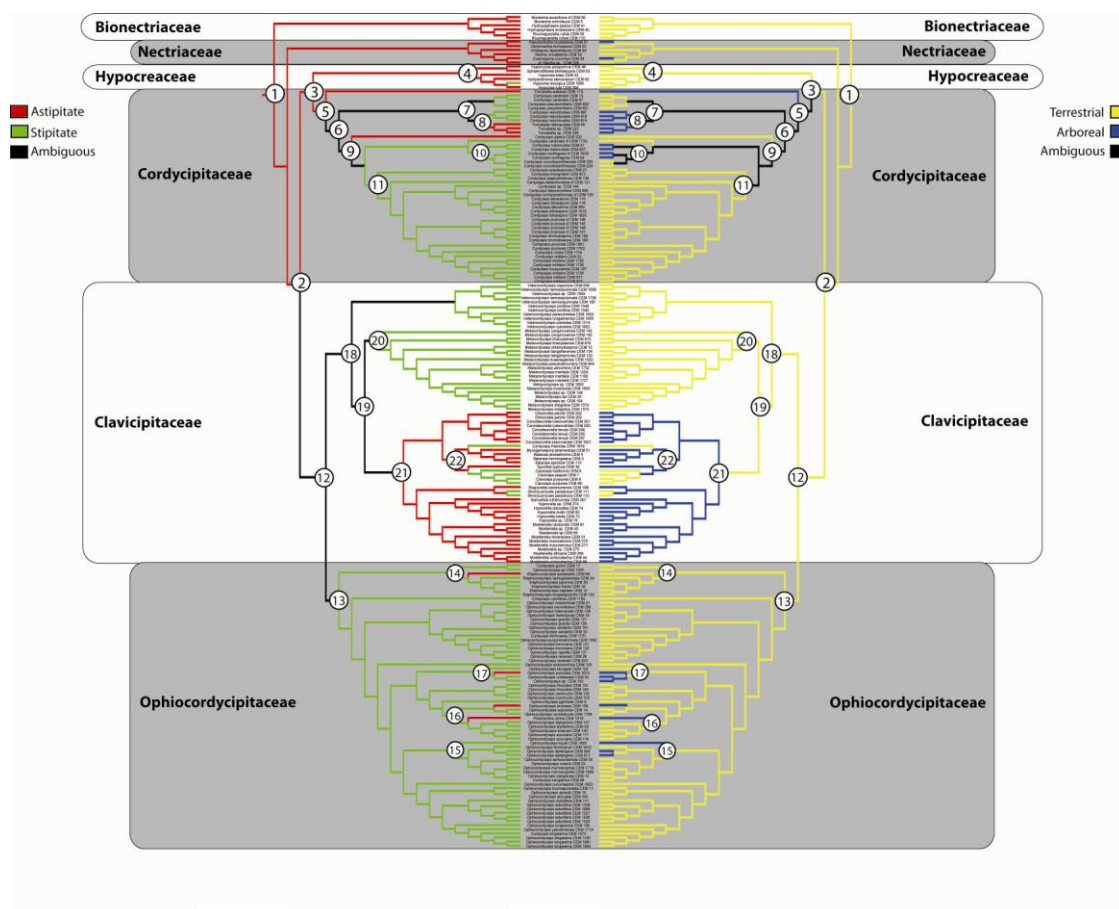


Figure 5.1 Maximum Likelihood mirror tree of the concatenated 5 gene data set. Numbers on branches indicate nodes of interest appearing in Table 3.

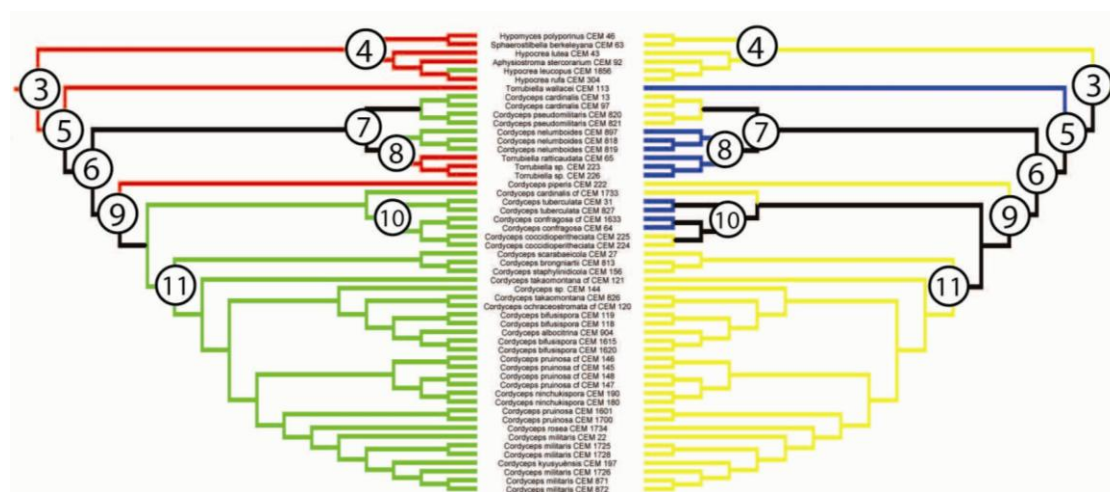


Figure 5.2 Detail of the families Hypocreaceae and Cordycipitaceae from the tree depicted in Fig. 5.1. Red and green branches denote astipitate and stipitate species, respectively. Yellow and blue branches denote terrestrial and arboreal species, respectively. Black branches are ambiguously reconstructed. Numbers on branches indicate nodes of interest appearing in Table 3.

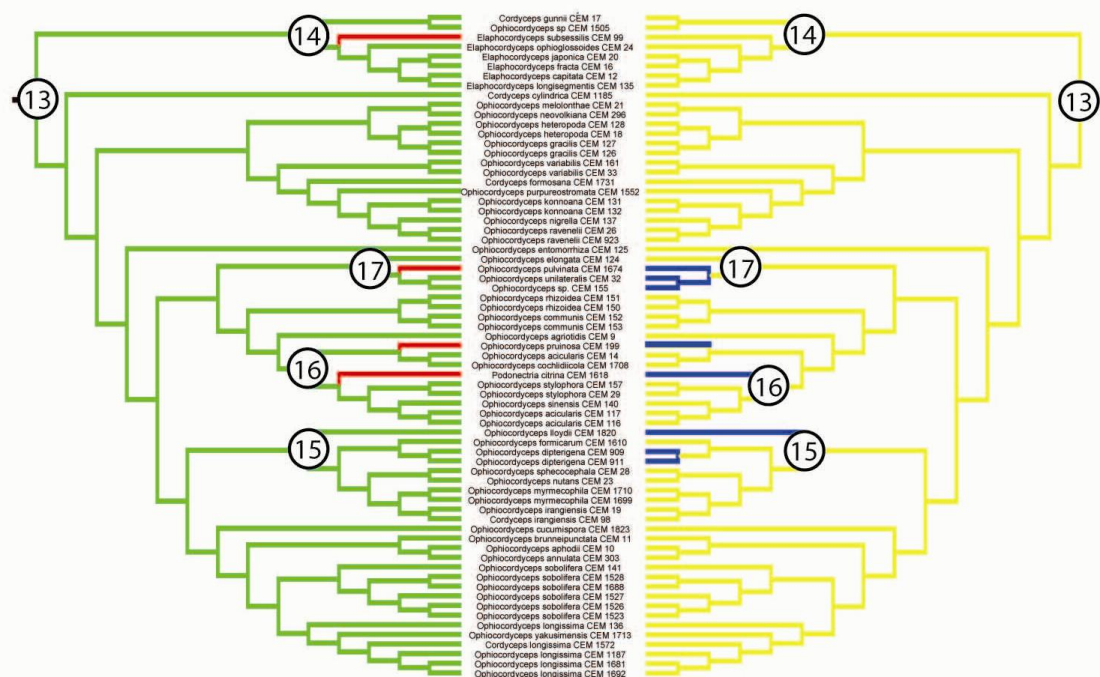


Figure 5.3 Detail of the family Ophiocordycipitaceae from the tree depicted in Fig. 5.1. Red and green branches denote astipitate and stipitate species, respectively. Yellow and blue branches denote terrestrial and arboreal species, respectively. Black branches are ambiguously reconstructed. Numbers on branches indicate nodes of interest appearing in Table 3.

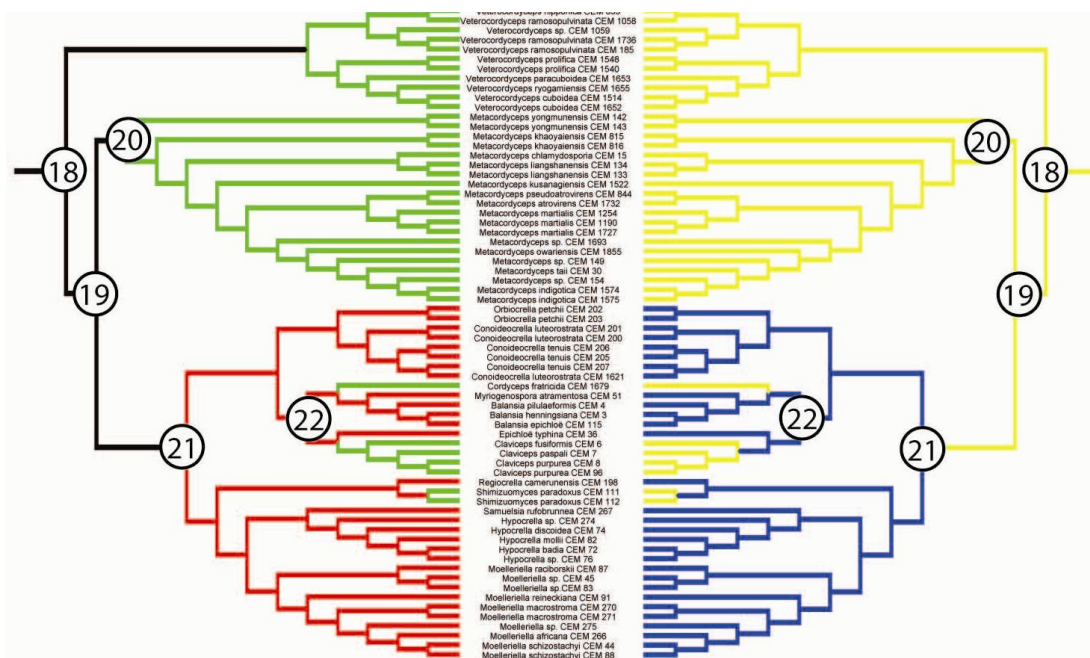


Figure 5.4 Detail of the family Clavicipitaceae from the tree depicted in Fig. 5.1. Red and green branches denote astipitate and stipitate species, respectively. Yellow and blue branches denote terrestrial and arboreal species, respectively. Black branches are ambiguously reconstructed. Numbers on branches indicate nodes of interest appearing in Table 3.

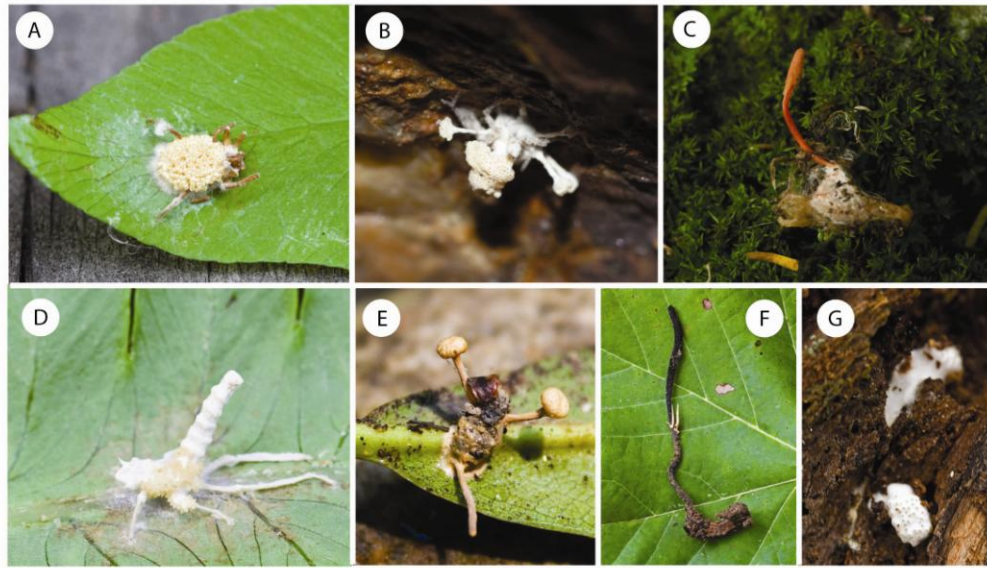


Figure 5.5 Species representative of the diversity of morphological types present in clavicipitaceous fungi. A. *Torrubiella* sp. B. *C. nelumboides*. C. *C. cf. pruinosa*. D. *P. citrina*. E. *O. dipterigena*. F. *O. ravenellii*. G. *O. subsessilis*

Table 5.1 Information for primers used in this study.

Gene	Primer	5'-Sequences-3'	source
TEF	983F	GCYCCYGGHCAYCGTGAYTTYAT	Carbone & Kohn (1999)
TEF	2218R	ATGACACCRACRGCRACRGTYTG	Steven Rehner, personal communication
LSU	LR5	ATCCTGAGGGAAACTTC	Vilgalys and Sun (1994)
LSU	LR0R	GTACCCGCTGAACTTAAGC	Vilgalys and Sun (1994)
SSU	SR7	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS4	CTTCCGTCAATTCCTTTAAG	White et al. (1990)
SSU	NS3	GCAAGTCTGGTGCCAGCAGCC	White et al. (1990)
SSU	NS1	GTAGTCATATGCTTGTCTC	White et al. (1990)
RPB1	RPB1Cr	CCNGCDATNTCRTTRTCCATRTA	Castlebury et al. (2004)
RPB1	CRPB1A	CAYCCWGGYTTYATCAAGAA	Castlebury et al. (2004)
RPB2	fRPB2-7cR	CCCATRGCTTGTYRCCCAT	Liu et al. (1999)
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al. (1999)

Table 5.2 Voucher information, character coding and genbank number for taxa used in this study

Species	Voucher_info	Habitat	Stipe	Genbank Accession Numbers					
				ITS	SSU	LSU	TEF	RPB1	RPB2
<i>Aphysiostroma stercorarium</i>	ATCC 62321	Terrstrial	No		AF543769	AF543792	AF543782	AY489633	EF469103
<i>Balansia epichloë</i>	AEG 96-15a	Arboreal	No	Pending	EF468949		EF468743	EF468851	EF468908
<i>Balansia henningsiana</i>	GAM 16112	Arboreal	No	Pending	AY545723	AY545727	AY489610	AY489643	DQ522413
<i>Balansia pilulaeformis</i>	AEG 94-2	Arboreal	No	Pending	AF543764	AF543788	DQ522319	DQ522365	DQ522414
<i>Bionectria aureofulva_cf</i>	GJS 71-328	Terrstrial	No		DQ862044	DQ862027	DQ862029		DQ862013
<i>Bionectria ochroleuca</i>	CBS 114056	Terrstrial	No		AY489684	AY489716	AY489611	DQ842031	DQ522415
<i>Claviceps fusiformis</i>	ATCC 26019	Terrstrial	Yes	Pending	DQ522539	U17402	DQ522320	DQ522366	
<i>Claviceps paspali</i>	ATCC 13892	Terrstrial	Yes	Pending	U32401	U47826	DQ522321	DQ522367	DQ522416
<i>Claviceps purpurea</i>	GAM 12885	Terrstrial	Yes	Pending	AF543765	AF543789	AF543778	AY489648	DQ522417
<i>Claviceps purpurea</i>	SA cp11	Terrstrial	Yes		EF469122	EF469075	EF469058	EF469087	EF469105
<i>Conoideocrella luteorostrata</i>	NHJ 12516	Arboreal	No	Pending	EF468994	EF468849	EF468800	EF468905	EF468946
<i>Conoideocrella luteorostrata</i>	NHJ 11343	Arboreal	No	Pending	EF468995	EF468850	EF468801	EF468906	
<i>Conoideocrella luteorostrata</i>	TNS 18531	Arboreal	No	Pending		Pending	Pending	Pending	
<i>Conoideocrella tenuis</i>	NHJ 6791	Arboreal	No	Pending	EU369113	EU369046	EU369028	EU369069	EU369089
<i>Conoideocrella tenuis</i>	NHJ 6293	Arboreal	No	Pending	EU369112	EU369044	EU369029	EU369068	EU369087
<i>Conoideocrella tenuis</i>	NHJ 345.01	Arboreal	No		EU369111	EU369045	EU369030		EU369088
<i>Cordyceps albocitrina</i>	OSC Pending	Terrstrial	Yes	Pending	Pending		Pending	Pending	
<i>Cordyceps atrovirens</i>	TNM F10184	Terrstrial	Yes	Pending	Pending	Pending		Pending	
<i>Cordyceps bifusispora</i>	EFCC 5690	Terrstrial	Yes		EF468952	EF468806	EF468746	EF468854	EF468909
<i>Cordyceps bifusispora</i>	EFCC 8260	Terrstrial	Yes		EF468953	EF468807	EF468747	EF468855	EF468910
<i>Cordyceps bifusispora</i>	TNS 18530	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps bifusispora</i>	TNS 18532	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Cordyceps brongniartii</i>	BCC 16585	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps cardinalis</i>	OSC 93610	Terrstrial	Yes	Pending	AY184974	AY184963	EF469059	EF469088	EF469106
<i>Cordyceps cardinalis</i>	OSC 93609	Terrstrial	Yes		AY184973	AY184962	DQ522325	DQ522370	DQ522422
<i>Cordyceps cardinalis_cf</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps coccidioperithecata</i>	NHJ 6709	Terrstrial	Yes	Pending	EU369110	EU369042	EU369025	EU369067	EU369086
<i>Cordyceps coccidioperithecata</i>	NHJ 5112	Terrstrial	Yes	Pending	EU369109	EU369043	EU369026	EU369066	
<i>Cordyceps confragosa</i>	CBS 101247	Arboreal	No	Pending	AF339604	AF339555	DQ522359	DQ522407	DQ522466
<i>Cordyceps confragosa_cf</i>	TNS 18579	Arboreal	No	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps cylindrica</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending		
<i>Cordyceps formosana</i>	TNM F13893	Terrstrial	Yes		Pending		Pending	Pending	Pending
<i>Cordyceps fraticida</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending		Pending	Pending
<i>Cordyceps gunnii</i>	OSC 76404	Terrstrial	Yes	Pending	AF339572	AF339522	AY489616	AY489650	DQ522426
<i>Cordyceps indigotica</i>	TNS F18554	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps indigotica</i>	TNS F18553	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps irangiensis</i>	OSC 128579	Terrstrial	Yes	Pending	EF469123	EF469076	EF469060	EF469089	EF469107

Table 5.2 cont.

<i>Cordyceps khaoyaiensis</i>	BCC 14290	Terrstrial	Yes	Pending		Pending	Pending	Pending	
<i>Cordyceps khaoyaiensis</i>	BCC 12687	Terrstrial	Yes	Pending		Pending	Pending	Pending	
<i>Cordyceps kusanagiensis</i>	TNS F18494	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Cordyceps kyusyuënsis</i>	EFCC 5886	Terrstrial	Yes		EF468960	EF468813	EF468754	EF468863	EF468917
<i>Cordyceps longissima</i>	TNS 18448	Terrstrial	Yes		Pending		Pending	Pending	
<i>Cordyceps martialis</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending		Pending	
<i>Cordyceps martialis</i>	EFCC 6863	Terrstrial	Yes			Pending	Pending		Pending
<i>Cordyceps martialis</i>	HMAS 197472(S)	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps militaris</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending		Pending	Pending
<i>Cordyceps militaris</i>	OSC 93623	Terrstrial	Yes	Pending	AY184977	AY184966	DQ522332	DQ522377	AY545732
<i>Cordyceps militaris</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps militaris</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps militaris</i>	TNS 16272	Terrstrial	Yes		Pending	Pending	Pending		Pending
<i>Cordyceps militaris</i>	TNS 16274	Terrstrial	Yes	Pending	Pending	Pending	Pending		Pending
<i>Cordyceps nelumboides</i>	TNS 16306	Arboreal	Yes	Pending	Pending		Pending		Pending
<i>Cordyceps nelumboides</i>	BCC 2190	Arboreal	Yes		Pending	Pending	Pending		
<i>Cordyceps nelumboides</i>	BCC 2093	Arboreal	Yes		Pending	Pending	Pending		Pending
<i>Cordyceps ninchukispora</i>	EGS 38.165	Terrstrial	Yes		EF468991	EF468846	EF468795	EF468900	
<i>Cordyceps ninchukispora</i>	EGS 38.166	Terrstrial	Yes		EF468992	EF468847	EF468794	EF468901	
<i>Cordyceps nipponica</i>	BCC 18108	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Cordyceps ochraceostromata_cf</i>	ARSEF 5691	Terrstrial	Yes	Pending	EF468964	EF468819	EF468759	EF468867	EF468921
<i>Cordyceps owariensis</i>	NBRC 33258	Terrstrial	Yes	Pending		Pending	Pending		Pending
<i>Cordyceps piperis</i>	CBS 116719	Arboreal	No			AY466442	DQ118749	DQ127240	EU369083
<i>Cordyceps pruinosa</i>		Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Cordyceps pruinosa</i>	TNS 18556	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps pruinosa_cf</i>	NHJ 10627	Terrstrial	Yes	Pending	EF468967	IEF468822	EF468763	EF468870	
<i>Cordyceps pruinosa_cf</i>	NHJ 10684	Terrstrial	Yes	Pending	EF468968	EF468823	EF468761	EF468871	
<i>Cordyceps pruinosa_cf</i>	EFCC 5197	Terrstrial	Yes	Pending	EF468965	EF468820	EF468760	EF468868	
<i>Cordyceps pruinosa_cf</i>	EFCC 5693	Terrstrial	Yes		EF468966	EF468821	EF468762	EF468869	
<i>Cordyceps pseudoatrovirens</i>	TNSF 16380	Terrstrial	Yes	Pending		Pending		Pending	Pending
<i>Cordyceps pseudomilitaris</i>	BCC 2091	Terrstrial	Yes	Pending	Pending	Pending	Pending		Pending
<i>Cordyceps pseudomilitaris</i>	BCC 1919	Terrstrial	Yes	Pending	Pending	Pending	Pending		Pending
<i>Cordyceps ramosopulvinata</i>	EFCC 5566	Terrstrial	Yes	Pending		Pending	Pending	Pending	
<i>Cordyceps ramosopulvinata</i>	EFCC 9072	Terrstrial	Yes		Pending	Pending	Pending	Pending	
<i>Cordyceps ramosopulvinata</i>	SU-65	Terrstrial	Yes			DQ118742	DQ118753	DQ127244	
<i>Cordyceps rosea</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cordyceps scarabaeicola</i>	ARSEF 5689	Terrstrial	Yes	Pending	AF339574	AF339524	DQ522335	DQ522380	DQ522431
<i>Cordyceps sp.</i>	EFCC 2535	Terrstrial	Yes		EF468980	EF468835	EF468772		
<i>Cordyceps sp.</i>	EFCC 12075	Terrstrial	Yes		Pending	Pending	Pending	Pending	
<i>Cordyceps sp.</i>	HMAS 199601	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending

Table 5.2 cont.

<i>Cordyceps staphylinidicola</i>	ARSEF 5718	Terrstrial	Yes	Pending	EF468981	EF468836	EF468776	EF468881	
<i>Cordyceps takaomontana</i>	BCC 12688	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Cordyceps takaomontana_cf</i>	NHJ 12623	Terrstrial	Yes	Pending	EF468984	EF468838	EF468778	EF468884	EF468932
<i>Cordyceps tuberculata</i>	OSC 111002	Arboreal	Yes	Pending	DQ522553	DQ518767	DQ522338	DQ522384	DQ522435
<i>Cordyceps tuberculata</i>	BCC 16819	Arboreal	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Cosmospora coccinea</i>	CBS 114050	Arboreal	No	Pending	AY489702	AY489734	AY489629	AY489667	DQ522438
<i>Elaphocordyceps capitata</i>	OSC 71233	Terrstrial	Yes	Pending	AY489689	AY489721	AY489615	AY489649	DQ522421
<i>Elaphocordyceps fracta</i>	OSC 110990	Terrstrial	Yes	Pending	DQ522545	DQ518759	DQ522328	DQ522373	DQ522425
<i>Elaphocordyceps japonica</i>	OSC 110991	Terrstrial	Yes	Pending	DQ522547	DQ518761	DQ522330	DQ522375	DQ522428
<i>Elaphocordyceps longisegmentis</i>	OSC 110992	Terrstrial	Yes			EF468816		EF468864	EF468919
<i>Elaphocordyceps ophioglossoides</i>	OSC 106405	Terrstrial	Yes		AY489691	AY489723	AY489618	AY489652	DQ522429
<i>Elaphocordyceps subsessilis</i>	OSC 71235	Terrstrial	No	Pending	EF469124	EF469077	EF469061	EF469090	EF469108
<i>Epichloë typhina</i>	ATCC 56429	Arboreal	No	Pending	U32405	U17396	AF543777	AY489653	DQ522440
<i>Hydropisphaera erubescens</i>	ATCC 36093	Terrstrial	No		AY545722	AY545726	DQ522344	DQ522390	AY545731
<i>Hydropisphaera peziza</i>	CBS 102038	Terrstrial	No		AY489698	AY489730	AY489625	AY489661	DQ522444
<i>Hypocrea leucopus</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Hypocrea lutea</i>	ATCC 208838	Terrstrial	No		AF543768	AF543791	AF543781	AY489662	DQ522446
<i>Hypocrea rufa</i>	CBS 114374	Terrstrial	No		AY489694	AY489726	AY489621	AY489656	EF692510
<i>Hypocrella africana</i>	P.C. 736	Arboreal	No			AY986917	AY986943	DQ000344	-
<i>Hypocrella badia</i>	BCC 8407	Arboreal	No	Pending	DQ372101		DQ384960	DQ384999	-
<i>Hypocrella discoidea</i>	BCC 8237	Arboreal	No	Pending	-	DQ384937	DQ384977	DQ385000	DQ452461
<i>Hypocrella macrostroma</i>	P.C. 605	Arboreal	No			AY986919	AY986946	DQ000347	-
<i>Hypocrella macrostroma</i>	J.B. 115	Arboreal	No			AY986920	AY986947	DQ000348	-
<i>Hypocrella mollii</i>	BCC 11738	Arboreal	No		DQ372086	DQ384940	DQ384963	DQ385003	-
<i>Hypocrella raciborskii</i>	BCC 8238	Arboreal	No	Pending	DQ372102		DQ384961	DQ385001	DQ452470
<i>Hypocrella reineckiana</i>	BCC 2355	Arboreal	No	Pending	DQ372092		DQ384970	DQ385011	DQ452474
<i>Hypocrella schizostachyi</i>	BCC 14123	Arboreal	No	Pending	DQ522557	DQ518771	DQ522346	DQ522392	DQ522447
<i>Hypocrella sp.</i>	GJS 89-104	Arboreal	No	Pending	U32409	U47832	DQ522347	DQ522393	DQ522448
<i>Hypocrella sp.</i>	P.C. 436.2	Arboreal	No			AY986922	AY986949	DQ000350	-
<i>Hypocrella sp.</i>	BCC 9483	Arboreal	No	Pending	DQ372085	DQ384938	DQ384962	DQ385002	-
<i>Hypocrella sp.</i>	P.C. 603	Arboreal	No			AY986923	AY986950	DQ000351	-
<i>Hypomyces polyporinus</i>	ATCC 76479	Terrstrial	No		AF543771	AF543793	AF543784	AY489663	
<i>Metacordyceps chlamydosporia</i>	CBS 101244	Terrstrial	Yes	Pending	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
<i>Metacordyceps liangshanensis</i>	EFCC 1452	Terrstrial	Yes		EF468962	EF468815	EF468756		
<i>Metacordyceps liangshanensis</i>	EFCC 1523	Terrstrial	Yes		EF468961	EF468814	EF468755		EF468918
<i>Metacordyceps sp.</i>	NHJ 12118	Terrstrial	Yes	Pending	EF468978	EF468829	EF468768	EF468878	EF468927
<i>Metacordyceps sp.</i>	OSC 110996	Terrstrial	Yes		EF468974	EF468832	EF468773	EF468880	EF468928
<i>Metacordyceps taii</i>	ARSEF 5714	Terrstrial	Yes	Pending	AF543763	AF543787	AF543775	DQ522383	DQ522434
<i>Metacordyceps yongmunensis</i>	EFCC 2135	Terrstrial	Yes		EF468979	EF468834	EF468769	EF468877	
<i>Metacordyceps yongmunensis</i>	EFCC 2131	Terrstrial	Yes	Pending	EF468977	EF468833	EF468770	EF468876	

Table 5.2 cont.

<i>Moelleriella mollii</i>	BCC 7963	Arboreal	No		DQ372087		DQ384964	DQ385004	DQ452466
<i>Moelleriella schizostachyi</i>	BCC 1985	Arboreal	No		DQ372105	DQ384939	DQ384959	DQ385012	DQ452471
<i>Myriogenospora atramentosa</i>	AEG 96-32	Arboreal	No	Pending	AY489701	AY489733	AY489628	AY489665	DQ522455
<i>Nectria cinnabarina</i>	CBS 114055	Terrstrial	No		U32412	U00748	AF543785	AY489666	DQ522456
<i>Nectria_cf_sp</i>	CBS 478.75	Terrstrial	No		U47842	U17404	EF469068	EF469097	EF469115
<i>Ophiocordyceps acicularis</i>	OSC 110988	Terrstrial	Yes		EF468951	EF468804	EF468745	EF468853	
<i>Ophiocordyceps acicularis</i>	OSC 110987	Terrstrial	Yes		EF468950	EF468805	EF468744	EF468852	
<i>Ophiocordyceps acicularis</i>	OSC 128580	Terrstrial	Yes	Pending	DQ522543	DQ518757	DQ522326	DQ522371	DQ522423
<i>Ophiocordyceps agriotidis</i>	ARSEF 5692	Terrstrial	Yes	Pending	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
<i>Ophiocordyceps annulata</i>	Pending	Terrstrial	Yes		Pending	Pending	Pending	Pending	
<i>Ophiocordyceps aphodii</i>	ARSEF 5498	Terrstrial	Yes		DQ522541	DQ518755	DQ522323		DQ522419
<i>Ophiocordyceps brunneipunctata</i>	OSC 128576	Terrstrial	Yes		DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
<i>Ophiocordyceps cochlidicola</i>	Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending		
<i>Ophiocordyceps communis</i>	NHJ 12582	Terrstrial	Yes	Pending	EF468975	EF468830	EF468771		EF468926
<i>Ophiocordyceps communis</i>	NHJ 12581	Terrstrial	Yes	Pending	EF468973	EF468831	EF468775		EF468930
<i>Ophiocordyceps cuboidea</i>	TNS 18487	Terrstrial	Yes		Pending	Pending	Pending		
<i>Ophiocordyceps cuboidea</i>	NBRC 101740	Terrstrial	Yes		Pending	Pending	Pending	Pending	
<i>Ophiocordyceps cucumispora</i>	OSC Pending	Terrstrial	Yes		Pending		Pending	Pending	
<i>Ophiocordyceps dipterigena</i>	OSC Pending	Arboreal	Yes		Pending	Pending	Pending	Pending	
<i>Ophiocordyceps dipterigena</i>	OSC Pending	Arboreal	Yes		Pending	Pending	Pending	Pending	
<i>Ophiocordyceps elongata</i>	OSC 110989	Terrstrial	Yes			EF468808	EF468748	EF468856	
<i>Ophiocordyceps entomorrhiza</i>	KEW 53484	Terrstrial	Yes	Pending	EF468954	EF468809	EF468749	EF468857	EF468911
<i>Ophiocordyceps formicarum</i>	TNS 18565	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Ophiocordyceps gracilis</i>	EFCC 8572	Terrstrial	Yes	Pending	EF468956	EF468811	EF468751	EF468859	EF468912
<i>Ophiocordyceps gracilis</i>	EFCC 3101	Terrstrial	Yes		EF468955	EF468810	EF468750	EF468858	EF468913
<i>Ophiocordyceps heteropoda</i>	EFCC 10125	Terrstrial	Yes	Pending	EF468957	EF468812	EF468752	EF468860	EF468914
<i>Ophiocordyceps heteropoda</i>	OSC 106404	Terrstrial	Yes		AY489690	AY489722	AY489617	AY489651	
<i>Ophiocordyceps irangiensis</i>	OSC 128577	Terrstrial	Yes	Pending	DQ522546	DQ518760	DQ522329	DQ522374	DQ522427
<i>Ophiocordyceps konnoana</i>	EFCC 7295	Terrstrial	Yes		EF468958			EF468862	EF468915
<i>Ophiocordyceps konnoana</i>	EFCC 7315	Terrstrial	Yes		EF468959		EF468753	EF468861	EF468916
<i>Ophiocordyceps lloydii</i>	OSC Pending	Arboreal	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Ophiocordyceps longissima</i>	Pending	Terrstrial	Yes		Pending		Pending	Pending	Pending
<i>Ophiocordyceps longissima</i>	EFCC 6814	Terrstrial	Yes			EF468817	EF468757	EF468865	
<i>Ophiocordyceps longissima</i>	Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps longissima</i>	Pending	Terrstrial	Yes	Pending		Pending		Pending	Pending
<i>Ophiocordyceps melolonthae</i>	OSC 110993	Terrstrial	Yes	Pending	DQ522548	DQ518762	DQ522331	DQ522376	
<i>Ophiocordyceps myrmecophila</i>	Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps myrmecophila</i>	Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps neovolkiana</i>	OSC Pending	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps nigrella</i>	EFCC 9247	Terrstrial	Yes	Pending	EF468963	EF468818	EF468758	EF468866	EF468920

Table 5.2 cont.

<i>Ophiocordyceps nutans</i>	OSC 110994	Terrstrial	Yes	Pending	DQ522549	DQ518763	DQ522333	DQ522378	
<i>Ophiocordyceps paracuboidea</i>	NBRC 101742	Terrstrial	Yes		Pending	Pending	Pending	Pending	Pending
<i>Ophiocordyceps prolifica</i>	TNS 18547	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	Pending
<i>Ophiocordyceps prolifica</i>	TNS 18481	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps pruinosa</i>	NHJ 12994	Arboreal	No		EU369106	EU369041	EU369024	EU369063	EU369084
<i>Ophiocordyceps pulvinata</i>	OSC Pending	Arboreal	No		Pending		Pending	Pending	
<i>Ophiocordyceps purpureostromata</i>	TNS 18430	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps ravenelii</i>	OSC Pending	Terrstrial	Yes		Pending		Pending	Pending	Pending
<i>Ophiocordyceps ravenelii</i>	OSC 110995	Terrstrial	Yes		DQ522550	DQ518764	DQ522334	DQ522379	DQ522430
<i>Ophiocordyceps rhizoidea</i>	NHJ 12529	Terrstrial	Yes	Pending	EF468969	EF468824	EF468765	EF468872	EF468922
<i>Ophiocordyceps rhizoidea</i>	NHJ 12522	Terrstrial	Yes	Pending	EF468970	EF468825	EF468764	EF468873	EF468923
<i>Ophiocordyceps ryogamiensis</i>	NBRC 101751	Terrstrial	Yes		Pending	Pending	Pending	Pending	
<i>Ophiocordyceps sinensis</i>	EFCC 7287	Terrstrial	Yes	Pending	EF468971	EF468827	EF468767	EF468874	EF468924
<i>Ophiocordyceps sobolifera</i>	TNS 18526	Terrstrial	Yes	Pending	Pending		Pending	Pending	
<i>Ophiocordyceps sobolifera</i>	TNS 18525	Terrstrial	Yes	Pending	Pending		Pending	Pending	
<i>Ophiocordyceps sobolifera</i>	TNS 18521	Terrstrial	Yes	Pending	Pending	Pending	Pending	Pending	
<i>Ophiocordyceps sobolifera</i>	Pending	Terrstrial	Yes	Pending	Pending			Pending	Pending
<i>Ophiocordyceps sobolifera</i>	KEW 78842	Terrstrial	Yes	Pending	EF468972	EF468828		EF468875	EF468925
<i>Ophiocordyceps sobolifera</i>	TNS 18524	Terrstrial	Yes	Pending	Pending		Pending	Pending	
<i>Ophiocordyceps sp</i>	TNS 18495	Terrstrial	Yes		Pending	Pending		Pending	
<i>Ophiocordyceps sp.</i>	OSC 110997	Terrstrial	Yes		EF468976		EF468774	EF468879	EF468929
<i>Ophiocordyceps sphecocephala</i>	OSC 110998	Terrstrial	Yes		DQ522551	DQ518765	DQ522336	DQ522381	DQ522432
<i>Ophiocordyceps stylophora</i>	OSC 110999	Terrstrial	Yes		EF468982	EF468837	EF468777	EF468882	EF468931
<i>Ophiocordyceps stylophora</i>	OSC 111000	Terrstrial	Yes	Pending	DQ522552	DQ518766	DQ522337	DQ522382	DQ522433
<i>Ophiocordyceps unilateralis</i>	OSC 128574	Arboreal	Yes		DQ522554	DQ518768	DQ522339	DQ522385	DQ522436
<i>Ophiocordyceps variabilis</i>	OSC 111003	Terrstrial	Yes		EF468985	EF468839	EF468779	EF468885	EF468933
<i>Ophiocordyceps variabilis</i>	ARSEF 5365	Terrstrial	Yes		DQ522555	DQ518769	DQ522340	DQ522386	DQ522437
<i>Ophiocordyceps yakusimensis</i>	Pending	Terrstrial	Yes		Pending	Pending		Pending	Pending
<i>Ophionectria trichospora</i>	CBS 109876	Terrstrial	No		AF543766	AF543790	AF543779	AY489669	DQ522457
<i>Orbiocrella petchii</i>	NHJ 5318	Arboreal	No		EU369105	EU369040	EU369021	EU369062	EU369080
<i>Orbiocrella petchii</i>	NHJ 6209	Arboreal	No	Pending	EU369104	EU369039	EU369023	EU369061	EU369081
<i>Podonectria citrina</i>	TNS 18537	Arboreal	No	Pending		Pending	Pending		Pending
<i>Pseudonectria rousseliana</i>	CBS 114049	Arboreal	No		AF543767	U17416	AF543780	AY489670	DQ522459
<i>Regiocrella camerunensis</i>	ARSEF 7682	Arboreal	No			DQ118735	DQ118743	DQ127234	
<i>Roumegueriella rufula</i>	CBS 346.85	Terrstrial	No		DQ522561	DQ518776	DQ522355	DQ522403	DQ522461
<i>Roumegueriella rufula</i>	GJS 91-164	Terrstrial	No		EF469129	EF469082	EF469070	EF469099	EF469116
<i>Samuelsia rufobrunnea</i>	P.C. 613	Arboreal	No			AY986918	AY986944	DQ000345	-
<i>Shimizuomyces paradoxus</i>	EFCC 6564	Terrstrial	Yes		EF469130	EF469083	EF469072	EF469101	EF469118
<i>Shimizuomyces paradoxus</i>	EFCC 6279	Terrstrial	Yes	Pending	EF469131	EF469084	EF469071	EF469100	EF469117
<i>Sphaerostilbella berkeleyana</i>	CBS 102308	Terrstrial	No		AF543770	U00756	AF543783	AY489671	DQ522465

Table 5.2 cont.

<i>Torrubiella ratticaudata</i>	ARSEF 1915	Arboreal	No	Pending	DQ522562	DQ518777	DQ522360	DQ522408	DQ522467
<i>Torrubiella sp.</i>	NHJ 7859	Arboreal	No		EU369107			EU369064	EU369085
<i>Torrubiella sp.</i>	DJ 29	Arboreal	No		EU369108		EU369027	EU369065	
<i>Torrubiella wallacei</i>	CBS 101237	Arboreal	No	Pending	AY184978	AY184967	EF469073	EF469102	EF469119
<i>Viridispora diparietispora</i>	CBS 102797	Terrtrial	No	Pending	AY489703	AY489735	AY489630	AY489668	DQ522471

Herbarium Codes: AEG, A. E. Glenn personal collection; ARSEF, USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; ATCC, American Type Culture Collection, Manassa, VA; BCC, BIOTEC Culture Collection, Klong Luang, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CUP, Cornell University Plant Pathology Herbarium; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; FAU, F. A. Uecker personal collection; GAM, Julian H. Miller Mycological Herbarium Athens, GA; HMAS, Chinese Academy of Sciences, Beijing, China; KEW, mycology collection of Royal Botanical Garden, KEW, Surrey, UK; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR; SA, S. Alderman personal collection; TNS, National Museum of Science and Nature, Tsukuba, Japan;

Table 5.3 Results of ancestral character state reconstructions. Proportional likelihood values are given for the state receiving 2 raw likelihood units or greater support for a node. For each node the number of bootstrap trees containing the node, as well as the number of trees scored for each character ambiguously are also given.

Node number	# Trees Node Present	Stipe				Habitat			
		Likelihood	Yes	No	Ambiguous	Likelihood	Terrestrial	Arboreal	Ambiguous
1	500	No 0.98362487	0	500	0	Terrestrial 0.98578090	388	0	112
2	464	No 0.93020187	0	458	6	Terrestrial 0.99447773	282	7	175
3	370	No 0.94321525	0	368	2	Terrestrial 0.98578652	172	6	192
4	500	No 0.99741555	0	500	0	Terrestrial 0.99846751	497	0	3
5	500	Ambiguous	0	469	31	Ambiguous	0	353	147
6	500	Ambiguous	1	5	494	Ambiguous	0	383	117
7	243	Ambiguous	4	0	239	Ambiguous	0	173	70
8	500	Ambiguous	4	5	491	Arboreal 0.98524945	0	500	0
9	500	Ambiguous	22	2	476	Ambiguous	1	231	268
10	499	Yes 0.99751242	431	0	68	Ambiguous	0	208	291
11	339	Yes 0.99976193	339	0	0	Ambiguous	338	0	1
12	484	Ambiguous	213	4	267	Terrestrial 0.99487119	372	9	103
13	500	Yes 0.99824226	499	0	1	Terrestrial 0.99987810	456	3	41
14	500	Yes 0.99896834	499	0	1	Terrestrial 0.99982989	500	0	0
15	500	Yes 0.99738505	500	0	0	Terrestrial 0.93457648	223	7	270
16	477	Yes 0.99736357	477	0	0	Terrestrial 0.99324249	290	0	187
17	500	Yes 0.96802292	500	0	0	Ambiguous	20	1	479
18	452	Ambiguous	229	1	222	Terrestrial 0.98149530	274	8	170
19	497	Ambiguous	201	51	245	Ambiguous	199	150	148
20	500	Yes 0.99714219	500	0	0	Terrestrial 0.99800389	475	0	25
21	243	No 0.92125554	3	92	148	Arboreal 0.94396362	2	149	92
22	495	No 0.91534206	9	158	328	Arboreal 0.94677991	0	405	90

CHAPTER SIX

Conclusion

This dissertation represents significant advances in understanding the systematics and evolution of clavicipitaceous fungi, particularly fungi associated with Clavicipitaceae. Initial assessments of species included in this study anticipated expansions of the genera *Cordyceps* and *Ophiocordyceps*, in line with diversity observed in previous studies, (e.g., Sung et al. 2007). This assessment was also limited by a paucity of characters able to circumscribe *Metacordyceps*. The unexpected placement of species in and around *Metacordyceps* and Clavicipitaceae has provided an opportunity to test evolutionary hypotheses and develop theories that extend beyond broadly to other groups of fungi, as well as general pathogen biology.

Chapter 2 dealt with the inclusion of previously unexamined species of *Cordyceps* sensu lato in the genus *Metacordyceps*. The inclusion of these taxa doubled the number of species associated with the genus and helps clarify associated morphological characters. Structure is also observed amongst the species of *Metacordyceps*. A strongly supported core clade of species displaying yellow, yellow-green to olive or reddish pigmentation and predominantly oblique perithecia associated with *Metarhizium* or green spored *Nomureae* anamorphs was identified. This core clade is subtended by a poorly supported phylogenetic grade of species lacking strong pigmentation and associated with *Pochonia* like anamorphs. Addition

of new taxa expands the known range of host associations for *Metacordyceps* and establishes several new anamorph-teleomorph connections.

Establishment of a new genus, *Veterocordyceps*, for species previously considered incertae sedis or placed in *Ophiocordyceps* on the basis of anamorph or teleomorph morphology alone was described in Chapter 3. Two well resolved clades within the genus were described as subgenera. *Veterocordyceps* subg.

Veterocordyceps contains species producing superficial perithecia, and express a *Hirsutella* or *Acremonium*-like anamorph when grown in culture. This feature was used by Ban et al. (2009) to place these species in *Ophiocordyceps*. However *Hirsutella* and *Acremonium* are not phylogenetically related and anamorphs produced by species in this subgenus may reflect the common verticillate anamorph morphology observed throughout Hypocreales. Species in *Veterocordyceps* subg. *Pulvinata* produce perithecia in terminal or lateral perithecial pads and were found to be associated with *Polycephalomyces* anamorphic states. Although the genus was well resolved as a unique clade of fungi, *Veterocordyceps* is considered a member of Clavicipitaceae s.l. A placement sister to Clavicipitaceae was suggested by maximum likelihood analyses, but this relationship does not receive support in Bayesian analyses. Expanded sampling of taxa, as well as the application of new molecular markers, will likely be necessary to fully resolve the familial relations of this group.

Chapter 4 presented a detailed analysis of the evolution of nutritional mode in Clavicipitaceae s.s. after inclusion of plant pathogenic *Ustilaginoidea virens* (Cooke) Takahashi and the fungal pathogen *Cordyceps fratricida* Tanda & Kobayasi.

Ustilaginoidea virens is the causal agent of rice false smut and placed at the base of the clade of scale insect and plant pathogens. *Cordyceps fraticida* is a pathogen of ergot sclerotia and is placed in the clade of grass symbionts as a close relative of *V. epiphytum*, a pathogen of coffee rust previously unassociated with any known teleomorphic state. The genus *Tyrannocordyceps* was proposed for pathogens of ergot sclerotia. Ancestral character-state reconstructions indicate that plant pathogens arose independently three times from a scale insect pathogenic background. This was inferred to be the result of close physical association of scale insects with plants facilitated by the permanent insertion of mouthparts. *Tyrannocordyceps fraticida* is reconstructed as having evolved from a plant pathogenic ancestor, possibly from competition arising during co-occurrence in the same host resulting not in exclusion but in a switch to direct infection of the competitor. This work suggests the host habitat theory of interkingdom host shifts, first proposed by Nikoh and Fukatsu (2000) for the jump from insects to deer truffles in *Elaphocordyceps*, should be applied more broadly throughout clavicipitaceous fungi as an explanation of the diversity of hosts.

Chapter 5 examined the evolution of stipe morphology throughout the order Hypocreales after the inclusion of previously unexamined species from Hypocreaceae, Cordycipitaceae and Ophiocordycipitaceae, as well as taxa in Clavicipitaceae examined in the previous chapters. Ancestral character-state analyses were performed for the characters of stipe morphology (absent or present) and habitat (terrestrial or arboreal). Correlation of these characters was also explored using the Pagel94 method available in Mesquite. Hypocreales was inferred to have evolved from a terrestrial,

astipitate ancestor. A stipitate morphology was inferred to have evolved independently in the Hypocreaceae and Cordycipitaceae, and either in the common ancestor for Ophiocordycipitaceae and Clavicipitaceae or separately for each, although there is some ambiguity as to when the stipitate morphology appeared for these families. The independent evolution of the stipitate morphologies throughout clavicipitaceous fungi explains the phylogenetic signal in stipe texture and color. Stipe morphology and habitat were strongly correlated, with the majority of astipitate forms occurring in arboreal species. This combination of characters is inferred to be from the retention of the ancestral stipe condition for arboreal species in Bionectriaceae and *Torrubiella wallacei* in Cordycipitaceae. However, reconstructions for the nodes subtending the core *Torrubiella*, as well as *C. piperis*, are ambiguous with regards to stipe morphology and understanding the trajectory of morphological changes must await more data for this family. All other astipitate species occurring in arboreal habitats were clearly derived from a stipitate, terrestrial ancestor. The stipe serves to elevate fertile tissues above the substrate to facilitate spore dispersal. The move to an arboreal habitat would make the utility of the stipe redundant, relieving the evolutionary pressure to expend energy incurred with stipe production. With the possible exception of *C. nelumboides*, there are currently no species definitely inferred to have acquired a stipitate morphology after the move to arboreal habitats.

In addition to the work presented here, an online repository, the *Cordyceps* Electronic Monograph, was created in an effort to bridge the gap between observation

and identification for an important group of animal, plant and fungal pathogens. This site is a collaborative effort for a globally distributed community of researchers and represents the current state of knowledge for the genus *Cordyceps* and its related taxa. Summaries of the ecology and systematics are provided to familiarize users encountering *Cordyceps* for the first time. Over 600 species in 14 genera are represented with taxonomic and synonymy information, host and geographical distribution data, and work is ongoing to populate content for macro- and microscopic morphological characters. A synoptic key is being developed to guide users to a subset of taxa representing the specimen in hand. We also provide a database of contemporary and historical literature, as well as numerous images of type specimens and field collected material to aid in the identification process. Finally, we bring techniques for species identification into the modern age by offering a BLAST search option for a curated database of sequences for genes used in published molecular studies of these taxa.

References

- Ban S, Sakane T, Toyama K, Nakagiri A, 2009 Teleomorph-anamorph relationships and reclassification of *Cordyceps cuboidea* and its allied species. *Mycoscience* **50**: 261-272.
- Nikoh N, Fukatsu T, 2000 Interkingdom Host Jumping Underground: Phylogenetic Analysis of Entomoparasitic Fungi of the Genus *Cordyceps*. *Molecular Biology and Evolution* **17**: 629-638.
- Sung G, Hywel-Jones NL, Sung J, Luangsa-ard JJ, Shrestha B, Spatafora JW, 2007 Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Stud Mycol* **57**: 5-59.