

## AN ABSTRACT OF THE THESIS OF

Desiree M. Johnson for the degree of Master of Science in Botany and Plant Pathology presented on March 20, 2006.

Title: Systematics of the Genus *Torrubiella*

Abstract approved:

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*Torrubiella* is a genus of entomopathogenic fungi in the Clavicipitaceae (Ascomycota). Species of the genus produce superficial perithecia on a loose mat of hyphae that forms directly on the host. The genus is estimated to contain about 70 species. *Torrubiella* is hypothesized to be a close relative of *Cordyceps* because of morphological similarities in perithecia, asci and ascospores. However, unlike *Cordyceps*, species of *Torrubiella* do not produce stipitate stromata. The two genera also differ in their host range. *Torrubiella* spp. attack spiders and scale insects, which are notably depauperate in the host profile of *Cordyceps*. Recent molecular studies rejected the monophyly of *Cordyceps* and resulted in the reclassification of *Cordyceps* (*Cordyceps* s.s., *Elaphocordyceps*, *Metacordyceps* and *Ophiocordyceps*) and the Clavicipitaceae s.l. into three families. Molecular analyses also did not support the monophyly of *Torrubiella*, but insufficient taxon sampling made conclusions about the genus difficult. To test and refine evolutionary hypotheses regarding the placement of *Torrubiella* in Clavicipitaceae and its relationship to *Cordyceps*, phylogenetic analyses of the Clavicipitaceae with an emphasis on *Torrubiella* were conducted. The phylogenetic informativeness of *anamorph* genera and host affiliation was also addressed. Weighted

parsimony analyses were conducted on sequence data from five nuclear loci including: nuclear small subunit ribosomal DNA (nrSSU), nuclear large subunit ribosomal DNA (nrLSU), the largest (RPB1) and second largest (RPB2) subunits of RNA polymerase II, and translation elongation factor 1 $\alpha$  (tef). The monophyly of *Torrubiella* was rejected by these analyses, with species of the genus present in each of the three families of clavicipitaceous fungi and often intermixed among *Cordyceps* species. The morphological characters traditionally used to define the genus are therefore not phylogenetically informative. Two new genera are proposed to accommodate two lineages of torrubielloid fungi. In addition, five species of *Torrubiella* are reclassified into different genera based on these phylogenetic analyses.

The majority of torrubielloid taxa sampled formed a well-supported clade of spider pathogens, however the taxonomic fate of the genus remains unresolved, as the type specimen was not sampled. The generic concept of a monophyletic *Cordyceps* was expanded to include two species of *Torrubiella* with anamorphs characteristic of the clade. One isolate of *Torrubiella* with distoseptate ascospores and a *Hirsutella* anamorph grouped with species of *Cordyceps* with similar ascospore and anamorph morphologies and was transferred to the genus *Ophiocordyceps*. In addition, two species of *Torrubiella* not sampled, with similar morphologies and anamorphs, were also transferred to *Ophiocordyceps*.

Some *anamorph* genera were restricted in their distribution and were phylogenetically informative in the classification of *Torrubiella*, especially in conjunction with host affiliation. Others were not and represent convergent or ancestral morphologies rather than natural, monophyletic groups. Host affiliation was phylogenetically informative and predictive of relationship for the spider pathogens and

armored scale insects. However, pathogens of soft scale insects were found in two of the three families and represented homoplastic ecologies.

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Systematics of the Genus *Torrubiella*

By

Desiree M. Johnson

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Desiree M. Johnson, Author

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# Systematics of the genus *Torrubiella*

## Chapter 1

### INTRODUCTION

The genus *Torrubiella* is a member of the Clavicipitaceae (Ascomycota: Hypocreales), a family of perithecial ascomycetes, well-known for their obligate symbioses with animals, plants, and other fungi, as well as for their role in human history. *Claviceps purpurea*, better known as ergot, is symbiotic with cereal grasses and produces numerous alkaloids which may have contributed to the Salem Witch Trials (Matossian and Caporael, 1976; Matossian, 1989; White *et al.*, 2003). *Beauveria bassiana*, a pathogen of Lepidopteran larvae, was the first organism demonstrated to be a causal agent of disease, which predates the development of the germ theory (White *et al.*, 2003). *Cordyceps sinensis* has a long history of use in traditional Chinese medicine and is thought to improve function in the immune system and increase stamina and endurance (Hobbs, 1995; White *et al.*, 2003). Some *anamorph* species, notably *Beauveria spp.* and *Metarhizium spp.*, and anamorphs of *Torrubiella*, have been used as biocontrol agents of agricultural pests (Evans, 2003; Samson *et al.*, 1988).

Fungi in the Clavicipitaceae are cosmopolitan in distribution, but are particularly diverse in the tropics. As entomopathogens, these fungi are important components in natural forest and agricultural settings, and are presumed to be significant agents of arthropod mortality. The Clavicipitaceae comprises more than 35 genera, with approximately 75% of species being arthropod pathogens

(Hawksworth *et al.*, 1995; Artjariyasripong *et al.*, 2001; Diehl, 1950; Rogerson, 1970; White *et al.*, 2003). The genus *Cordyceps* comprises more than 400 species and is morphologically and ecologically diverse, representing much of the phylogenetic diversity in the family. *Anamorph* forms (asexual states) also play an important role in the life cycles of these fungi, yet few have been linked to teleomorphs (sexual states) (Hodge, 2003). The genus *Torrubiella*, which is closely related to *Cordyceps*, is an arthropod pathogen primarily found on spiders and scale insects. These fungi produce superficial perithecia directly on their hosts rather than on stipitate stromata, as in *Cordyceps*. Flask-shaped perithecia are produced on a weft of hyphae that covers the host and also attaches them to the surface upon which they died. There are approximately 60 accepted species of *Torrubiella*, with new species being discovered often following the continual examination of tropical forests (see Table 1). *Torrubiella* has been hypothesized to be closely related to *Cordyceps* because of similarities in perithecia, ascus, and ascospore morphology, as well as its occurrence on arthropods (Petch, 1923; Mains, 1949; Kobayasi, 1982). Petch (1923) discussed the validity of the genus, in regards to intermediate species (which produce superficial perithecia on stromata), but retained the genus for species which produce perithecia astromatically. Previous phylogenetic studies have shown that *Torrubiella* is not monophyletic and integrates at multiple points within *Cordyceps* (Sung, 2005; Artjariyasripong *et al.*, 2001).

## ENTOMOPATHOGENS IN THE KINGDOM FUNGI

Arthropod associated fungi are found within all major fungal lineages. The symbiotic relationships of these fungi and their arthropod hosts range from mutualistic to antagonistic. More than 700 species of entomopathogenic fungi have been described. (Hajek and St. Leger, 1994; Samson *et al.*, 1988). Discussed here are those relationships that involve fungi deriving their nutrition directly from the body of the host and not relationships with fungus-cultivating insects.

### **Chytridiomycota**

Members of the genus *Coelomomyces* are obligate parasites, with aquatic Dipterans typically serving as their primary hosts. The genus is the only group of fungi, besides plant pathogenic rusts, that are heteroecious, typically having a crustacean as an alternate host (Whisler *et al.*, 1974, 1975). Infected larvae normally die in the fourth instar stage and resting sporangia develop in the corpse. *Coelomomyces stegomyiae* is a pathogen of the yellow fever mosquito. Larvae with high levels of infection die; however those with only a light infection live to reproduce (Lucarotti, 1992). Egg production requires a blood meal, which is also required for the formation of spores. Infected females release fungal resting spores instead of eggs when they attempt to oviposit, thereby dispersing the fungus to new habitats (Lucarotti, 1992). Species of *Coelomomyces* are capable of causing significant epizootics, with mortality rates greater than 50%, often exceeding 90%, that can persist in a population for several years (Apperson *et al.*, 1992).

## **Zygomycota**

Entomophthoralean fungi attack Dipteran hosts and are common in both forests and urban settings. Many have a narrow host range and some are even restricted to a single host species (Alexopoulos *et al.*, 1996). In *Entomophthora muscae*, a pathogen of flies, conidia are explosively discharged and surrounded by a mucilaginous substance that facilitates adhesion. After a conidium comes in contact with a fly, death occurs in approximately five to eight days (Brobyn and Wilding, 1983). Flies are attached to the substrate by mycelia and are often surrounded by conidia forcibly discharged from their abdomen. Male flies are attracted to dead infected flies (Møler, 1993), attempt to copulate with them and become infected themselves. Spores are capable of multiple germinations, increasing chances of contact with a host (Humber, 1984).

*Massospora cicada* is a pathogen of periodical cicadas. The cicadas emerge from the soil once every 13 or 17 years in masses as adults, a reproductive strategy that presumably evolved to evade predation. Initial infection occurs in the soil as nymphs approach the surface. The fungus disorganizes the abdomen, which fills with conidia. Sections of the abdomen begin falling off, starting with the genitalia (Soper, 1974). The cicada continues to disperse, congregate, call and attempts to copulate with females, all the while, dispersing spores for secondary infection. Resting spores develop in secondarily infected individuals that disperse spores as they sift out through breaks in the abdomen (Soper, 1976).

Trichomycetes are a class of obligate symbionts found primarily in the hindgut of aquatic arthropods that feed on living algae or decaying vegetation, attached to the gut lining by a holdfast (Lichtwardt, 1986). The nature of the symbiosis depends on the species, environmental conditions, and the developmental stage of the host. Mosquito larvae infected with *Smittium culisetae* have been shown to derive nutritional benefit in nutrient depleted environments (Horn and Lichtwardt, 1981). The same fungus in black fly larvae provides nutritional benefit in stressed environments and no apparent effect in non-stressed environments; however, in adult populations, the ovary is infected, resulting in reduced fecundity (McCreadie *et al.*, 2005). In contrast, *Smittium morbosum* is often lethal to mosquito larvae (Sweeney, 1981).

### **Basidiomycota**

The nature of the interactions between fungi in the Septobasidiales and scale insects range from parasitic to mutualistic. These fungi are related to the plant pathogenic rusts (Blanz and Gottschalk, 1986). *Septobasidium spp.* are usually considered to have a mutualistic relationship with Diaspidid scale insects. The hosts are infected as first instar larvae, during the dispersal phase (crawlers). When the infected insects metamorphose into adults, the fungus invades the haemocoel through natural openings, producing coiled haustoria. Infected insects remain small and sterile, but live as long as non-parasitized insects. The insect continues to feed on the plant, providing continuous nutrition for the fungus, which continues to grow, encasing other members of the colony that do not

become infected. These non-infected insects are presumed to benefit from this interaction, as they are still able to reproduce and are protected from desiccation and predation by parasitoid wasps, which are a major cause of mortality. The fungal colony expands due to spore dispersal by the crawlers (Couch, 1938).

### **Ascomycota**

Members of the Lauboulbeniales are primarily ectoparasites of beetles. The fungi form thalli with determinate growth of only a few cells (1-15). Species exhibit host specificity, as well as specificity for infecting particular parts of the body, sometimes directly related to points of contact during mating (Evans, 1988).

Members of the genus *Myriangiium* are pathogens of armored scale insects (Diaspididae) and are particularly common in the southern United States (Miller, 1940; Evans and Prior, 1990). These Loculoascomycetes produce a black stroma of indeterminate growth over their hosts with asci imbedded in the tissue. The stroma gelatinizes in response to moisture and the asci swell, forcibly ejecting their ascospores (Miller, 1938, 1940).

An untapped diversity of microfungi has been found within some species of arthropods. These obligate endosymbionts have been found in the guts of Hemipteran (true bugs) and Coleopteran (beetles) insects, and are thought provide necessary enzymes and detoxify plant material (Nardon and Grenier, 1989). Most known fungal endosymbionts of arthropods are true yeasts of the Saccharomycotina, but a recent phylogenetic analysis revealed a lineage of yeast-like symbionts in the Clavicipitaceae associated with planthoppers (Suh *et al.*,

2001). Clavicipitalean fungi are also important agents of arthropod mortality and represent a diverse group, both morphologically and ecologically. The oldest known fossil of a Clavicipitalean fungus is from Burmese amber, approximately 100 million years old (Poinar *et al.*, in prep.), suggesting a Cretaceous radiation and an ancient origin of the insect symbiosis in the Clavicipitaceae (Sung 2005).

## **ECOLOGY OF ENTOMOPATHOGENS**

The most important environmental factors contributing to the distribution of entomopathogenic fungi are temperature, humidity, solar radiation, and wind. These fungi are usually found in forest habitats, where they are better protected from solar radiation, the local humidity is higher and the environment is buffered from temperature extremes. These fungi are often found in microhabitats with higher microhumidity, such as along side streams. They also are more diverse in areas that exhibit high insect diversity. *Torrubiella spp.* and other aerial entomopathogens, generally use strands of hyphae to anchor their host in an aerial position on the underside of leaves, which is advantageous for spore dispersal because it protects the host from predation and scavenging (Evans, 1989). It is also suspected that this keeps the hydrophobic propagules dry, allowing for aerial dispersal in an often moist environment. Similar attachment methods are found in the Entomophthorales (Evans, 1989). Diversity of these fungi typically decreases after forest disturbance, either due to the loss of hosts or the changes in environmental factors (Samson *et al.*, 1988).

Differing characteristics of propagules allow for dispersal in a variety of conditions. For instance, Hywel-Jones, notes finding *T. luteorostrata* in the rainy season and its anamorph, *Paecilomyces cinnamomeus* in the dry season (1993). *Anamorph* fungi produce large numbers of mitotic spores in a short period of time, which presumably allows the fungus to quickly colonize new hosts (Hodge, 2003). Conidia of *anamorph* clavicipitalean genera, such as *Akanthomyces*, *Gibellula*, *Beauveria*, *Metarhizium*, and *Paecilomyces* are dry and hydrophobic, characteristics that presumably are suitable for long-distance dispersal by wind. These dry spores exhibit electrostatic forces and structural components (eg. lipoproteins) which facilitate attachment to the cuticle of insects (Samson *et al.*, 1988; Evans and Hywel-Jones, 1997a). In contrast, spores of *Hypocrella*, *Hirsutella*, and *Verticillium* are slime spores that are dispersed via rainwater facilitated by the drip tips common on tropical forest leaves. Hydrophilic slime conidia dispersed by rain splash can further be transmitted via wind (Hajek and St. Leger, 1994). The mucus coating is thought to help resistance to desiccation, assist in adhesion to and degradation of the cuticle (Boucias and Pendland, 1991). Interestingly, there are few examples of hydrophobic *anamorph* spores produced on scale insects, most likely because slime spores are an effective way to spread infection across colonies (Samson *et al.*, 1988). Filiform aco-spores, which are not actively discharged, are adapted for dispersal in aquatic microhabitats (on leaves) during the wet season in a forest; the shape also increases probability of contact with insect hosts (Bandoni and Koske, 1974; Cox, 1983). In many species,

ascospores divide into many partspores, producing a kind of shotgun approach to finding a host.

It is commonly observed that arthropods infected with entomopathogenic fungi exhibit behavior that is not normal. "Summiting disease" refers to the phenomenon of infected insects climbing from the relative safety of their normal habitation, exposing themselves to predation. It is not clear whether this behavior is under fungal control as a means of aiding dispersal, or a tactic employed by the insect to minimize infection to kin (Evans, 1989).

## **MECHANISM OF INFECTION**

The infection process begins with a spore or conidium coming in contact with the cuticle of an appropriate host. Infection is through the integument; a process unique to fungi, as other parasitic micro-organisms, such as bacteria, viruses and microsporidia, must penetrate through the host's mid-gut (Samson *et al.*, 1988). The first obstacle an entomopathogenic fungus must overcome is the cuticle, composed of three non-cellular layers excreted above the epidermis. The first layer is the epicuticle, composed of lipoproteins and wax: a hard, impervious, inhospitable structure that in addition contains antifungal compounds that may be harmful to germinating spores (Smith and Grula, 1982). In order to penetrate the cuticle, the fungus must produce a series of enzymes: lipases, proteases, and chitinases (St. Leger, 1991; Samson *et al.*, 1988). Upon infection, the host releases haemocytes, which attempt to engulf the intruding fungal cells, and prophenoloxidasases, resulting in melanization (Evans, 1988). Once in the

haemocoel, the fungus quickly disseminates as yeast-like hyphal bodies. This tactic of rapid colonization overwhelms the haemocytes and toxins produced by fungus make the haemocytes function improperly (Evans, 1988). The host is killed by toxins circulated with the yeast-like hyphal bodies. After death, the fungus feeds saprobically, consuming all internal organs.

## SECONDARY METABOLITES

Many entomopathogens, particularly Clavicipitalean fungi, produce a suite of biologically active compounds necessary to overcome their host's immune systems, kill the host, and ward off competitive saprobes. Destruxins in *Metarhizium anisopliae* affect organelles, paralyzing cells and causing disfunction (Hajek and St. Leger, 1994). Beauvericin, originally isolated from *Beauveria spp.* and *Paecilomyces spp.*, forms Na<sup>+</sup> and K<sup>+</sup> complexes, resulting in increased permeability across membranes (Vey *et al.*, 2001; Ovchinnikov *et al.*, 1971) and can induce programmed cell death similar to apoptosis and cause cytolysis and DNA fragmentation (into multiples of 200 base pairs) (Vey *et al.*, 2001; Ojcius *et al.*, 1991). Cyclosporin A, an immunosuppressant used in organ transplant surgery and treatment of autoimmune diseases, was originally isolated from *Tolyocladium inflatum*, the asexual stage of *Cordyceps subsessilis* (Isaka *et al.*, 2003).

The only Entomophthoralean fungi that produce mycotoxins are members of the genus *Conidiobolus* (Prasertphon and Tanada, 1969). There is no evidence of antibiotic production in other members of order, which kill their host by means

of nutrient depletion and mechanical damage from hyphae; as it is not advantageous to kill the host initially with no means of protecting the cadaver from microbial infection (Samson *et al.*, 1988).

## HOST BIOLOGY AND ECOLOGY

Species of *Torrubiella* are primarily pathogens of spiders and hemipteran insects, particularly scale insects. Additional hosts include ants (Hymenoptera), moths (Lepidoptera), crickets (Orthoptera), and *Cordyceps* species. Host identification is often limited to the ordinal or familial level. The host is usually overgrown by the fungus, making identification difficult, if not impossible, to such a degree that scale insect fungi were thought to be pathogens of plants. Even if any host remains visible, most mycologists are not entomologists, and entomologists are not often consulted for identification.

Aside from spiders, *Torrubiella spp.* are historically said to be pathogens of “Homopterans”, a paraphyletic group (Bourgoin and Campbell, 2002; Campbell *et al.*, 1995), which includes scale insects, leafhoppers, aphids, white flies, and cicadas. All these insects are terrestrial plant feeders that share a similar biology and morphology; many produce honeydew and are attended by ants. In accordance with the most recent systematics of these insects, *Torrubiella spp.* are pathogens of the order Hemiptera, and more specifically the Suborders Sternorrhyncha (aphids, scale insects and white flies) and Cicadomorpha (cicadas and leafhoppers) (Bourgoin and Campbell, 2002 ). No species of *Torrubiella* are known to occur on cicadas, however, many species of *Cordyceps*, such as

*Cordyceps sinclarii*, are found exclusively on cicadas. Only a few species of *Cordyceps* has been reliably identified as pathogens of scale insects. In addition, a fossil of the genus *Hymenostilbe* on a male scale was recently found in 100 million year old Burmese amber (Poinar *et al.*, in prep).

*Torrubiella* is confirmed to parasitize two families of scale insects, the soft scales (Coccidae) and the armored scales (Diaspididae). Many species accounts though, specify only “scale insect” or the author had a very broad interpretation of ‘coccid’, which could also include Aleyrodidae (white flies) (Evans and Hywel-Jones, 1997). The Coccidae uptake nutrients from the phloem of host plants and produce honeydew excretions that are often tended by ants (Ben-Dov, 1997). The Diaspididae do not produce honeydew, as they take up nutrients from the content of cells rather than from phloem vessels (Ben-Dov, 1990). Adult female armored scale insects live under a scale cover which is a product of their body, but not part of it. The scale is formed by secreted wax filaments, cemented by anal secretion, and embedded with the skin of the two preceding molts (Ben-Dov, 1990). The Diaspididae are parasitized by only three species of *Torrubiella*, representing a minor host. Diaspidid pathogenicity is notably scarce in the Clavicipitaceae *s.l.*, however *Nectria* (Nectriaceae, Hypocreales) and *Podonectria* (Tubeufiaceae, Dothidiomycetes) are important pathogens of armored scale insects (Evans and Prior, 1990; Evans and Hywel-Jones, 1997). Scale insects exhibit extreme sexual dimorphism. Females are stationary, wingless, legless, eyeless, have a complete fusion of abdomen, head and thorax, and are long-lived; where as males have ‘normal’ insect bodies, are winged, motile, ephemeral and have no mouth parts, as

they do not feed as adults (Ben-Dov, 1990). Little reference is made of which life stage or sex of scale insects is parasitized by *Torrubiella*, but immature stages are only present for a limited time and males adults are smaller, shorter lived and randomly located. Based on host life histories it can be concluded that the adult females are the primary hosts, as they are stationary and gregarious; a life style more conducive to fungal dispersal. "Plant suckers" do not often fall victim to bacteria and viruses, which are gut pathogens, as the possibility of ingestion is very low, therefore, entomopathogenic fungi, after parasitoid and parasitic insects, are major pathogens of these insects (Evans and Hywel-Jones, 1997).

Spiders (Araneae) also represent a major group of hosts of *Torrubiella*. Spiders are the seventh largest order amongst all living organisms (with over 38,000 species), yet few members of the Clavicipitaceae parasitize them outside of *Torrubiella* (less than ten species of *Cordyceps*) (Kobayasi, 1941; Mains, 1954). Little is known of spider cuticular biochemistry in comparison to insects, though it is generally considered to be similar. Spiders, like insects, possess an epi- and endocuticle, however spiders have an additional layer, the mesocuticle, which is excreted prior to the endocuticle. The endo- and mesocuticle do not differ structurally, but the mesocuticle is thought to be a more sclerotized form of cuticle and differs in reaction to certain dyes (Foelix, 1996). The hard exocuticle is absent in the abdominal region of spiders, which is thought to be the site of infection (Foelix, 1996; Evans and Samson, 1987).

## **TAXONOMY OF THE GENUS *TORRUBIELLA***

All members of the Clavicipitaceae possess distinctive, long, cylindrical asci with a pronounced apical cap, that contain long, filiform and multiseptate ascospores, which in many species, disarticulate into partspores at maturity (Rogerson, 1970; Spatafora and Blackwell, 1993). *Torrubiella* was erected to accommodate arthropod pathogens similar to *Cordyceps* that produced superficial perithecia on a weft of hyphae. The genus was established in 1885 by Boudier (*Rev. Myco*, VII). The type species is *T. aranicida*, was collected in Montmorency, France on a spider:

*Torrubiella aranicida* Boud.: Perethicia elongated-conoid, subflexuose, 0.65-0.7 mm high, 0.3-0.35mm in diameter, smooth, ochraceous or ochraceous orange, scattered or caespitose, on a thin white, byssoid stroma. Paraphyses conspicuous, as long as the asci, very slender, apex clavate and 3  $\mu$  thick. Asci linear, very long, eight-spored, 330-350 X 5-6 $\mu$ , apex rounded and not swollen; ascospores filiform, very slender, as long as or longer than asci, 300-400X0.5-2  $\mu$ , obscurely septate and granular.

Following this description, about 80 species names have been added to the genus (Petch, 1923; Mains, 1949; Kobayasi and Shimizu, 1982; Kobayasi, 1982; O'Donnell, Common and Imshaug, 1977; Samson, Reenen-Hekstra van and Evans, 1989; Hywel-Jones, 1993, 1995, 1997b; Zare, R., and W. Gams, 2001; Bischoff and White Jr., 2004) representing about 70 valid species (see Table 1). Petch (1923) presents detailed species accounts for the pathogens of scale insects. Kobayasi and Shimizu (1982) and Kobayasi (1982) reviewed 59 species of

**Table 1 - List of *Torrubiella* spp. with their hosts and anamorphs**

Taxon	Host	Anamorph
1 <i>Torrubiella alba</i> Petch	Araneae	<i>Lecanicillium araneorum</i> (Petch) Zare & W. Gams
2 <i>Torrubiella alboglobosa</i> Kobayasi & Shimizu	Araneae	
3 <i>Torrubiella albolanata</i> Petch	Araneae	<i>Gibellula petchii</i> Humber & Rombach
4 <i>Torrubiella albotomentosa</i> Petch	pupae of Diptera	<i>Cylindrophora araneorum</i> Petch = ? <i>Gibellula petchii</i>
5 <i>Torrubiella arachnophila</i> (J.R. Johnst.) Mains	Araneae	<i>Gibellula arachnophila</i> (Ditmar) Vuill.
6 <i>Torrubiella arachnophila f. alba</i> Kobayasi & Shimizu	Araneae	<i>Gibellula pulchra</i> Cavara
<i>Torrubiella arachnophila f. arachnophila</i> (J.R. Johnst.) Mains	Araneae	
<i>Torrubiella arachnophila var. arachnophila</i> (J.R. Johnst.) Mains	Araneae	
<i>T. arachnophila var. pleiopus</i> = <i>T. pleiopus</i>	Araneae	<i>Gibellula pleiopus</i> (Vuill.) Mains
7 <i>Torrubiella arachnophila var. pulchra</i> Mains	Araneae	
8 <i>Torrubiella aranicida</i> Boud.	Araneae	<i>Hirsutella cuneispora</i> (Boudier) Petch?
9 <i>Torrubiella aurantia</i> Kobayasi & Shimizu	Araneae	
10 <i>Torrubiella barda</i> Petch	Coccoidea	
11 <i>Torrubiella blattae</i> Petch	pupae of Diptera	
12 <i>Torrubiella brunnea</i> Keissl.	Coccoidea	
<i>T. carnata</i> = <i>Cordyceps carnata</i>	Formicidae	<i>Hirsutella liberiana</i> Mains (1949) = <i>Tilachlidium brachiatum</i> (Batsch) Petch
13 <i>Torrubiella clavata</i> Samson & H.C. Evans	Araneae	<i>Gibellula clavata</i> Samson & H.C. Evans
<i>Torrubiella colombiana</i> Kobayasi	Insecta	
14 <i>Torrubiella confragosa</i> Mains	Coccidae	<i>Lecanicillium lecanii</i> (Zimm.) Zare & W. Gams
<i>T. cordyceps</i> Dingley = <i>Torrubiella lloydi</i>	<i>Cordyceps</i>	
15 <i>Torrubiella corniformis</i> Kobayasi & Shimizu	Araneae	
16 <i>Torrubiella dabieshanensis</i> B. Huang, M.Z. Fan & Z.Z. Li	Araneae	
17 <i>Torrubiella dimorpha</i> Tzean, L.S. Hsieh & W.J. Wu	Araneae	<i>Gibellula dimorpha</i> Tzean, L.S. Hsieh & W.J. Wu
18 <i>Torrubiella ellipsoidea</i> Kobayasi & Shimizu	Araneae	
19 <i>Torrubiella falklandica</i> O'Donnell, Common & Imshaug	Araneae	
20 <i>Torrubiella farinacea</i> Kobayasi & Shimizu	Araneae	
21 <i>Torrubiella flava</i> Petch	Araneae	<i>Akanthomyces arachnophilus</i> (Petch) Samson & H.C. Evans
22 <i>Torrubiella flavoviridis</i> (A. Møller) Kobayasi	Araneae	
23 <i>Torrubiella formicarum</i> Samson, Reenen & H.C. Evans	Formicidae	<i>Pseudogibellula formicarum</i> (Mains) Samson & H.C. Evans

Table 1 (continued)

24	<i>Torrubiella formosana</i> Kobayasi & Shimizu	Araneae	
25	<i>Torrubiella fusiformis</i> Kobayasi & Shimizu	Araneae	
26	<i>Torrubiella gibellulae</i> Petch	Araneae	<i>Gibellula araneorum</i> P. Syd.
27	<i>Torrubiella globosa</i> Kobayasi & Shimizu	Araneae	<i>G. globosa</i> Kobayasi & Shimizu = <i>Gibellula pulchra</i> Cavara
28	<i>Torrubiella globosoides</i> Kobayasi & Shimizu	Araneae	
29	<i>Torrubiella globosostipitata</i> Kobayasi & Shimizu	Araneae	<i>G. globosostipitata</i> Kobayasi & Shimizu = <i>Gibellula pulchra</i> Cavara
30	<i>Torrubiella gonylepticida</i> (A. Møller) Petch	Araneae-Gonyleptidae	<i>Paecilomyces longipes</i> (Petch) A.H.S. Br. & G. Sm.
31	<i>Torrubiella hemipterigena</i> Petch	Cicadellidae	<i>Verticillium hemipterigenum</i> Petch (1932)
32	<i>Torrubiella hirsutellae</i> (Petch) Rossman	Cicadellidae	<i>Hirsutella</i> sp.
33	<i>Torrubiella inegoensis</i> Kobayasi & Shimizu	Araneae	
34	<i>Torrubiella iriomoteana</i> Kobayasi & Shimizu	Coccoidea	<i>Hirsutella</i> sp.
35	<i>Torrubiella lecanii</i> J.R. Johnst.	Coccidae	
36	<i>Torrubiella leiopus</i> (Mains) Kobayasi & Shimizu	Araneae	<i>Gibellula leiopus</i> Cavara (1950)
	<i>T. liberiana</i> Mains = <i>Cordyceps carnata</i>	Formicidae	<i>Hirsutella liberiana</i> Mains (1949) = <i>Tilachlidium brachiatum</i> (Batsch) Petch
37	<i>Torrubiella lloydii</i> (Mains) Rossman	<i>Cordyceps</i>	
38	<i>Torrubiella longissima</i> Kobayasi & Shimizu	Araneae	
39	<i>Torrubiella luteorostrata</i> Zimm.	Diaspididae	<i>Paecilomyces cinnamomeus</i> (Petch) Samson & W. Gams
40	<i>Torrubiella mammillata</i> Kobayasi & Shimizu	Araneae	
41	<i>Torrubiella minuta</i> Kobayasi & Shimizu	Araneae	
	<i>Torrubiella minutissima</i> Lagarde	<i>Cordyceps</i>	
42	<i>Torrubiella minutissima</i> Kobayasi & Shimizu	Araneae	
43	<i>Torrubiella miyagiana</i> Kobayasi & Shimizu	Araneae	
44	<i>Torrubiella neofusiformis</i> Kobayasi & Shimizu	Thomisidae	
45	<i>Torrubiella oblonga</i> Kobayasi & Shimizu	Araneae	
	<i>T. ochracea</i> Pat. = <i>Cordyceps tuberculata</i>	moth	
46	<i>Torrubiella ooniansis</i> Kobayasi & Shimizu	Araneae	
47	<i>Torrubiella pallida</i> Kobayasi & Shimizu	Araneae	
48	<i>Torrubiella paxillata</i> Petch	Cicadellidae	<i>Pleurodesmospora coccorum</i> (Petch) Samson, W. Gams & H.C. Evans
49	<i>Torrubiella petchii</i> Hywel-Jones	larva of <i>Coccus</i>	<i>Hirsutella</i> sp.
50	<i>Torrubiella piperis</i> Bischoff & White	Coccoidea	<i>Lecanicillium</i> sp.

**Table 1 (continued)**

	<i>T. pleiopus</i> = <i>T. leiopus</i>	Araneae	<i>Gibellula pleiopus</i> (Vuill.) Mains
51	<i>Torrubiella pruinosa</i> (Petch) Minter & B.L. Brady	Cicadellidae	<i>Hirsutella versicolor</i> Petch (1932)
52	<i>Torrubiella pseudogibellulae</i> Samson, Reenen & H.C. Evans	Formicidae	
53	<i>Torrubiella psyllae</i> Sawada	psyllid	
	<i>T. pulchra</i> (Mains) Koval = <i>T. arachnophila</i> var. <i>pulchra</i>	Araneae	
54	<i>Torrubiella pulvinata</i> Mains	Opiliones	
55	<i>Torrubiella ratticaudata</i> Humber & Rombach	Araneae	<i>Gibellula clavulifera</i> var. <i>alba</i>
56	<i>Torrubiella rhynchotocola</i> (A. Møller) Kobayasi	Hemiptera	
57	<i>Torrubiella rokkiana</i> Kobayasi & Shimizu	Araneae	
58	<i>Torrubiella rosea</i> Kobayasi & Shimizu	Araneae	
59	<i>Torrubiella rostrata</i> (Henn.) Henn.	Lepidoptera	
60	<i>Torrubiella rubra</i> Pat. & Lagerh.	Coccoidea	
61	<i>Torrubiella ryogamimontana</i> Kobayasi & Shimizu	Araneae	
62	<i>Torrubiella ryukyuensis</i> Kobayasi & Shimizu	Araneae	
63	<i>Torrubiella sericicola</i> Höhn.	Lepidoptera cocoon	
64	<i>Torrubiella siamensis</i> Hywel-Jones	Coccoidea	<i>Hirsutella</i> sp.
65	<i>Torrubiella sphaerospora</i> Samson, Reenen & H.C. Evans	Coccidae	
66	<i>Torrubiella sublintea</i> Petch	Coccoidea	
67	<i>Torrubiella superficialis</i> Kobayasi & Shimizu	Coccidae	
68	<i>Torrubiella tenuis</i> Petch	Diaspididae	
69	<i>Torrubiella tomentosa</i> Pat.	Diaspididae	
70	<i>Torrubiella tomentosa</i> var. <i>citrina</i> Pat.	?diaspidid	
71	<i>Torrubiella tomentosa</i> var. <i>tomentosa</i> Pat.	?diaspidid	
72	<i>Torrubiella truncata</i> (Petch) K.T. Hodge	Cicadellidae	<i>Hirsutella floccosa</i> Speare (1920)
73	<i>Torrubiella wallacei</i> H.C. Evans	larval Lepidoptera	<i>Simplicillium wallacei</i> H.C. Evans

*Torrubiella*, focusing on the spider pathogens. There have been no modern reviews of the genus.

*Torrubiella* has been hypothesized to be closely related to *Cordyceps* because of morphological and ecological similarities to the genus (Petch, 1923; Mains, 1949; Kobayasi, 1982). Petch (1923) discussed the validity of the genus *Torrubiella*, because of intermediate species that had characteristics of both *Cordyceps* and *Torrubiella*, but retained the genus for species which produce perithecia astromatically.

## ANAMORPH TAXONOMY

There are 8 *anamorph* genera known to be associated with *Torrubiella*. These include: *Gibellula*, *Granulomanus*, *Akanthomyces*, *Paecilomyces*, *Hirsutella*, *Pseudogibellula*, *Simplicillium*(=*Verticillium*) and *Lecanicillium* (=*Verticillium*). *Gibellula*, *Granulomanus*, and *Pseudogibellula* are exclusively linked to *Torrubiella*; the remaining are also potential or documented anamorphs of *Cordyceps*. Variation in anamorph structures has presumably evolved in response to selection pressures toward more effective dispersal and infection (Hodge, 2003).

### ***Akanthomyces* Lebert (Z. Wiss. Zoo. 9:4447, 1858)**

Colonies slow-growing, typically white to cream, becoming setose with synnemata. Conidiomata synnematous; terete, usually whit to cream, sometimes darkened toward base, bearing a hymenium-like palisade of phialidic conidiogenous cells over their entire surface, or over a fertile region surmounting a short stipe. Mononematous conidiogenous cells sometimes produced sparsely in culture, and when present, longer and narrower than those found on conidomata. Synnematous conidogenous cells subglobose to

ellipsoid to conical with a short, narrow neck or without a neck, sometimes verruculose. Conidia single-celled, hyaline, shape variable among species (ellipsoid to clavate to cylindrical), produced in dry chains (Hodge, 2003).

Most species of *Akanthomyces* are associated with *Torrubiella* and are pathogens of spiders; however species on other hosts may be linked to *Torrubiella*. Besides its association with *Torrubiella*, *Akanthomyces* has been observed as an anamorph of *Cordyceps* species, where it exhibits a much broader host range.

***Gibellula* Cavara (*Atti Ist. Bot. Pavia* 2:347, 1894)**

Colonies very slow growing on standard media, white to pinkish tan. Sporulation typically synnematosus but sometimes mononematous. Conidiophores typically arising at right angles from the surface of the synnema, *Aspergillus*-like, consisting of a simple verruculose stipe bearing an inflated apical vesicle from which a series of cylindrical or inflated metulae arise, each metula bearing one or a few cylindrical to flask-shaped conidiogenous cells. Conidiogenous cells flask-shaped phialides which taper abruptly at the apex and produce conidia in dry chains. Conidia single-celled, hyaline, smooth-walled, and ellipsoid to fusiform. A *Granulomanus* synanamorph is frequently present (Hodge, 2003).

*Gibellula* spp. are pathogens of spiders and are not known to be associated with any other teleomorphic genera. The function of synnematal production may be more than elevation of conidia for aerial dispersal, it may also augment sporulation capacity, providing a greater surface for production, particularly on small spiders (Evans and Samson, 1987). The *Gibellula* morphology is unique among the other Clavicipitalean *anamorph* genera and is hypothesized to be monophyletic.

***Granulomanus* de Hoog and Samson in de Hoog (*Persoonia* 10:70, 1978)**

Cultural characters unknown. Sporulation typically mononematous but sometimes synnematosus. Conidiogenous cells typically arising from the host surface, cylindrical, polyblastic, developing many

short necks or denticles, each of which produces a single, dry conidium. These conidiogenous cells sometimes arise on *Aspergillus*-like heads typical of the *Gibellula* synanamorph. Conidia single-celled, hyaline, smooth-walled, and narrow ellipsoid to fusoid. A *Gibellula* synanamorph is usually present (Hodge, 2003).

It has been hypothesized that production of *Granulomanus*, in conjunction with *Gibellula* may be for transmission to spiders coming in direct contact with the cadaver, rather than for aerial dispersal (Evans and Samson, 1987).

***Hirsutella* Pat. (Rev. Mycol. 14:67, 1892)**

Colonies slow- to fast-growing, white to tan to dark brown. Sporulation synnematosus in some species with known or suspected *Cordyceps* anamorphs, strictly mononematous in others. Conidiogenous cells phialides, cylindrical to flask-shaped, tapering abruptly into one or several slender necks, on which one or a few conidia accumulate in a persistent subglobose slime drop. In some species the slime drop darkens with age; in others, it is so scanty that it appears as a roughening of the conidial wall. Conidia one- or two-celled, mostly either bluntly fusiform or subglobose, hyaline, smooth-walled, or appearing rough due to irregular distribution of the slime. Insecticolous (Hodge, 2003).

Six species of *Torrubiella* are documented with a *Hirsutella* anamorph: *T. hirsutellae* (Petch, 1937), *T. iriomoteana* (Hywel-Jones, 1995), *T. petchii* (Hywel-Jones, 1997), *T. pruinosa* (Hywel-Jones, 1997; Petch, 1932), *T. siamensis* (Hywel-Jones, 1995), *T. truncata* (Petch) K.T. Hodge (Hodge, 1998). Species of *Cordyceps* have also been connected to *Hirsutella* anamorphs. Previous molecular studies have shown that *Hirsutella* is restricted in its phylogenetic distribution (Hodge, 1998; Sung, 2005) and that some anamorphs previously described as *Hirsutella* are more appropriately described as *Simplicillium* (Sung, 2005).

***Lecanicillium* W. Gams and Zare (*Nova Hedwigia* 72:50, 2001)**

Colonies fast-growing, white to cream, frequently forming octahedral crystals in the medium. Conidiophores little differentiated from the subtending hyphae, commonly arising from aerial hyphae, initially erect with one or two whorls of phialides, becoming prostrate and bearing large numbers of phialide whorls or single phialides. Phialides aculeate, with conidia accumulating at the tips in bundles, stacks, or chains. Short, flask-shaped, rapidly collapsing phialides which bear single conidia are present in some isolates. Conidia single-celled, hyaline, ellipsoid to falcate. Lacking dictyochlamyospores (Hodge, 2003).

*Lecanicillium* is the known anamorph of three species of *Torrubiella* (*Torrubiella alba*, *Torrubiella confragosa* and *Torrubiella piperis*), as well as the anamorph of the type species of *Cordyceps*.

***Paecilomyces* Bainier, sect. *Isariodea* Samson (*Stud. Mycol.* 6:31, 1974)**

Colonies medium- or fast-growing, usually appearing fluffy or powdery. Conidiophores erect, simple or with complex whorled branching structure. Conidiogenous cells short flask-shaped phialides tapering to a short, narrow neck. Conidia one-celled, hyaline, dry, adhering in long chains (Hodge, 2003).

*Paecilomyces* is a morphologically, ecologically and phylogenetically diverse genus. Previous molecular studies have shown the genus to be a polyphyletic (Sung, 2005; Luangsa-Ard *et al.*, 2005; Luangsa-ard *et al.*, 2004; Obornik *et al.*, 2001). *Paecilomyces* spp. can be found on a wide range of hosts (Samson, 1974).

***Pseudogibbellula* Samson and Evans (*Acta Bot. Neerl.* 22:524, 1973)**

With pale, terete synnemata arising from the body of an arthropod host. Conidiophores produced on the synnemata and on the host body, verruculose, terminating in a small subglobose, *Aspergillus*-like vesicle. The cylindrical conidiogenous cells arise from one or two series of subglobose branches arising from the vesicle. Conidia produced singly from multiple loci surrounding the upper

part of the conidiogenous cell. Conidia one-celled, ellipsoid to apiculate, dry (Hodge, 2003).

*Pseudogibbellula* is a monotypic genus. The type species is *Pseudogibbellula formicarum*; its known teleomorph is *Torrubiella pseudogibbellula*. This species occurs on ants. *Pseudogibbellula* superficially resembles *Gibbellula*, and was originally described in the genus, but differs in conidial ontogeny (Samson and Evans, 1973).

***Simplicillium* W. Gams and Zare (Nova Hedwigia 73: 38, 2001)**

Conidiophores prostrate and little-differentiated. Conidiogenous cells phialides, arising singly from aerial hyphae, aculeate with a narrow tip. Conidia adhering in globose slimy heads or imbricate chains. Occurring mainly on fungi and insects (Hodge, 2003).

*Simplicillium* is the known anamorph of one species of *Torrubiella* (*T. wallacei*) and *Simplicillium lanosoniveum* produces a *Torrubiella* spp. in culture (Zare and Gams, 2001).

**SYSTEMATICS OF THE GENUS *TORRUBIELLA***

*Torrubiella* has been hypothesized to be closely related to *Cordyceps* because of similarities in perithecia, ascus and ascospore morphology, as well as its occurrence on arthropods (Petch, 1923; Mains, 1949; Kobayasi, 1982).

*Cordyceps* is a morphologically and ecologically diverse genus that produces perithecia immersed (typically) on a stipitate stroma. Some species of *Cordyceps* (eg. *C. tuberculata*) produce superficial perithecia on a stroma; these have been hypothesized to be closely related to *Torrubiella* and possibly represent an intermediate morphology (Mains, 1958). Previous phylogenetic studies have

shown that *Torrubiella* is not monophyletic and integrates at multiple points within *Cordyceps* (Sung, 2005; Artjariyasripong *et al.*, 2001), but sampling of *Torrubiella* spp. was limited. Using a multi-gene analysis, Sung (2005) demonstrated that there are three main clades in the Clavicipitaceae, which do not collectively form a monophyletic group. Based on these results, the Clavicipitaceae was divided into separate families corresponding to the three clades (Clavicipitaceae *s.s.*, Cordycipitaceae and Ophiocordycipitaceae), and *Cordyceps* was reclassified into *Cordyceps s.s.*, *Elaphocordyceps*, *Metacordyceps* and *Ophiocordyceps* (Sung, 2005). These studies did not sample a large number of *Torrubiella* species and did not attempt to address the phylogenetic diversity of the genus. Thus, the phylogenetic structure of the genus *Torrubiella* and its anamorphs remains unclear and species of genus have not been integrated into the new classification. In this study we increased the sampling of *Torrubiella* spp. and associated *anamorph* taxa for a more robust multi-gene phylogeny in order to address our questions. The main objectives of this study were to 1) test the monophyly of *Torrubiella*, 2) investigate the relationship of *Torrubiella* with *Cordyceps* and other genera of the Clavicipitaceae, 3) determine the phylogenetic distribution and taxonomic utility of *anamorph* genera and 4) to investigate the phylogenetic distribution and taxonomic utility of the host affiliation.

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## **Chapter 2**

### **SYSTEMATICS AND EVOLUTION OF THE GENUS *TORRUBIELLA***

**(Clavicipitaceae; Hypocreales; Ascomycota)**

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## ABSTRACT

*Torrubiella* is a genus of entomopathogenic fungi that comprises pathogens of spiders and scale insects. Based on morphology of perithecia, asci and ascospores, it is classified in Clavicipitaceae as a close relative of *Cordyceps*. *Torrubiella* is distinguished from *Cordyceps* morphologically by superficial perithecia and the absence of a stroma. Previous molecular phylogenetic studies of Clavicipitaceae revealed that the family comprises three well-supported clades, each of which contain species of *Cordyceps*. To test and refine evolutionary hypotheses regarding the placement of *Torrubiella* in Clavicipitaceae and its relationship to *Cordyceps*, phylogenetic analyses of the Clavicipitaceae with an emphasis on *Torrubiella* were conducted. Weighted parsimony analyses were conducted on sequence data from five nuclear loci including: nuclear small subunit ribosomal DNA (nrSSU), nuclear large subunit ribosomal DNA (nrLSU), the largest (RPB1) and second largest (RPB2) subunits of RNA polymerase II, and translation elongation factor 1 $\alpha$  (tef). Taxon sampling included 33 isolates from seventeen teleomorphic species and sixteen representatives of the anamorph genera *Akanthomyces*, *Gibellula*, *Paecilomyces*, and *Verticillium*, which are known to be associated with *Torrubiella*. The monophyly of *Torrubiella* was rejected by these analyses, with species of the genus present in each of the three clades of clavicipitaceous fungi and were often intermixed among *Cordyceps* species. The morphological characters traditionally used to define the genus are therefore not phylogenetically informative. Two new genera *Petchea* and *Armaphthora* are proposed to accommodate two separate lineages of

torrubiellioid fungi in the Clavicipitaceae *s.s.* In addition, two species are reclassified in *Cordyceps* and three are reclassified in *Ophiocordyceps* based on the phylogeny. The remaining residual species of *Torrubiella* are retained in the genus and await future study. Phylogenetic importance of *anamorph* genera and host affiliation are discussed.

## INTRODUCTION

The genus *Torrubiella* is a member of the Clavicipitaceae (Ascomycota: Hypocreales), a family of perithecial ascomycetes, well-known for their obligate symbioses with animals, plants, and other fungi, as well as for their role in human history (White *et al.*, 2003). Species of *Torrubiella*, like other members of the Clavicipitaceae *sensu lato*, possess cylindrical asci, thickened ascus apices, and filiform ascospores, which in many species disarticulate into partspores (Kobayasi, 1941, Rogerson, 1970; Spatafora and Blackwell, 1993). The genus was erected in 1885 by Boudier and since then, about 80 names have been added, representing about 70 species (Petch, 1923; Mains, 1949; Kobayasi and Shimizu, 1982; Kobayasi, 1982; O'Donnell, Common and Imshaug, 1977; Samson, Reenen-Hekstra van and Evans, 1989; Hywel-Jones, 1993, 1995, 1997b; Zare, R., and W. Gams, 2001; Bischoff and White Jr., 2004). Petch (1923) presents detailed species accounts for the pathogens of scale insects. Kobayasi and Shimizu (1982) and Kobayasi (1982) reviewed 59 species of *Torrubiella*, focusing on the spider pathogens. There have been no modern reviews of the genus.

*Torrubiella* has traditionally been defined as an entomopathogen that produces superficial perithecia on a loose mat of hyphae (subiculum), but some species produce perithecia on a non-stipitate stroma. The genus was established to accommodate species similar to *Cordyceps* that lacked a stipitate stroma. The greatest diversity of *Torrubiella* is found in tropical to sub-tropical, humid, undisturbed forests.

Eight *anamorph* genera have been linked to *Torrubiella* spp. These include: *Gibellula*, *Granulomanus*, *Akanthomyces*, *Paecilomyces*, *Hirsutella*, *Pseudogibellula*, *Lecanicillium* (= *Verticillium*) and *Simplicillium* (= *Verticillium*). The *anamorph* genera *Gibellula*, *Granulomanus*, and *Pseudogibellula* are exclusively linked to *Torrubiella*; the remaining are also potential or confirmed anamorphs of *Cordyceps*. The taxonomic utility of anamorphs varies according to genus (Hodge 2003). Some *anamorph* genera (e.g., *Aschersonia*, *Beauveria*, *Hirsutella*, *Hymenostilbe* and *Metarhizium*) are restricted in their phylogenetic distribution and phylogenetically informative in characterizing *Cordyceps* species (Sung, 2005). In contrast, other anamorphs such as *Paecilomyces* and *Verticillium* are morphologically and phylogenetically diverse and previous molecular studies have shown them to be a polyphyletic (Sung *et al.*, 2001; Luangsa-Ard *et al.*, 2005; Luangsa-ard *et al.*, 2004; Obornik *et al.*, 2001).

*Torrubiella* spp. infect a wide range of arthropods, but are primarily pathogens of spiders (Arachnida) and scale insects (Hemiptera). Most species are restricted to a particular host order or family, but hosts are often difficult to identify to the genus or species level, so the degree to which these fungi are host

specific is poorly known. In addition, the host specificity of the *anamorph* genera varies from narrow (eg. *Gibellula* is restricted to spiders) to broad (eg. *Paecilomyces*, *Akanthomyces* on at least six orders of Athropoda). *Torrubiella* is confirmed to parasitize two families of scale insects, the soft scales (Coccidae) and the armored scales (Diaspididae). Many species accounts though, specify only “scale insect” or the author had a very broad interpretation of ‘coccid’, which could also include Aleyrodidae (white flies) (Evans and Hywel-Jones, 1997). In addition, scale insects exhibit extreme sexual dimorphism. Females are stationary, wingless, legless, eyeless, have a complete fusion of abdomen, head and thorax, and are long-lived; where as males have ‘normal’ insect bodies, are winged, motile, ephemeral and have no mouth parts, as they do not feed as adults (Ben-Dov, 1990). Little reference is made of which life stage or sex of scale insects is parasitized by *Torrubiella*, but based on host life histories it is assumed that the adult females are the primary hosts, as they are stationary and gregarious (Evans and Hywel-Jones, 1997).

Spiders (Aranae) are the seventh largest order amongst all living organisms and represent the other major group of hosts of *Torrubiella*. Surprisingly, few teleomorphic taxa of the Clavicipitaceae parasitize them outside of *Torrubiella*. For example, less than 10 species of *Cordyceps* are reported from spider hosts (Kobayasi, 1941; Mains, 1954). Little is known of spider cuticular biochemistry in comparison to insects, though it is generally considered to be similar. Spiders, like insects, possess an epi- exo- and endocuticle, however spiders have an additional layer, the mesocuticle, which is excreted prior to the

endocuticle. The endo- and mesocuticle do not differ structurally, but the mesocuticle is thought to be a more sclerotized form of cuticle and differs in reaction to certain dyes (Foelix, 1996). The hard exocuticle is absent in the abdominal region of spiders, which is thought to be the site of infection (Foelix, 1996; Evans and Samson, 1987). The major hosts of *Torrubiella* – scales and spiders – are thus unique compared to *Cordyceps* and while the phylogenetic distribution of host affiliation for *Torrubiella* is not known, phylogenetic host affiliation studies in *Cordyceps* suggest little correlation (Sung, 2005; Nikoh *et al.*, 2000).

*Torrubiella* has been hypothesized to be closely related to *Cordyceps* because of similarities in perithecia, ascus and ascospore morphology, as well as its occurrence on arthropods (Petch, 1923; Mains, 1949; Kobayasi, 1982). *Cordyceps* is a morphologically and ecologically diverse genus that produces superficial to immersed perithecia (according to species) on a stipitate stroma. Some species of *Cordyceps* (e.g., *C. tuberculata*) that produce superficial perithecia on a stroma have been hypothesized to be closely related to *Torrubiella* and possibly represent an intermediate morphology (Mains, 1958). Previous phylogenetic studies have shown that *Torrubiella* is not monophyletic and integrates at multiple points within *Cordyceps* (Artjariyasripong *et al.*, 2001), but sampling of *Torrubiella* species was limited. Multi-gene analysis have demonstrated that there are three main clades in the Clavicipitaceae which do not collectively form a monophyletic group (Sung *et al.*, 2001, 2004; Sung 2005; Casltebury *et al.*, 2004). Based on these results, the Clavicipitaceae was divided

into separate families corresponding to the three clades (Clavicipitaceae *s.s.*, Cordycipitaceae and Ophiocordycipitaceae), and *Cordyceps* was reclassified into *Cordyceps s.s.*, *Elaphocordyceps*, *Metacordyceps* and *Ophiocordyceps* (Sung, 2005). These studies did not sample a large number of *Torrubiella* species and did not attempt to address the phylogenetic diversity of the genus. Thus, the phylogenetic structure of the genus *Torrubiella* and its anamorphs remain unclear and species of genus have not been integrated into the new classification. In this study, we increased the sampling of *Torrubiella spp.* and associated *anamorph* taxa for a more robust multi-gene phylogeny to test the monophyly of *Torrubiella* and its relationship with *Cordyceps* and other genera of the Clavicipitaceae. In addition, the phylogenetic distribution and taxonomic utility of the host affiliation are explored.

## **MATERIALS AND METHODS**

### **Taxon Sampling**

Thirty-three isolates of *Torrubiella* and associated anamorphs were sampled including five isolates from a previous study (Sung, 2005). These taxa were appended to a 90 taxa dataset from the Hypocreales, which included 74 sexual and asexual taxa from the Clavicipitaceae (Sung, 2005). Voucher and host information for each species is provided in Table 2. Based on previous phylogenetic analyses, *Glomerella cingulata* (Glomerellaceae) and *Verticillium dahliae* (Phyllachoraceae) were used as outgroups (Castlebury *et al.*, 2004; Spatafora *et al.*, 1998).

**Table 2** - The list of cultures and specimens used in this study

Taxon	Specimen voucher	Host/Substrate	GeneBank accession number <sup>2</sup>				
			<i>nrSSU</i>	<i>nrLSU</i>	<i>EF-1<math>\alpha</math></i>	<i>nrRPB1</i>	<i>nrRPB2</i>
<i>Akanthomyces arachnophilus</i>	NHJ 10469	Spider (Arachnida)	*	*	*	*	
<i>Akanthomyces cinereus</i>	NHJ 3510	Spider (Arachnida)	*		*	*	*
<i>Akanthomyces novoguineensis</i>	NHJ 4314	Spider (Arachnida)	*		*	*	*
<i>Akanthomyces novoguineensis</i>	NHJ 13117	Spider (Arachnida)	*		*	*	*
<i>Akanthomyces novoguineensis</i>	NHJ 13161	Spider (Arachnida)	*		*	*	
<i>Akanthomyces novoguineensis</i>	NHJ 11923	Spider (Arachnida)	*	*	*	*	*
<i>Akanthomyces</i> sp.	NHJ 3582	on pentatomid	*	*	*	*	*
<i>Akanthomyces</i> sp.	NHJ 3497	on pentatomid	*	*	*		
<i>Aphysiostroma stercorearium</i>	ATCC 62321	on cow dung	AF543769	AF543792	AF543782	AY489633	GS
<i>Aschersonia badia</i>		Scale insect (Hemiptera)	GS	GS	GS	GS	GS
<i>Aschersonia placenta</i>		Scale insect (Hemiptera)	GS	GS	GS	GS	GS
<i>Aschersonia placenta</i>		Scale insect (Hemiptera)	GS	GS	GS	GS	GS
<i>Balansia henningsiana</i>	GAM 16112	<i>Panicum</i> sp. (Poaceae)	AY545723	AY545727	AY489610	AY489643	GS
<i>Balansia pilulaeformis</i>	AEG 97-2	Poaceae	AF543764	AF543788	GS	GS	GS
<i>Beauveria caledonica</i>	ARSEF 2567		AF339570	AF339520	GS	GS	
<i>Bionectria</i> cf. <i>aureofulva</i>	GJS 71-328	Poaceae	GS	GS	GS		GS
<i>Bionectria ochroleuca</i>	CBS 114056	on bark	AY489684	AY489176	AY489611		GS
<i>Claviceps fusiformis</i>	ATCC 26019	Poaceae	GS	GS	GS	GS	
<i>Claviceps paspali</i>	ATCC 13892	Poaceae	GS	GS	GS	GS	GS
<i>Claviceps purpurea</i>	GAM 12885	<i>Dactylis glomerata</i> (Poaceae)	AF543765	AF543789	AF543778	AY489648	GS
<i>Claviceps purpurea</i>		Poaceae	GS	GS	GS	GS	GS
<i>Cordyceps cardinalis</i>	OSC93609	Larva (Lepidoptera)	AY184973	AY184962	GS	GS	GS
<i>Cordyceps cardinalis</i>	OSC93610	Larva (Lepidoptera)	AY184974	AY184963	GS	GS	GS
<i>Cordyceps gunnii</i>	OSC 76404	Larva (Lepidoptera)	AF339572	AF339522	AY489616	AY489650	GS
<i>Cordyceps irangiensis</i>	NHJ 12572	Ant (Hymenoptera)	GS	GS	GS	GS	GS
<i>Cordyceps irangiensis</i>	NHJ 12575	Ant (Hymenoptera)	GS	GS	GS	GS	GS
<i>Cordyceps militaris</i>	OSC 93623	moth (Lepidoptera)	AY184977	AY184966	GS	GS	AY545731
<i>Cordyceps nutans</i>	OSC 110994	Stink bug (Hemiptera)	GS	GS	GS	GS	

**Table 2 (continued)**

<i>Cordyceps scarabaeicola</i>	EFCC 5689	Scarab (Coleoptera)	AF339574	AF339524	GS	GS	GS
<i>Cordyceps sphecocephala</i>	OSC 110998	Bee (Hymenoptera)	GS	GS	GS	GS	GS
<i>Cordyceps subsessilis</i>	OSC 111001	Larva (Coleoptera)	GS	GS	GS	GS	GS
<i>Cordyceps tuberculata</i>	OSC 111002	Moth (Lepidoptera)	GS	GS	GS	GS	GS
<i>Cosmospora coccinea</i>	CBS 114050	<i>Inonotus nodulosus</i> (Hymenomycetes)	AY489702	AY489734	AY489629	AY489667	GS
<i>Elaphocordyceps capitata</i>	OSC 71233	<i>Elaphomyces</i> sp. (Euscomycetes)	AY489689	AY489721	AY489615	AY489649	GS
<i>Elaphocordyceps fracta</i>	OSC 110990	<i>Elaphomyces</i> sp. (Euscomycetes)	GS	GS	GS	GS	GS
<i>Elaphocordyceps japonica</i>	OSC 110991	<i>Elaphomyces</i> sp. (Euscomycetes)	GS	GS	GS	GS	GS
<i>Elaphocordyceps ophioglossoides</i>	OSC 106405	<i>Elaphomyces</i> sp. (Euscomycetes)	AY489691	AY489723	AY489618	AY489652	GS
<i>Engyodontium aranearum</i>	CBS 309.85	Spider (Arachnida)	AF339576	AF339526	GS	GS	GS
<i>Epichloë typhina</i>	ATCC 56429	<i>Festuca rubra</i> (Poaceae)	U32405	U17396	AF543777	AY489653	GS
<i>Gibellula cf. alba</i>	NHJ 11679	Spider (Arachnida)			*	*	
<i>Gibellula longispora</i>	NHJ 12014	Spider (Arachnida)	*		*	*	*
<i>Gibellula pulchra</i>	NHJ 10808	Spider (Arachnida)	*	*	*	*	*
<i>Gibellula pulchra</i>	AB 327399	Spider (Arachnida)	AB 327399	AB 327391			
<i>Gibellula sp.</i>	AB 237661	Spider (Arachnida)	AB 237661				
<i>Gibellula sp. 04</i>	NHJ 10788	Spider (Arachnida)	*	*	*	*	*
<i>Gibellula sp. 04</i>	NHJ 13158	Spider (Arachnida)	*	*	*	*	*
<i>Gibellula sp.10</i>	NHJ 5401	Spider (Arachnida)	*			*	*
<i>Glomerella cingulata</i>	CBS114054	<i>Fragaria</i> sp.	AF543762	AF543786	AF543773	AY489659	GS
<i>Glomerella cingulata</i>	FAU 513	<i>Fragaria</i> sp.	U48427	U48428	AF543772	GS	GS
<i>Haptocillium balanoides</i>	CBS 250.82	Nematoda	AF339588	AF339539	GS	GS	GS
<i>Haptocillium sinense</i>	CBS 567.95	Nematoda	AF339594	AF339545	GS	GS	GS
<i>Haptocillium zeosporum</i>	CBS 335.80	Nematoda	AF339589	AF339540	GS	GS	GS
<i>Hirsutella</i> sp.	NHJ 12527	Coleoptera	GS	GS	GS	GS	GS
<i>Hirsutella</i> sp.	NHJ 12525	Coleoptera	GS	GS	GS	GS	GS
<i>Hydropisphaera erubescens</i>	ATCC 36093	Plant	AY545722	AY545726	GS	GS	AY545732
<i>Hydropisphaera peziza</i>	CBS 102038	on bark	AY489698	AY489730	AY489625	AY489661	GS
<i>Hymenostilbe aurantiaca</i>	NHJ 12574	Ant (Hymenoptera)	GS	GS	GS	GS	GS
<i>Hypocrea lutea</i>	ATCC 208838	on decorticated conifer wood	AF543768	AF543791	AF543781	AY489662	GS

**Table 2 (continued)**

<i>Hypocrella schizostachyi</i>	NHJ 12605	Scale insect (Hemiptera)	GS	GS	GS	GS	GS
<i>Hypocrella</i> sp.	GJS 89104	Scale insect (Hemiptera)	GS	GS	GS	GS	GS
<i>Hypomyces polyporinus</i>	ATCC 76479	<i>Trametes versicolor</i> (Hymenomycetes)	AF543771	AF543793	AF543784	AY489663	
<i>Isaria farinosa</i>	OSC 111005	Pupa (Lepidoptera)	GS	GS	GS	GS	
<i>Isaria farinosa</i>	OSC 111006	Pupa (Lepidoptera)	GS	GS	GS	GS	
<i>Isaria tenuipes</i>	OSC 111007	Larva (Lepidoptera)	GS	GS	GS	GS	GS
<i>Lecanicillium antillanum</i>	CBS 350.85	Agaric mushroom (Hymenomycetes)	AF339585	AF339536	GS	GS	GS
<i>Lecanicillium psalliotae</i>	CBS 532.81	forest soil	AF339609	AF339560	GS	GS	GS
<i>Lecanicillium psalliotae</i>	CBS 101270	Soil	GS	GS	GS	GS	GS
<i>Leuconectria clusiae</i>	ATCC 22228	Soil	AY489700	AY489732	AY489627	AY489664	GS
<i>Mariannaea pruinosa</i>	ARSEF 5413	Pupa (Lepidoptera)	AY184979	AY184968	GS	GS	GS
<i>Metacordyceps chlamydosporia</i>	CBS 101244	mollusc eggs (Diplopoda)	GS	GS	GS	GS	GS
<i>Metacordyceps taii</i>	ARSEF 5714	Larva (Coleoptera)	AF543763	AF543787	AF543775	GS	GS
<i>Metarhizium album</i>	ARSEF 2082	Hemiptera	GS	GS	GS	GS	GS
<i>Metarhizium anisopliae</i> var. <i>majus</i>	ARSEF 3145	<i>Oryctes rhinoceros</i> (Coleoptera)	AF339579	AF339530	AF543774	GS	GS
<i>Metarhizium flavoviride</i> var. <i>minus</i>	ARSEF 2037	<i>Nilaparvata lugens</i> (Homoptera)	AF339580	AF339531	GS	GS	GS
<i>Myriogenospora atramentosa</i>	AEG 96-32	<i>Andropogon virginicus</i> (Poaceae)	AY489701	AY489733	AY489628	AY489665	GS
<i>Nectria cinnabarina</i>	CBS 114055	<i>Betula</i> sp. (Betulacaceae)	U32412	U00748	AF543785	AY489666	GS
cf. <i>Nectria</i> sp.	CBS 478.75	?	U47842	U17404	GS	GS	GS
<i>Nomuraea atypicola</i>	CBS 744.73	Spider (Arachnida)	GS	GS	GS	GS	
<i>Ophiocordyceps agriota</i>	ARSEF 5692	Larva (Coleoptera)	GS	GS	GS	GS	GS
<i>Ophiocordyceps aphodii</i>	ARSEF 5498	<i>Aphodius hewitti</i> (Coleoptera)	GS	GS	GS		GS
<i>Ophiocordyceps brunneapunctata</i>	NHJ 12565	Coleoptera	GS	GS	GS	GS	GS
<i>Ophiocordyceps</i> cf. <i>acicularis</i>	NHJ 12592	Coleoptera	GS	GS	GS	GS	GS
<i>Ophiocordyceps heteropoda</i>		Nymph of cicada (Hemiptera)	GS	GS	GS	GS	GS
<i>Ophiocordyceps melolonthae</i>	OSC 110993	<i>Phyllophaga</i> sp. (Coleoptera)	GS	GS	GS	GS	
<i>Ophiocordyceps ravenelli</i>	OSC 110995	<i>Phyllophaga</i> sp. (Coleoptera)	GS	GS	GS	GS	GS
<i>Ophiocordyceps stylophora</i>	OSC 110999	Larva (Coleoptera)	GS	GS	GS	GS	GS
<i>Ophiocordyceps unilateralis</i>	NHJ 12523	Ant (Hymenoptera)	GS	GS	GS	GS	GS
<i>Ophiocordyceps variabilis</i>	OSC 111003	Diptera	GS	GS	GS	GS	GS

**Table 2 (continued)**

<i>Ophionectria trichospora</i>	CBS 109876	Liana	AF543766	AF543790	AF543779	AY489669	GS
<i>Paecilomyces lilacinus</i>	CBS 284.36	Soil					
<i>Paecilomyces lilacinus</i>	CBS 431.87	Egg mass of Meloïdogyne					
<i>Pochonia chlamydosporia</i> var. <i>catenulata</i>	CBS 504.66	Soil in mixed forest	AF339593	AF339544	GS	GS	GS
<i>Pochonia gonioides</i>	CBS 891.72	Nematoda	AF339599	AF339550	GS	GS	GS
<i>Pseudonectria rousseliana</i>	CBS 114049	<i>Buxus sempervirens</i> (Buxaceae)	AF543767	U17416	AF543780	AY489670	GS
<i>Rotiferophthora angustispora</i>	CBS 101437	Rotifer (Rotifera)	AF339584	AF339535	AF543776	GS	GS
<i>Roumeguieriella rufula</i>	CBS 346.85	<i>Globodera rostochiensis</i> (Nematoda)	GS	GS	GS	GS	GS
<i>Roumeguieriella rufula</i>	GJS 91-164	<i>Globodera rostochiensis</i> (Nematoda)	GS	GS	GS	GS	GS
<i>Shimizuomyces paradoxa</i>	EFCC 6279	<i>Smilax sieboldii</i> (Smilacaceae)	GS	GS	GS	GS	GS
<i>Shimizuomyces paradoxa</i>	EFCC 6564	<i>Smilax sieboldii</i> (Smilacaceae)	GS	GS	GS	GS	GS
<i>Simplicillium lamellicola</i>	CBS 116.25	<i>Agaricus bisporus</i> (Hymenomycetes)	AF339601	AF339552	GS	GS	GS
<i>Simplicillium lanosoniveum</i>	CBS 101267	<i>Hemileia vastatrix</i> (Urediniomycetes)	AF339603	AF339554	GS	GS	GS
<i>Simplicillium lanosoniveum</i>	CBS 704.86	<i>Hemileia vastatrix</i> (Urediniomycetes)	AF339602	AF339553	GS	GS	GS
<i>Sphaerostilbella berkeleyana</i>	CBS102308	Polypore (Hymenomycetes)	AF543770	U00756	AF543783	GS	GS
<i>Torrubiella confragosa</i>	CBS 101247	<i>Coccus viridis</i> (Hemiptera)	AF339604	AF339555	GS	GS	GS
<i>Torrubiella luteostrata</i>	NHJ 12516	Scale insect (Hemiptera)	*	*			*
<i>Torrubiella luteostrata</i>	NHJ 11343.1	Scale insect (Hemiptera)	*	*		*	
<i>Torrubiella petchii</i>	NHJ 5318	Scale insect (Hemiptera)	*	*	*	*	*
<i>Torrubiella petchii</i>	NHJ 6240	Scale insect (Hemiptera)	*	*	*	*	*
<i>Torrubiella petchii</i>	NHJ 6209	Scale insect (Hemiptera)	*	*	*	*	*
<i>Torrubiella piperis</i>	JB209	Scale insect (Hemiptera)		GS	GS	GS	GS
<i>Torrubiella pruinosa</i>	NHJ 12994	Scale insect (Hemiptera)	*	*	*	*	*
<i>Torrubiella ratticaudata</i>	ARSEF 1915	Spider (Arachnida)	GS	GS	GS	GS	GS
<i>Torrubiella sp.</i>	NHJ 5112.1	Spider (Arachnida)	*	*	*	*	
<i>Torrubiella sp.</i>	NHJ 6709	Spider (Arachnida)	*	*	*	*	*
<i>Torrubiella sp.</i>	NHJ 7859	Spider (Arachnida)	*			*	*
<i>Torrubiella longissima</i>	DJ 29	Spider (Arachnida)	*		*	*	
<i>Torrubiella tenuis</i>	NHJ 345.04	Scale insect (Hemiptera)	*	*	*		*
<i>Torrubiella tenuis</i>	NHJ 6293	Scale insect (Hemiptera)	*	*	*	*	*

**Table 2 (continued)**

<i>Torrubiella tenuis</i>	NHJ 6791	Scale insect (Hemiptera)	*	*	*	*	*
<i>Torrubiella wallacei</i>	CBS 101237	Larva (Lepidoptera)	AY184978	AY184967	GS	GS	GS
<i>Verticillium dahliae</i>	ATCC 16535	<i>Crataegus crus-galli</i> (Rosaceae)	AY489705	AY489737	AY489632	AY489673	GS
<i>Verticillium epiphytum</i>	CBS 384.81	<i>Hemileia vastatrix</i> (Urediniomycetes)	AF339596	AF339547	GS	GS	GS
<i>Verticillium incurvum</i>	CBS 460.88	<i>Ganoderma lipsiense</i> (Hymenomycetes)	AF339600	AF339551	GS	GS	GS
<i>Viridispora diparietispora</i>	CBS 102797	<i>Crataegus crus-galli</i> (Rosaceae)	AY489703	AY489735	AY489630	AY489668	GS

**Table 2** - The list of cultures and specimens used in this study. Bold indicates *Torrubiella* and linked asexual genera.

<sup>1</sup>AEG, A. E. Glenn personal collection; ARSEF, USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; ATCC, American Type Culture

Collections, Manassas, VA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; EFCC, Entomopathogenic Fungal Culture

Collection, Chuncheon, Korea; GAM, Julian H. Miller Mycological Herbarium Athens, GA; FAU, F. A. Uecker personal collection,

GJS, G. J. Samuels personal collection; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR.

<sup>2</sup>GS, sequences obtained from Sung, 2005; \*, sequences obtained in this study

### **DNA isolation, PCR amplification, and Sequencing**

Total genomic DNA was extracted from specimens grown in a malt extract liquid media following the CTAB protocol (Doyle and Doyle, 1990). Five nuclear gene regions were amplified and sequenced in order to create a robust phylogeny of Clavicipitaceae and *Torrubiella*. Gene regions were selected based on a study by Sung (2005). Regions sequenced were from nuclear ribosomal small and large subunit (*nrSSU* and *nrLSU*), elongation factor 1 $\alpha$  (*tef*), and the largest and second largest subunits of RNA polymerase II (*RPB1* and *RPB2*), totaling approximately 5000 base pairs of data.

The PCR amplification of *nrSSU*, *nrLSU*, and *tef* was performed in a BIORAD iCycler thermocycler programmed as follows: 94 °C for 2 min; 4 cycles of 94 °C for 30 sec, 55 °C for 1 min, and 72 °C for 2 min; 35 cycles of 94 °C for 30 sec, 50.5 °C for 1 min, and 72 °C for 2 min; 72 °C for 3 min. The *nrSSU* was amplified with either the primer pairs NS1 and NS4 or NS1 and NS6. All taxa were sequenced using the primers NS1, NS3, SR7 and NS4 (Table 3). The *nrLSU* was amplified and sequenced with the primers LR0R and LR5 (Table 3). The *tef* was amplified and sequenced with the primers 983F and 2218R (Table 3). The PCR amplification of *RPB1* and *RPB2* was performed in a BIORAD iCycler thermocycler programmed as follows: 94 °C for 2 min; 4 cycles of 94 °C for 30 sec, 47 °C for 1 min, and 72 °C for 2 min; 35 cycles of 94 °C for 30 sec, 47 °C for 1 min, and 72 °C for 2 min; 72 °C for 3 min. The *RPB1* was amplified and sequenced using the primers CRPB1A and RPB1CR (Table 3). For the amplification of *RPB2*, primer pairs fRPB2-5F and fRPB2-7cR or RPB2F1 and

Table 3. List of primers used in this study

Genes	Primers	Sequence, 5'- 3'	References
<i>NrSSU</i>	NS1	5'-GTAGTCATATGCTTGTCTC- 3'	White et al. (1990)
	SR7	5'-GTTCAACTACGAGCTTTTAA- 3'	White et al. (1990)
	NS3	5'-GCAAGTCTGGTGCCAGCAGCC- 3'	White et al. (1990)
	NS4	5'-CTTCCGTCAATTCCTTTAAG- 3'	White et al. (1990)
	NS6		
<i>NrLSU</i>	LR0R	5'-GTACCCGCTGAACTTAAGC- 3'	Vilgalys and Sun (1994)
	LR5	5'- ATCCTGAGGGAAACTTC- 3'	Vilgalys and Sun (1994)
<i>EF-1<math>\alpha</math></i>	983F	5'-GCYCCYGGHCAYCGTGAYTTYAT-3'	Castlebury et al. (2004)
	2218R	5'-ATGACACCRACRGCRCRGTGTG-3'	Castlebury et al. (2004)
<i>RPB1</i>	RPB1CR	5'-CCNGCDATNCRTRTRTCCATRTA- 3'	Castlebury et al. (2004)
	CRPB1A	5'-CAYCCWGGYTTYATCAAGAA-3'	Castlebury et al. (2004)
<i>RPB2</i>	fRPB2-5F	5'-GAYGAYMGWGATCAYTTYGG- 3'	Liu et al. (1999)
	RPB2-5F1	5'-TKGCIGTIGGYATCAARCC- 3'	Sung (2005)
	RPB2-5F2	5'-GGGGWGAYCAGAAGAAGGC- 3'	Sung (2005)
	fRPB2-7cR	5'-CCCATRGTGTGYRCCCAT- 3'	Liu et al. (1999)

RPB2R2 were used. Sequencing was performed using the same primers as amplification (Table 3).

All PCR products were purified using GeneClean III purification kits (Qbiogene, Irvine, CA) according to the manufacturer's instructions and eluted in 10 $\mu$ L of TE. Sequencing reactions were conducted on a BIORAD iCycler Thermal Cycler (BIORAD, Hercules, CA) using ABI Big-Dye Ready-reaction kit (Applied Biosystems Inc., Foster City, CA) following manufacturer's instruction.

### Phylogenetic Analyses

Sequences were edited using CodonCode, version 1.4.4 (Dedham, MA).

Edited sequences were manually aligned in BioEdit Sequence Alignment Editor,

version 7.0.5.2 (Hall, 1999). Ambiguously aligned regions of *nrSSU*, *nrLSU*, and *RPB1* were excluded from phylogenetic analyses and gaps were treated as missing data.

Weighted parsimony (WP) analyses were performed using PAUP\* 4.0b10 (Swofford, 2002), using calculated substitution rates for the five gene concatenated data set. The multigene data set was divided into eleven partitions, which were defined as *nrSSU*, *nrLSU* and nine codon positions of the three protein-coding genes (*tef*, *RPB1* and *RPB2*). Data for each partition was subjected to a symmetric step matrix after ambiguously aligned regions were excluded. Character changes were summarized using the “Show character status/full detail/hide excluded characters” option under the “Data” menu in PAUP\* 4.0b10 (Swofford, 2002). The proportional frequency of changes between character states was calculated using the program STMatrix 2.1 (available at <http://www.lutzonilab.net/pages/download.shtml>) and converted into cost of changes using a negative natural logarithm (Felsenstein, 1981; Wheeler, 1990). Heuristic searches were performed using the following options: 100 replicates of random sequence addition, TBR (Tree bisection-reconnection) branch swapping, and MulTrees ON. Nonparametric bootstrap analyses (Felsenstein, 1985) were performed on parsimony informative characters only using the identical settings as the weighted parsimony analyses with 500 replicates, each with five replicates of random sequence addition.

## RESULTS

## Sequence Alignment

The five-gene, 125-taxon dataset included 4947 base pairs of sequence data (*nrSSU*: 1103 bp, *nrLSU*: 964 bp, *tef*: 1029 bp, *RPB1*: 803 bp, *RPB2*: 1052 bp). After ambiguously aligned sites were excluded, the data set included 4437 sites of which 1782 characters were parsimony informative (*nrSSU*: 217 bp, *nrLSU*: 249 bp, *EF-1 $\alpha$* : 1<sup>st</sup>-90 bp, 2<sup>nd</sup>-55 bp, 3<sup>rd</sup>-257 bp, *RPB1*: 1<sup>st</sup>-109 bp, 2<sup>nd</sup>-75 bp, 3<sup>rd</sup>-212 bp, *RPB2*: 1<sup>st</sup>-135 bp, 2<sup>nd</sup>-79 bp, 3<sup>rd</sup>-303 bp).

## Phylogenetic analyses

Weighted parsimony (WP) analyses of the dataset resulted in seven equally parsimonious trees of 33481.86 steps. One of the seven equally parsimonious trees is shown in Figure 1. In this study, nodes were considered strongly supported when associated with bootstrap proportions greater than 70 percent, however all values greater than 50 are shown. Collapsed nodes are denoted with asterisks.

## Phylogenetic relationships of the *Torrubiella*

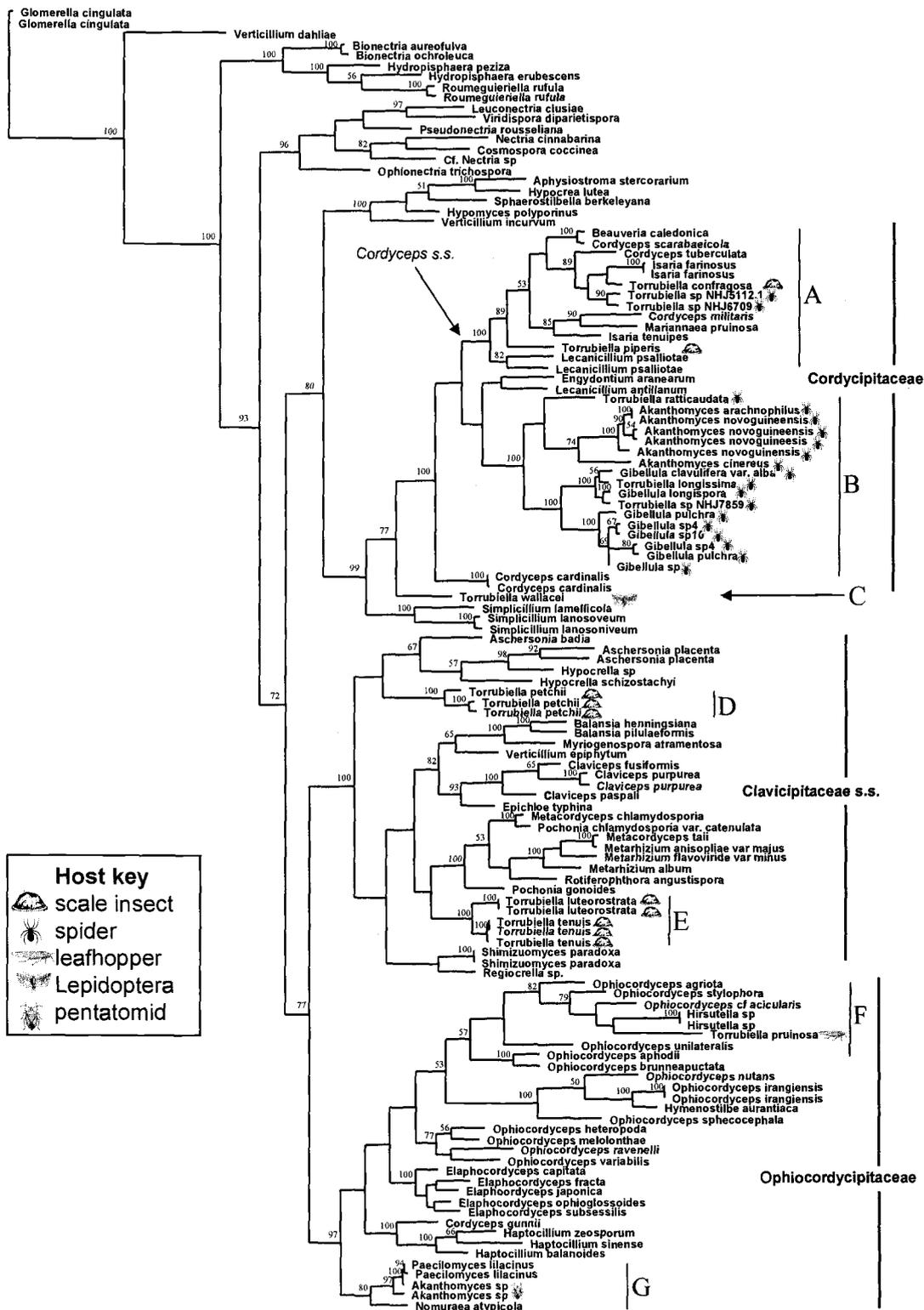
WP analyses of the five-gene data set inferred three well-supported, monophyletic clades of clavicipitaceous fungi (Figure 1). These results are consistent with the findings of Sung (2005) and support the recognition of three separate families of clavicipitaceous fungi, Clavicipitaceae *s.s.*, Cordycipitaceae and Ophiocordycipitaceae (Sung and Spatafora, 2004; Sung, 2005). These clades are statistically well-supported in these analyses by the bootstrap proportions of

the WP analyses (BP) (Cordycipitaceae: BP = 99, Clavicipitaceae s.s.: BP = 100, Ophiocordycipitaceae: BP = 97). Species of *Torrubiella* are found in all three families and are members of clades or lineages designated A - G.

The Cordycipitaceae contains 22 isolates of *Torrubiella* fungi, within two well-supported subclades and the isolated species *T. wallacei*. These are labeled in Figure 1 as: clade A (BP=100), clade B (BP =100), and lineage C, respectively. Clade A contains three species of *Torrubiella* that are closely related to *Cordyceps* species including *C. militaris* and *C. tuberculata*. Clade B is biphyletic, with a well-supported (BP=74) *Akanthomyces* subclade (BP=74) and a well-supported *Gibellula* subclade (BP=100). *Torrubiella wallacei* (C) is an isolated branch and is among the most basal lineages of the Cordycipitaceae.

The Clavicipitaceae s.s. contains two statistically well-supported clades of *Torrubiella* fungi. These are labeled in Figure 1 as clade D (BP=100) and clade E (BP = 100). The internal relationships among the subclades of Clavicipitaceae s.s., including the two *Torrubiella* clades, are not strongly supported in these analyses.

The Ophiocordycipitaceae contains two groups of *Torrubiella* fungi (F and G). Lineage F consists of the single species *T. pruinosa* that is statistically well-supported (BP=82) as a member of a subclade that includes numerous species of *Cordyceps*. Clade G forms a statistically well-supported (BP=80) subclade of *anamorph* genera presumably linked to *Torrubiella*. The relationships



50 changes

**Figure 1** – Phylogenetic relationships among *Torrubiella* spp. and Clavicipitaceae s.l. Results are based on combined data from five genes (i.e. *nrSSU*, *nrLSU*, *EF-1 $\alpha$* , *RPB1* and *RPB2*) from 125 taxa. This phylogeny is one of 7 trees sampled from Weighted Parsimony analyses with a length of 33481.86 steps. Bootstrap proportions were obtained in weighted parsimony (WP) analyses and shown above their corresponding nodes for  $\geq 50\%$ .

within clade G are well-supported and include two isolates of *Paecilomyces lilacinus* on nematodes and two isolates of *Akanthomyces* on pentatomids. The internal relationships among the subclades of the Ophioicordycipitaceae and are not strongly supported in these analyses.

## DISCUSSION

Previous studies have shown that *Torrubiella* is not monophyletic, but lack of sampling made drawing any definitive conclusions about the genus difficult (Sung, 2005; Artjariyasripong *et al.*, 2001). Though substantial progress has been made in clavicipitalean systematics and *Cordyceps s.l.* has been segregated into four genera, the placement of *Torrubiella* remained largely unknown (Sung, 2005). Our results show that species of *Torrubiella* are members of each of the three families of clavicipitaceous fungi, representing multiple derivations of the combined morphology of superficial perithecia produced on a highly reduced stroma or subiculum.

The morphological characters most consistent with the classification of the three families of clavicipitaceous fungi are texture, pigmentation and morphology of the stromata (Sung, 2005). In addition, *anamorph* genera (e.g., *Aschersonia*, *Beauveria*, *Hirsutella*, *Hymenostilbe* and *Metarhizium*) were restricted in their phylogenetic distribution and phylogenetically informative in characterizing *Cordyceps* species (Sung, 2005). Since stromata are typically lacking in *Torrubiella spp.*, these morphological characters, which proved useful in *Cordyceps*, are limited in their utility in systematics of *Torrubiella*. We did,

however, find that some *anamorph* genera (eg. *Gibellula*, *Hirsutella*) were restricted in their phylogenetic distribution and are phylogenetically informative.

### ***Torrubiella* of Cordycipitaceae**

The family Cordycipitaceae is characterized as containing species that produce pallid to brightly colored, superficial to completely immersed perithecia. Species of Cordycipitaceae most typically attack lepidopteran or coleopteran hosts on exposed substrates or shallowly buried in leaf litter (e.g. *C. militaris*, *C. tuberculata*, *C. scarabaeicola*) and rarely occur on host deeply buried in soil or wood (Sung, 2005). Anamorphs in this family include: *Lecanicillium*, *Simplicillium*, *Paecilomyces*, *Akanthomyces*, *Gibellula*, and *Beauveria*. This family contains the type species of *Cordyceps* (*C. militaris*) that parasitizes various Lepidoptera pupae and has a *Lecanicillium* anamorph. *Cordyceps s.s.* was circumscribed to create a monophyletic *Cordyceps* with the remaining species of genus reclassified as *Metacordyceps*, *Elaphocordyceps* or *Ophiocordyceps* according to the phylogeny of the Hypocreales (Sung, 2005). These results support the conclusion that some species of *Torrubiella* (Clade A) as being members of *Cordyceps s.s.* and that they should be re-classified as *Cordyceps*. These species include two scale insect pathogens with a *Lecanicillium* anamorphs (*T. confragosa* and *T. piperis*) and the two unnamed spider pathogens. Preliminary analyses of *Torrubiella alba* Petch, which has also been linked to *Lecanicillium* anamorph (*L. aranearum*), also place it in the Cordycipitaceae (data

not shown). However, this placement was based on incomplete data, thus *T. alba* is not synonymized in *Cordyceps* at this time and awaits further analyses.

Additional *Torrubiella* species and associated *anamorph* genera sampled were members of Cordycipitaceae but placed outside of the *Cordyceps s.s.* clade. The majority of these species form a well-supported biphyletic clade (Clade B) that contains all species of *Torrubiella* on spiders with either a *Gibellula* or *Akanthomyces* anamorph. *Gibellula* is specific to spiders and restricted to this clade with species forming a distinct, well-supported subclade. Likewise, the spider pathogens of *Akanthomyces* form a subclade although *Akanthomyces* on other hosts are distributed throughout the tree (Figure 1). Though morphological species concepts of *Akanthomyces* may vary among mycologists (eg. *A. arachnophilous*, *A. novoguineensis*), the spider associated taxa form a well-supported clade. *Akanthomyces cinereus*, is distinct among the *Akanthomyces* spider pathogens in lacking the production of pigment in culture, a finding consistent with the topology of Figure 1. In this *Gibellula-Akanthomyces* clade, anamorph morphology in conjunction with host affinity is a good predictor of phylogeny.

This clade of spider-associated *Torrubiella* (Clade B) along with *T. wallacei* (lineage C) form a paraphyletic group, which we recognize as *Torrubiella*. We were not able to include material of the type species of *Torrubiella* (*T. aranicida* Boud.), which was originally collected in France on a spider in 1885. Although Boudier did not describe an anamorph in his original description, he later (Boudier, 1887) described an anamorph which grew from a specimen of *T.*

*arancida* placed in an incubating chamber and named it *Isaria cuneispora* Boud., which Petch later suggested it to be reminiscent of *Hirsutella* (1922). The original drawings (Figure 2, Boudier, 1887) depict a simple morphology similar to species of *Lecanicillium* and *Simplicillium* with fusiform-falcate conidia (Zare and Evans, 2001) like those produced by the anamorph of *T. wallacei*. Though we cannot place *T. arancida* at this time, based on anamorph morphology and host affiliation, it is provisionally classified in the Cordycipitaceae, but outside the genus *Cordyceps* s.s. The classification of these fungi awaits further investigation involving definitive *T. arancida* material.



Figure 2 - Drawing of *Isaria cuneispora* by Boudier depicting conidiophores and conidia

As previously shown (Zare and Gams, 2001; Zare *et al.*, 2000; Sung, 2005), the *Simplicillium* anamorph is among the first diverging lineages of the Cordycipitaceae. Species of *Torrubiella* with a *Simplicillium* anamorph place

here (*T. wallacei* = *Simplicillium wallacei* and *Simplicillium lanosoniveum*) and are outside of the clade of the *Gibellula/Akanthomyces* clade and *Cordyceps s.s.* *Simplicillium lanosoniveum* is associated mainly with rust fungi, but has been recorded on various arthropods and has been observed to have a *Torrubiella*-like teleomorph in culture (Zare and Gams, 2001). *Torrubiella wallacei* was originally described from a lepidopteran pupae. Evans noted that the perithecia of teleomorphs with *Simplicillium* anamorphs differ in being thin-walled, delicate and hyaline or lightly pigmented, where as species with *T. confragosa*-type teleomorphs are thick-walled, robust and strongly pigmented (Zare and Gams, 2001). Only two species of *Simplicillium* have been linked to a *Torrubiella* teleomorph (Zare and Gams, 2001).

### ***Torrubiella* of Clavicipitaceae**

The Clavicipitaceae s.s includes grass-associated genera (e.g., *Balansia*, *Claviceps*, and *Epichloë*) in addition to species of *Cordyceps s.l.* (*Metacordyceps*), *Torrubiella* and *Hypocrella*. This family includes arthropod-associated species that are pathogens of scale insects (*Torrubiella*, *Hypocrella*, and *Regiocrella*), Lepidoptera (eg. *Metacordyceps taii*, *Metacordyceps liangshanensis*), and generalists used in biocontrol (eg. *Metarhizium spp.*). Notably, there are no spider pathogens in this family.

*Torrubiella tenuis* and *T. luteorostata* represent a unique, well-supported lineage (Clade E) in the Clavicipitaceae s.s. These species share similar morphological characters (eg. elongated, flask-shaped perithecia and planar

stromata) (Hywel-Jones, 1993, 1997) and are two out of only three species of *Torrubiella* confirmed to parasitize the diaspidid (armored) scale insects (Evans and Prior, 1990). Diaspidids are a derived group of scale insects that are protected by a scale covering produced from wax filaments and anal excretions embedded with the skin of the two preceding molts. *Torrubiella tomentosa* is the other species that is confirmed on armored scales, but was not included here. The anamorph of *T. luteorostata* is *Paecilomyces cinnamomeus*, but no anamorph has been observed in *T. tenuis* (Hywel-Jones, 1993). A new genus, *Armaphthora* (see below) is proposed below to accommodate *T. tenuis* and *T. luteorostrata*.

*Torrubiella petchii* is a pathogen of scale insects found on bamboo. The anamorph has been reported as *Hirsutella*, but the morphology is atypical of *Hirsutella* species of the *Ophiocordyceps* clade (see below). Isolates of this species form a well-supported clade (Clade D) that is distinct from all other generic lineages of the family, including the genera *Hypocrella* and *Regiocrella*, which also produce superficial perithecia on a reduced stroma and parasitize scale insects. *Hypocrella* is a monophyletic genus that produces an *Aschersonia* anamorph. *Aschersonia spp.* produce pycnidia or acervuli, filled with often brightly colored, slimy conidia, on a stroma (Hodge, 2003). However, some species of *Hypocrella* produce a synanamorph that is coelomycetous (Hywel-Jones and Samuels, 1998). *Regiocrella*, a new genus proposed by Chaverri *et al.*, has a *Hypocrella* morphology and ecology (on scale insects and white flies), but produces a pycnidial non-*Aschersonia* anamorph (2006). *Torrubiella petchii* and *Torrubiella* species of clade E are phylogenetically distinct from the other scale

insect pathogens of the Clavicipitaceae *s.s.* Parasitism of scale insects is a prominent ecology in this family, and interestingly, is the only clade that contains clavicipitaceous plant symbionts. A new genus, *Petchea* (see below), is proposed to accommodate *T. petchii*.

### ***Torrubiella* of Ophiocordycipitaceae**

The Ophiocordycipitaceae contains species previously classified as *Cordyceps* that are pathogens of arthropods (e.g., *Ophiocordyceps unilateralis*, *O. sinensis*), as well as species that parasitize truffles (*Elaphocoryceps ophioglossoides*, *E. capitata*). The truffle parasites are part of a monophyletic clade that also includes pathogens of cicada nymphs (Nikoh and Fukatsu, 2000; Sung, 2005) and represent an interkingdom host shift between fungi and animals facilitated by co-occurrence deep in the soil (Nikoh and Fukatsu, 2000).

*Ophiocordyceps* is the only part of the tree where the *Hirsutella s.s.* anamorph is found. It corresponds to the well-supported monophyletic genus, which includes species previously classified as *Cordycepioideus* in addition to *Cordyceps*.

*Hirsutella* has been documented and described as the anamorph in six species of *Torrubiella*: *T. hirsutellae* (Petch, 1937), *T. iriomotiana* (Hywel-Jones, 1995), *T. petchii* (Hywel-Jones, 1997), *T. pruinosa* (Hywel-Jones, 1997; Petch, 1932), *T. siamensis* (Hywel-Jones, 1995), *T. truncata* (Petch) K.T. Hodge (Hodge, 1998).

*Torrubiella pruinosa* (Petch) Minter and Brady is a member of the *Ophiocordyceps* clade (F). It was originally described by Petch in the genus *Calonectria* with a *H. versicolor* Petch anamorph, which is often found

accompanying teleomorphic specimens (Hywel-Jones, 1997c). Where as other species of the clavicipitaceous fungi, including *Torrubiella*, have *filiform*, thin-walled, hyaline ascospores (Kobayasi and Shimizu, 1982) that usually break into partspores, the ascospores of *T. pruinosa* are fusiform, distoseptate and faintly pigmented (Blackwell and Gilbertson, 1984; Hywel-Jones, 1997c). The lack of fit of the genus *Torrubiella* has been previously recognized and the relationship of *T. pruinosa* to the genus *Cordycepioideus* (Hywel-Jones, 1997c, Hodge, 2001), which also has distoseptate, pigmented ascospores and a *Hirsutella* anamorph (Stifler, 1941), has been suggested. Species of *Cordyceps* in this part of the tree also possess fusiform, septate ascospores (e.g., *C. acicularis*, *C. stylophora*, *C. clavulata*) (Mains, 1958). It is anticipated that *Torrubiella truncata* (Petch) K.T. Hodge and *T. hirsutellae* (Petch) A.Y. Rossman, which like *T. pruinosa* were previously classified in *Calonectria* because of ascospore morphology and have *Hirsutella* anamorphs are also part of clade F. These species will be transferred to *Ophiocordyceps* as well.

*Hirsutella* was originally described as synnematus and characterized by basally inflated phialides in a discontinuous hymeneal layer with conidia embedded in mucous sheaths (Speare, 1920; Hodge, 1998). More recently the concept of *Hirsutella* was expanded to include mononematous species and those with polyphialides, phialides without significant basal inflation, capitate synnemata, conidia without a mucous coat, and didymoconidia (Hodge, 1998). Hodge described the genus as “a constellation of species that differ widely in their morphology” (1998). She recognized a broad concept, including mononematous

and synnematosus species and found the genus to be monophyletic with the inclusion of *Harposporium* (1998). The Clavicipitaceae s.s. and the Cordycipitaceae also contains species of *Torrubiella* and *Cordyceps* that have been assigned *Hirsutella* anamorphs which are atypical (e.g., *C. pseudomilitaris*), but upon further investigation were more accurately characterize in other anamorph genera (e.g. *Simplicillium* W.Gams and Zare) (Sung, 2005). The anamorphs of *T. iriomotiana* (Hywel-Jones, 1995), *T. petchii* (Hywel-Jones, 1997b), and *T. siamensis* (Hywel-Jones, 1995) have been described as *Hirsutella*, but the morphology is atypical of other species described in the genus (phialides not basally inflated). Hywel-Jones recognized this and suggested a needed revision of the genus (1997c). Unfortunately, Hodge did not include any of these *Torrubiella* spp. in the molecular component of the revision of *Hirsutella* (1998). However, *T. petchii* was included in our sampling and does not place in the same family as the *Hirsutella* s.s. clade and does not have the spores characteristic of the *Ophiocordyceps-Hirsutella* clade. The morphology of the anamorph of *T. petchii* is more similar to verticillate anamorphs (*Simplicillium*-like), than *Hirsutella*. While a more thorough investigation of *Torrubiella*-associated anamorphs of the of the Clavicipitaceae is necessary, these results along with those of Sung (2005) support a narrow definition of *Hirsutella* as being phylogenetically informative.

Clade G contains an assemblage of species with three *anamorph* genera on disparate hosts: *Akanthomyces* sp. on pentatomids, *Paecilomyces lilacinus* on a nematode (and isolated from soil), and *Nomuraea atypicola* on a spider. Though

*Nomuraea atypicola* is phylogenetically distinct, there is little sequence variation between the two isolates of *Akanthomyces* and the two isolates of *Paecilomyces lilacinus*. *Akanthomyces* and *Paecilomyces* are morphologically, ecologically and phylogenetically diverse. Previous molecular studies have shown *Paecilomyces* to be a polyphyletic (Sung, 2005; Luangsa-Ard *et al.*, 2005; Luangsa-ard *et al.*, 2004; Obornik *et al.*, 2001) and the genus can be found on a wide range of hosts (Samson, 1974). *Paecilomyces spp.* produce simple or complex whorled branching conidiophores supporting short, flask-shaped phialides that taper to a short, narrow neck (Hodge, 2003). In this study, *Akanthomyces* is found in two different families (Cordycipitaceae and Ophiocordycipitaceae). *Akanthomyces spp.* are characterized by synnemata covered with a hymenium of ellipsoidal or cylindrical phialides that taper to a neck and produce unicellular, hyaline conidia in chains (Mains, 1950; Hsieh *et al.*, 1997). The host range of some species of *Akanthomyces* is broad, ranging from ants (Hymenoptera) to Lepidoptera (Samson and Evans, 1974), and *A. johnsonii*, a presumed a saprotroph, is regularly isolated from soil (Vincent *et al.*, 1988); however, the spider pathogens are restricted. *Akanthomyces* is separated from *Paecilomyces* by the production of phialides in a hymenium, where as in *Paecilomyces*, the phialides are verticillate and more spaciouly arranged (Samson, 1974; Samson and Evans, 1974). *Akanthomyces gracilis* has been suggested as an intermediate form between the two genera (Samson and Evans, 1974). Isolates of *P. lilacinus* used in this study were obtained from nematodes and a soil sample, however the host range of the species is rather broad, having been found on a range of insects

(including pentatomids) and the intestine of a chameleon (Samson, 1974; CBS database). These data are consistent with these isolates representing a set of closely related taxa that exhibit different morphologies, perhaps in relation to different hosts. *Nomuraea* produces simple conidiophores with whorled, short, flask-shaped phialides that taper to a short, narrow neck (Samson, 1974; Hodge, 2003). Previous phylogenetic studies have shown *Nomuraea* to be polyphyletic, with another species, *N. rileyi* (Farl.) Samson grouping with species of *Metarhizium* (Sung, 2005). Species of *Metarhizium* produce greenish colored conidia as does *N. rileyi*, and interestingly, *N. atypicola* (anamorph of *C. cylindrica*) possesses lavender colored conidia similar to those of *P. lilacinus* (Coyle *et al.*, 1990; Evans, 2003; Hywel-Jones and Sivichai, 1995).

Our results were similar to Sung (2005) in that anamorphs with restricted phylogenetic distribution were informative in characterizing *Torrubiella* species (e.g., *Hirsutella* and *Gibellula*) when genera are unique and well-defined. However, *Akanthomyces* and *Paecilomyces* represent broad morphological concepts that provide little phylogenetic, predictive power. Host affiliation in conjunction with *anamorph* morphology is phylogenetically informative for some hosts, *i.e.*, spiders, but host affinity is not a reliable predictor of phylogeny for the numerous pathogens of scale insects, which are found in all three families with the exception of the Diaspididae.

### **Evolution of Astipitate Stromata**

Species of *Torrubiella* do not produce perithecia on a stipitate stroma.

This morphology has been derived multiple times among these fungi, presumably from stipitate ancestors. Neither *Torrubiella* nor *Cordyceps* represent monophyletic groups, but instead artificial groupings of similar morphologies and homoplastic ecologies. Most *Torrubiella spp.* are pathogens of spiders and scale insects, where as important hosts of *Cordyceps* include Lepidoptera, Coleoptera, Hymenoptera, and Hemiptera with rare occurrences on scale insects and spiders. On these latter two hosts, *Torrubiella* species produce a subiculum with superficial perithecia, whereas *Cordyceps* species produce stipitate stroma with imbedded or superficial perithecia. There are numerous examples in fungal evolution of the derivation of simple morphologies from more complex fruiting bodies or vice-versa (e.g. evolution of resupinate and mushroom sporocarps in the Basidiomycota) (Hibbett and Binder, 2002). We propose that *Cordyceps* and *Torrubiella* represent another example of this general phenomenon of convergent and dynamic stromata evolution, and furthermore, the derivation of the more simple morphology is strongly correlated with host affiliation, *i.e.*, spiders and scale insects.

Hosts of *Torrubiella* are generally small and one explanation for the lack of a stroma may be that they do not provide enough nutrition to produce a stipitate stroma in addition to perithecia. However, species of *Cordyceps* (e.g., *C. unilateralis*) manage to produce stipitate stromata on species of ants, which also have small bodies and presumably limited nutrition. In addition, large species of *Hypocrella* produce stroma 1000 times the weight of their host scale insect

(Hywel-Jones and Samuels, 1998), but these may represent a *Septobasidium*-like association, where the insect is a means of deriving nutrition from the plant. *Hyperdermium* species also produce a stroma much larger than their hosts and are suggested to be epibionts of plants that derive their nutrition from the plant via that stylet of the scale insect after it is killed (Sullivan *et al.*, 2000). *Torrubiella piperis* also produces a stroma that is much larger than its scale insect host (Bischoff and White, 2004). Spiders and scale insects can be found freely exposed on leaf surfaces, so there is presumably no need for the fungus to elevate the propagules for dispersal, therefore selection pressure for spore dispersal may vary. However, *Cordyceps clavulata*, that parasitizes scale insects and *Cordyceps* species on exposed ants produce stipitate stromata. Similarly, *Cordyceps cylindrica*, which occurs on buried trapdoor spiders, does produce a stipitate stroma (Hywel-Jones and Sivichai, 1995). Therefore, selection for loss of the stipitate stroma likely stems from a mix of ecological and biochemical characteristics of some hosts that result in the homoplastic trait of astipitate or subiculate stromata among the clavicipitaceous fungi. Thus, historical taxonomic treatments that have classified the majority of astromatic scale insect or spider infecting species in *Torrubiella* rely on a variable phenotype and do not accurately reflect evolutionary history.

## TAXONOMIC REVISIONS

Two new genera and combinations for the species that were previously classified in *Torrubiella* are listed below. We only propose new combinations that

were confidently assigned based on these analyses or supported by morphologies reassessed as synapomorphies.

***Armaphthora* Johnson and Spatafora *gen. prov.***

*Type species-* *Torrubiella luteorostrata* Zimm.

*Description-* Stromata pulvinate to planar, not stipitate, variously colored. Hypothallus hyaline. Perithecia produced on the stroma or hypothallus, elongated flask-shaped to elongated conic, yellow-brown to purple-red. Asci cylindrical with thickened ascus apex. Ascospores filiform, multiseptate, disarticulating into partspores. Hosts include diaspidid scale insects.

*Anamorph genera-* *Paecilomyces*

The genus *Armaphthora* is proposed for species of *Torrubiella s. l.* in Clavicipitaceae *s.s.* based on the phylogenetic placement of *T. luteorostrata* and *T. tenuis* (Clade E) (Figure 1). The genus is applied to Clade E which is strongly supported (BP =100). Members of this genus are the only recorded pathogens of diaspidid scale insects in the Clavicipitaceae *s.l.* The morphological characters shared by these two species are elongated, flask-shaped perithecia and planar stromata (Hywel-Jones, 1993, 1997). *Torrubiella tomentosa* has also been recorded as a pathogen of diaspidid scales, however based on morphological characters alone, we cannot confidently transfer this species to *Armaphthora*.

Included species:

*Armaphthora tenuis* (Petch) Johnson and Spatafora *comb. prov.*  
 ≡ *Torrubiella tenuis* Petch (1922) *Ann. Perad.* 7: 323.

*Armaphthora luteorostata* (Zimm.) Johnson and Spatafora *comb. prov.*  
 ≡ *Torrubiella luteorostrata* Zimm. (1901) *Centralb. F. Bakt. Abt.* **2(7)**: 872.

***Petchea* Johnson and Spatafora *gen. prov.***

*Type species-* *Torrubiella petchii* Hywel-Jones

*Description-* Stroma covering the host body, ring-like, color ochraceous, stipe lacking. Perithecia crowded, superficial, flask-shaped. Asci cylindrical with thickened ascus apex. Ascospores filiform, not disarticulating into partspores.

*Anamorph genera-* verticillate, *Simplicillium*-like

The genus *Petchea* is proposed for species of *Torrubiella s. l.* in Clavicipitaceae *s.s.* based on the phylogenetic placement of *T. petchii* (Clade D) (Figure 1). All isolates of *Torrubiella petchii* are grouped with strong support (BP =100). Ecologically, this species is unique, in that it is only found on bamboo and may be specific to a bamboo scale insect (Hywel-Jones, 1997).

Included species:

*Petchea petchii* (Hywel-Jones) D.M. Johnson, Hywel-Jones and Spatafora *comb. prov.*  
 ≡ *Torrubiella petchii* Hywel-Jones (1997) *Mycol. Res.* **101(2)**: 143.

***Cordyceps* (L.: Fr.) Link emend. Sung et Spatafora**

Sung (2005) defined *Cordyceps s.s.* as a monophyletic genus that was restricted to the type, *C. militaris*, and closely related species with pallid to brightly colored stromata and *Lecanicillium*, *Beauveria*, *Isaria* and *Microhilum*

anamorphs. In light of this present study, *Cordyceps s.s.* can be expanded to include species of *Torrubiella* with *Lecanicillium* anamorphs.

Included species:

*Cordyceps confragosa* (Mains) D.M. Johnson, Hywel-Jones and Spatafora *comb. prov.*

≡ *Torrubiella confragosa* Mains (1949) *Mycologia* **41(3)**: 305.

*Cordyceps piperis* (Bischoff and White) D.M. Johnson, Hywel-Jones and Spatafora *comb. prov.*

≡ *Torrubiella piperis* Bischoff and White (2004) *Studies in Mycology* **50**: 91.

### ***Ophiocordyceps* Petch emend. Sung et Spatafora**

Sung (2005) redefined *Ophiocordyceps* Petch as a monophyletic genus with species possessing dark pigmentation in at least its some part of the stroma and the occurrence of *Hirsutella* and *Hymenostilbe* anamorphs. Our results confidently place one species in this genus (*T. pruinosa*) and two others are transferred based on synapomorphies of ascospore and anamorph morphologies.

Included species:

*Ophiocordyceps pruinosa* (Petch) D.M. Johnson, Hywel-Jones and Spatafora *comb. prov.*

≡ *Torrubiella pruinosa* (Petch) Minter and B.L. Brady (1980) *Trans. Br. mycol. Soc.* **74**: 278.

≡ *Calonectria pruinosa* Petch (1932) *Trans. Br. mycol. Soc.* **16(4)**: 226.

*Ophiocordyceps truncata* (Petch) D.M. Johnson, Hywel-Jones and Spatafora *comb. prov.*

≡ *Torrubiella truncata* (Petch) K.T. Hodge (1998) Ph.D. dissertation: 75.

≡ *Calonectria truncata* Petch (1942) *Trans. Br. mycol. Soc.* **25**: 257.

*Ophiocordyceps hirsutellae* (Petch) D.M. Johnson, Hywel-Jones and Spatafora *comb. prov.*

- ≡ *Torrubiella hirsutellae* (Petch) A.Y. Rossman (1983) *Mycol. Pap.* **150**: 100.  
 ≡ *Calonectria hirsutellae* Petch (1937)

We propose to retain the remaining species of *Torrubiella* not treated above within *Torrubiella sensu lato*. These residual species were either not included in this study and their placement in the phylogeny of clavicipitaceous fungi is unclear based on morphology alone or their taxonomic status is unclear because the placement of the type species is unknown. Until such time that further analyses, which include the type species *T. aranicida*, clarify the phylogenetic placement of these species, they will be retained in *Torrubiella sensu lato*.

***Torrubiella* Boudier 1885**

- Torrubiella alba* Petch (1931) *Trans. Br. mycol. Soc.* **16**: 224.
- Torrubiella alboglobosa* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus., Tokyo, Bot.* **8**(2): 63.
- Torrubiella albolanata* Petch (1944) *Trans. Br. mycol. Soc.* **27**: 85.
- Torrubiella albotomentosa* Petch (1944) *Trans. Br. mycol. Soc.* **27**: 86.
- Torrubiella arachnophila* (J.R. Johnst.) Mains (1950) *Mycologia* **42**: 316.
- Torrubiella arachnophila f. alba* Kobayasi and Shimizu (1977) *Kew Bull.* **31**(3): 561.
- Torrubiella aranicida* Boud. (1885) *Revue mycol., Toulouse* **7**.
- Torrubiella aurantia* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus., Tokyo, Bot.* **8**(2): 67.
- Torrubiella barda* Petch (1922) *Ann. Peradeniya* **7**: 324.
- Torrubiella blattae* Petch (1941) *Trans. Br. Mycol. Soc.* **25**: 258.
- Torrubiella brunnea* Keissl. *Ann. Myc.* **7**: 292.

- Torrubiella clavata* Samson and H.C. Evans (1992) *Mycologia* **84**(3): 306.
- Torrubiella colombiana* Kobayasi (1982) *Trans. Mycol. Soc. Japan* **23**(2): 114.
- Torrubiella corniformis* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*, Tokyo, Bot. **8**(2): 59.
- Torrubiella dabieshanensis* B. Huang, M.Z. Fan and Z.Z. Li (1998) in Huang, Ding, Fan & Li, *Mycosystema* **17**(2): 110.
- Torrubiella dimorpha* Tzean, L.S. Hsieh and W.J. Wu (1998) *Mycol. Res.* **102**(11): 1350.
- Torrubiella ellipsoidea* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*, Tokyo, Bot. **8**(2): 50.
- Torrubiella falklandica* O'Donnell, Common and Imshaug (1977) *Mycologia* **69**: 619.
- Torrubiella farinacea* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*, Tokyo, Bot. **8**(2): 68.
- Torrubiella flava* Petch (1923) *Trans. Br. mycol. Soc.* **9**: 127.
- Torrubiella flavoviridis* (A. Møller) Kobayasi (1983) *Trans. Mycol. Soc. Japan* **23**(3): 361.
- Torrubiella formicarum* Samson, Reenen and H.C. Evans (1989) *Stud. Mycol.* **31**: 127.
- Torrubiella formosana* Kobayasi and Shimizu (1982) *Bull. Natn. Sci. Mus.*, Tokyo, Bot. **8**(2): 53.
- Torrubiella fusiformis* Kobayasi and Shimizu (1981) *Bull. Natn. Sci. Mus.*, Tokyo, Bot. **7**(4): 117.
- Torrubiella gibellulae* Petch (1932) *Annls mycol.* **30**: 391.
- Torrubiella globosa* Kobayasi and Shimizu (1982) *Bull. Natn. Sci. Mus.*, Tokyo, Bot. **8**(2): 45.
- Torrubiella globosoides* Kobayasi and Shimizu (1982) *Bull. Natn. Sci. Mus.*, Tokyo, Bot. **8**(2): 51.
- Torrubiella globosostipitata* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*, Tokyo, Bot. **8**(2): 49.

- Torrubiella gonylepticida* (A. Møller) Petch (1937) *Trans. Br. Mycol. Soc.* **21**: 50.
- Torrubiella hemipterigena* Petch (1931) *Trans. Br. mycol. Soc.* **16**: 236.
- Torrubiella inegoensis* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 56.
- Torrubiella iriomoteana* Kobayasi and Shimizu (1982) *Bull. Natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 73.
- Torrubiella lecanii* J.R. Johnst. (1918) *Mem. Soc. Cubana Hist.* **3**: 80.
- Torrubiella lloydii* (Mains) Rossman (1977) *Mycologia* **69**(2): 373.  
≡ *Torrubiella cordyceps* Dingley (1953) *Trans. Roy. Soc. New Zealand* **81**(3):  
340.
- Torrubiella longissima* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 69.
- Torrubiella mammillata* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 54.
- Torrubiella minuta* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*, Tokyo,  
Bot. **8**(2): 62.
- Torrubiella minutissima* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 57.
- Torrubiella miyagiana* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 58.
- Torrubiella neofusiformis* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 51.
- Torrubiella oblonga* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*, Tokyo,  
Bot. **8**(2): 68.
- Torrubiella ooaniensis* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*,  
Tokyo, Bot. **8**(2): 58.
- Torrubiella pallida* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus.*, Tokyo,  
Bot. **8**(2): 65.
- Torrubiella paxillata* Petch (1937) *Trans. Br. Mycol. Soc.* **21**: 49.

- Torrubiella pseudogibellulae* Samson, Reenen and H.C. Evans (1989) *Stud. Mycol.* **31**: 127.
- Torrubiella psyllae* Sawada (1914) *Bot. Mag. Tokyo* **28**: 275.
- Torrubiella pulvinata* Mains (1949) *Mycologia* **41(3)**: 303.
- Torrubiella ratticaudata* Humber and Rombach (1987) *Mycologia* **79(3)**: 376.
- Torrubiella rhynchotica* (A. Møller) Kobayasi (1982) *Trans. Mycol. Soc. Japan* **23**: 287.
- Torrubiella rokkiana* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus., Tokyo, Bot.* **8(2)**: 53.
- Torrubiella rosea* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus., Tokyo, Bot.* **8(2)**: 61.
- Torrubiella rostrata* (Henn.) Henn. *Hedwigia* **41**: 162.
- Torrubiella rubra* Pat. and Lagerh. (1893) *Bull. Soc. Myc. France* **9**: 154.
- Torrubiella ryogamimontana* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus., Tokyo, Bot.* **8(2)**: 66.
- Torrubiella ryukyuensis* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus., Tokyo, Bot.* **8(2)**: 55.
- Torrubiella sericicola* Höhnelt (1909) *Sitzbl. Akad. Wiss. Wien* **188**: 302.
- Torrubiella siamensis* Hywel-Jones (1995) *Mycol. Res.* **99(3)**: 331.
- Torrubiella sphaerospora* Samson, Reenen and H.C. Evans (1989) *Stud. Mycol.* **31**: 124.
- Torrubiella sublintea* Petch (1922) *Ann. Peradeniya* **7**: 324.
- Torrubiella superficialis* Kobayasi and Shimizu (1982) *Bull. natn. Sci. Mus., Tokyo, Bot.* **8(2)**: 74.
- Torrubiella tomentosa* Pat. *Bull. Soc. Myc. France* **8**: 133.
- Torrubiella tomentosa* var. *citrina* Pat. *Bull. Soc. Myc. France* **8**: 133.
- Torrubiella tomentosa* var. *tomentosa* Pat. *Bull. Soc. Myc. France* **8**: 133.

*Torrubiella wallacei* H.C. Evans (2001) *Nova Hedwigia* 73(1-2): 46.

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## Chapter 3

### CONCLUSIONS

#### Systematics and evolution of the genus *Torrubiella*

Molecular systematics support the close relationship between *Torrubiella* and *Cordyceps*, a finding consistent with similarities in ascospore, ascus and perithecial morphology. Unlike *Cordyceps*, however, *Torrubiella* does not produce imbedded perithecia on a stipitate stroma, but forms superficial perithecia on a loose mat of hyphae. The two genera also differ in their host range. *Torrubiella* spp. attack spiders and scale insects, which are notably depauperate in the host profile of *Cordyceps*. Recent molecular studies rejected the monophyly of *Cordyceps* and resulted in the reclassification of *Cordyceps* (*Cordyceps* s.s., *Elaphocordyceps*, *Metacordyceps* and *Ophiocordyceps*) and the Clavicipitaceae s.l. (Clavicipitaceae s.s., Cordycipitaceae and Ophiocordycipitaceae) (Sung *et al.*, 2001, Sung and Spatafora, 2004, Sung, 2005). Molecular analyses also did not support the monophyly of *Torrubiella*, but insufficient taxon sampling made conclusions about the genus difficult (Sung, 2005; Artjariyasripong *et al.*, 2001). Here, we increased the sampling of *Torrubiella* spp. and associated *anamorph* taxa to produce a more robust multi-gene phylogeny based on nucleotide sequence data from five loci. In addition, the phylogenetic utility of the unique host affiliation of *Torrubiella* was tested. Weighted parsimony analyses of 125 taxa yielded seven most equally parsimonious trees with polytomies restricted to

highly similar isolates, resulting in a robust assessment of the *Torrubiella* systematics.

Phylogenetic analyses rejected the monophyly of *Torrubiella*. It is present in all three clades of clavicipitaceous fungi and has likely evolved from stipitate, stromatic taxa, i.e., *Cordyceps s.l.*, at least three times. The majority of torrubielloid taxa sampled formed a well-supported clade of spider pathogens, however the taxonomic fate of the remains unresolved, as the type specimen was not sampled. A number of advancements were made, however, that resulted in taxonomic revisions. The generic concept of a monophyletic *Cordyceps* was expanded to include two species of *Torrubiella*, *T. confragosa* and *T. piperis*, with *Lecanicillium* anamorphs characteristic of the clade. *Torrubiella pruinosa* with distoseptate ascospores and *Hirsutella* anamorph grouped with species of *Cordyceps* with similar ascospore and anamorph morphologies and will be transferred to the genus *Ophiocordyceps*. Two separate clades of *Torrubiella* were resolved within the Clavicipitaceae and represent two new generic lineages. The genus *Petchea* is erected to accommodate the species *T. petchii* and the genus *Armaphthora* is erected to accommodate the two Diaspididae pathogens, *T. luteorostrata* and *T. tenuis*.

Some *anamorph* genera (*Gibellula* and *Hirsutella*) were phylogenetically informative in the classification of *Torrubiella*, especially in conjunction with host affiliation. Others were not and represent convergent or ancestral morphologies rather than natural, monophyletic groups. *Gibellula spp.* and *Akanthomyces spp.* on spiders were restricted to the Cordycipitaceae. However,

*Akanthomyces spp.* occurring on other hosts were distributed throughout the three clades of clavicipitaceous fungi, as were species of *Paecilomyces*. *Hirsutella* associated species of *Torrubiella* and *Cordyceps* formed a monophyletic group after re-examination of some atypical morphologies, which are more accurately described as simple verticillate anamorphs (e.g., *Simplicillium*). Host affiliation was phylogenetically informative and predictive of relationship for the spider pathogens (Cordycipitaceae) and armored scale insects (Clavicipitaceae *s.s.*). Pathogens of soft scale insects, however, were found in each of the three families and represented homoplastic ecologies.

### **Recommendations for future study**

In order to better assess the systematics of the genus *Torrubiella*, the evolution of host affiliation and the taxonomic utility of *anamorph* genera, additional taxa need to be sampled including species of *Torrubiella*, *Cordyceps*, *Gibellula*, and *Akanthomyces*. In particular, the type species *T. aranicida* and preferably the type specimen (though greater than 100 years old), needs to be sequenced in order to determine the taxonomic fate of *Torrubiella*, as well as the classification of the spider pathogens. Besides the need for more general sampling across the genus, some species are of particular interest for future taxonomic studies. More spider associated taxa need to be sequenced in order to more confidently assess the restricted distribution of the *anamorph* genera and host affiliation, in particular, those with a *Lecanicillium* anamorph (*T. alba*). In addition, species of *Cordyceps spp.* that parasitize spiders and produce little

stroma (eg. *C. thaxteri*), as well as the few scale insect pathogens (*C. clavulata*), are of interest to the classification of *Torrubiella*. The phylogenetic affinity of *Verticillium pseudohemipteran*, a pathogen of scale insects, remains unknown (Zare *et al.*, 2000; Sung *et al.*, 2001), and though not linked to a *Torrubiella* teleomorph, it may likely be phylogenetically associated because of its ecology and the link to a teleomorph of a similar species, *V. hemipteran* (anamorph of *T. hemipteran*) (Petch, 1923; Hywel-Jones *et al.*, 1997). The sampling of additional species of *Torrubiella* with distoseptate ascospores and *Hirsutella* anamorph is needed to better assess the monophyly of *Hirsutella* and the classification of this group with a unique ascospore morphology.

To better address the phylogenetics of the genus *Akanthomyces*, more species need to be sampled from a broader ecological and morphological scope. *Akanthomyces gracilis*, which has a broad host range, has been suggested as an intermediate form between other species of *Akanthomyces* and the genus *Paecilomyces* (Samson and Evans, 1974) (One isolate of *A. gracilis* was sampled in this study, however it could not be confidently placed within the Clavicipitaceae *s.l.*). Also the phylogenetic placement of *A. johnsonii*, an apparently saprobic fungus (Vincent *et al.*, 1988), needs to be assessed in order to better understand the ecology of these fungi.

In addition, the evolution of host affiliation in the Clavicipitaceae *s.s.*, which includes all plant symbionts of the group, is an interesting question. Many of the arthropod pathogens of the family occur on scale insects, which are intimately associated with plants for food and habitat. Pathogenicity of scale

insects may represent an ancestral ecology for this family and an important link in the host jump from animals to plant. But any definitive conclusions await future study involving more indepth character and taxon sampling and more exhaustive analysis of character state evolution.

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