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Regiocrella, a new entomopathogenic genus with a pycnidial anamorph and its phylogenetic placement in the Clavicipitaceae

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Abstract: A new genus, *Regiocrella*, is described with two species, *R. camerunensis* and *R. sinensis*, based on specimens collected in Cameroon and China. Both species are parasitic on scale insects (Coccidae, Homoptera). Morphological and molecular evidence place the new genus in the Clavicipitaceae (Hypocreales), despite its combination of characters that are atypical of that family; *Regiocrella* is characterized by having perithecia partly immersed in a subiculum, noncapitate asci, unicellular fusiform ascospores and pycnidial-acervular conidiomata. The two new species, *R. camerunensis* and *R. sinensis*, are distinguished based on ascospore and perithecium size. Morphological characters were evaluated and compared to other genera in the Clavicipitaceae, especially those parasitic on scale insects or with pycnidial-acervular anamorphs or synanamorphs (i.e. *Aschersonia*, *Ephelis* or *Sphacelia*): *Atkinsonella, Balansia, Claviceps*, *Epichlöe, Hypocrella, Myriogenospora* and *Neoclaviceps*. The phylogenetic relationships of *Regiocrella* were examined with three gene loci: large subunit nuclear ribosomal DNA (LSU), translation elongation factor 1-α (TEF), and RNA polymerase II subunit 1 (RPB1). The results of this study confirm that *Regiocrella* is distinct from other genera in the Clavicipitaceae and that its two species form a monophyletic group. *Regiocrella* is shown to be closely related to the scale insect pathogen *Hypocrella* and the plant-associated genera *Balansia, Claviceps*, *Epichlöe, Myriogenospora* and *Neoclaviceps*. This study also provides insights into the evolution of pycnidial-acervular conidiomata and scale insect parasitism within the Clavicipitaceae. Plant-associated genera form a monophyletic group correlated with Clavicipitaceae subfamily Clavicipitoideae *sensu* Diehl. We also demonstrate that scale insect parasites have multiple evolutionary origins within the family and genera with pycnidial-acervular anamorphs or synanamorphs have a single origin.

Key words: Ascomycota, evolution, Hypocreales, molecular phylogenetics, systematics

INTRODUCTION

The family Clavicipitaceae (Ascomycota, Hypocreales) comprises 30–40 genera and 160 species (Kirk et al 2001); however this number is conservative considering that in the genus *Cordyceps* (Fr.) Link alone there are at least 115 species. The majority of the genera in the Clavicipitaceae are parasitic on insects; a few genera are associated with plants or other fungi. The teleomorphs in the Clavicipitaceae are generally conspicuous, with brightly colored fleshy stromata that can be stipitate to effuse, clavate to round, and with perithecia superficial or immersed in the stroma. The asci are generally long cylindrical with a prominent apical cap and contain ascospores that are filiform and multiseptate. Characters of both the teleomorph and anamorph have contributed to classification schemes in the Clavicipitaceae. As is the case for many other ascomycetous teleomorph genera, general teleomorphic characteristics are highly conserved and variation between genera and species often can be observed in the anamorph (e.g. the anamorphs of *Cordyceps sensu lato* fall into more than 10 genera including *Akanthomyces* Lebert, *Beauveria* Vuillemin, *Hirsutella* Pat., *Hymenostilbe* Petch, *Isaria* Fr., *Metarrhizium* Sorokin, *Nomuraea* Maublanc, *Paecilomyces* Bainier and *Tolypocladium* W. Gams, among others). Systematics research suggests that *Cordyceps s. l.* should be segregated into more than one genus. About 40 anamorph genera have been linked to the Clavicipitaceae (Hodge 2003).

Some classification systems in the Clavicipitaceae have been based on anamorph characteristics. Diehl's (1950) study formalized the classification system of Gäumann (1926) and Gäumann and Dodge (1928) (i.e. *Oomyces-Ascopolyporus* group [*Oomyces* Berk. & Broome has been excluded from the Clavicipitaceae],

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Epichlöe-Claviceps group and *Cordyceps* group) that was based on the premise that the anamorphic states and other characters were indicative of ''three divergent evolutionary trends'' (Diehl 1950). These three groups were classified respectively in subfamilies Clavicipitaceae subf. Oomycetoideae, Clavicipitaceae subf. Clavicipitoideae and Clavicipitaceae subf. Cordycipitoideae. This same study recognized three tribes within subfamily Clavicipitoideae (i.e. Clavicipiteae, Balansiae and Ustilaginoideae) based mainly on the conidial states. The tribe Clavicipiteae included *Claviceps* Tul., with *Sphacelia* Léveillé or sphacelial anamorphs; the Balansiae included genera that had typhodial (*Neotyphodium* Glenn et al or *Acremonium*-like), ephelidial (*Ephelis*-like) or both anamorphs (i.e. *Atkinsonella* Diehl, *Balansia* Speg. and *Epichlöe* [Fr.] Tul. & C. Tul.); the Ustilaginoideae included *Munkia* Speg. and *Ustilaginoidea* Bref. Recent studies suggest that many genera in the subfamily Clavicipitoideae form a monophyletic group based on DNA sequence data (Bischoff et al 2005, Kuldau et al 1997, Pažoutová et al 2004, Sullivan et al 2001). In addition some of these studies reported that the form genera *Sphacelia, Ephelis* and *Neotyphodium* are closely related, thus providing support for Diehl's subfamilies and tribes.

In this study we report on two specimens of an unidentified genus collected in Cameroon and China. These specimens resemble *Hypocrella* Sacc. (anamorph *Aschersonia* Mont.) in that they grow on scale insects and have pycnidial-acervular conidiomata that release slimy orange cirrhi of conidia. The conidiomata are also similar to those in the plant pathogenic genera *Atkinsonella, Balansia, Myriogenospora* Atk. and *Neoclaviceps* J. White et al with ephelidial anamorphs and *Claviceps* and *Epichlöe*, with sphacelial anamorphs. Other genera that are parasitic on scale insects are *Ascopolyporus* Møller, *Dussiella* Pat., *Hyperdermium* J. White et al and *Torrubiella* Boud. *Torrubiella sensu stricto* is parasitic on spiders; therefore it is possible that other taxa in *Torrubiella* belong to a different genus. The unidentified genus also is morphologically similar to *Torrubiella s. l.* in the almost naked obpyriform perithecia that are partially embedded in an effuse stroma or subiculum composed of very loosely interwoven hyphae. Of interest, the asci and ascospores of the new genus are not typical of the Clavicipitaceae. The asci are not capitate, and the ascospores are unicellular and short fusiform. Similar characteristics have been observed in a few other clavicipitaceous species such as *Torrubiella pruinosa* (Petch) Minter & Brady and *''Calonectria'' truncata* Petch (probably also a *Torrubiella*) and *T. hirsutellae* (Petch) A.Y. Rossman, which have fusiform, 7–14-septate ascospores; these species are on not found on scale insects and do not have pycnidial-acervular anamorphs.

The present study addresses these questions: (i) Are the Cameroonian and Chinese specimens members of a new genus? (ii) Where does this new genus fit in relation to other clavicipitaceous genera with pycnidial-acervular conidiomata and scale insect parasitism? This study also will provide preliminary insights into the evolution of conidiomata and scale insect parasitism within the Clavicipitaceae. Morphological and molecular phylogenetic analyses were used to answer the above questions. Three genetic loci were studied (i.e. large subunit nuclear ribosomal DNA [LSU], translation elongation factor 1-α [TEF] and RNA polymerase II subunit 1 [RPB1]).

MATERIALS AND METHODS

Morphological examination.—Two specimens of the unidentified genus were examined: CUP 67512 from Cameroon (collected by H.C. Evans) and CUP CH-264 from China (collected by B. Huang). The specimens have been deposited at the Cornell University Plant Pathology Herbarium (CUP). Ascomata of both specimens were rehydrated briefly in distilled water with a trace of Tween® 80 (J.T. Baker Chemical Co., Phillipsburg, New Jersey). Germination of ascospores was attempted by isolating asci containing ascospores and placing them on Difco™ potato-dextrose agar (PDA) (BD Diagnostic Systems, Franklin Lakes, New Jersey) with antibiotics; only CUP 67512 ascospores germinated. The culture obtained was deposited in the ARS Collections of Entomopathogenic Fungi (ARSEF) (Ithaca, New York). Stromata, perithecia, asci and ascospores were characterized by light microscopy. Morphological observations of the colonies and anamorph were based on cultures grown on PDA for 4 wk in an incubator at 25 C with alternating 12 h fluorescent light and 12 h darkness. Color terminology in anamorph and teleomorph descriptions is from Kornerup and Wanscher (1978). Scion Image beta version 4.0.2 (Scion Corp., Frederick, Maryland) was used to measure continuous characters such as ascospore and conidium length. Confidence intervals ($\alpha = 0.05$), minimum and maximum values for 10–30 anamorph and teleomorph measurements were calculated using Systat 8.0 (SPSS Inc., Chicago, Illinois).

DNA extraction, PCR, and sequencing.—The culture derived from CUP 67512 and cultures of various other representative clavicipitaceous genera used in the phylogenetic analyses (TABLE I) were grown on potato-dextrose broth in a 6 cm diam Petri plate for about 1 wk. The mycelial mat was harvested in a laminar flow hood and dried with clean, absorbent paper towels. DNA was extracted with Ultra Clean™ Plant DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, California) and DNeasy™ Plant Protocol (QIAGEN

TABLE I. Specimens and cultures used in phylogenetic analyses and GenBank numbers

Genus and species	Voucher/culture number	LSU	TEF	RPB1
Aschersonia napoleonae	P.C. 737	AY986910	AY986936	DQ000337
Ascopolyporus polychrous	P.C. 546	DQ118737*	DQ118745*	DQ127236*
Ascopolyporus villosus	ARSEF 6355	AY886544	DQ118750*	DQ127241*
Balansia henningsiana	GAM 16112[2]	AY489715	AY489610	AY489643
Claviceps purpurea	GAM 12885[2]	AF543789	AF543778	AY489648
Cordyceps capitata	OSC 71233[2]	AY489721	AY489615	AY489649
Cordyceps gunnii	OSC 76404[2]	AF339522	AY489616	AY489650
Cordyceps heteropoda	OSC 106404[2]	AY489722	AY489617	AY489651
Cordyceps inegoensis	SU-15	DQ118741*	DQ118752*	DQ127243
Cordyceps ophioglossoides	OSC 106405[2]	AY489723	AY489618	AY489652
Cordyceps ramosopulvinata	SU-65	DQ118742*	DQ118753*	DQ127244*
Epichlöe elymi	C. Schardl 760	AY986924	AY986951	DQ000352
Epichlöe typhina	ATCC 56429[2]	U17396	AF543777	AY489656
Hyperdermium pulvinatum	P.C. 602	DQ118738*	DQ118746*	DQ127237*
Hypocrea lutea	ATCC 208838[2]	AF543791	AF543781	AY489662
Hypocrea rufa	CBS 114374[2]	AY489726	AY489621	AY489656
Hypocrella viridans	M.L. 202i	AY986913	AY986939	DQ000340
Myriogenospora atramentosa	AEG 96–32[2]	AY489733	AY489628	AY489665
Neoclaviceps monostipa	ATCC MYA-621	AF245293	AY986983	DQ000353
Podocrella harposporifera	G.J.S. 81–358	AF339519	DQ118747*	DQ127238*
Polycephalomyces formosus	ARSEF 1424	AY259544	DQ118754*	DQ127245*
Regiocrella camerunensis	CUP 67512, ARSEF 7682	DQ118735*	DQ118743*	DQ127234*
Regiocrella orientalis	CUP CH-2640	DQ118736*	DQ118744*	DQ127235*
Torrubiella piperis	CBS 116719	AY466442	DQ118749*	DQ127240*
Torrubiella sp.	P.C. 385	DQ118739*	DQ118748*	DQ127239*
"Torrubiella" sp.[1]	J.B. 207	DQ118740*	DQ118751*	DQ127242*

*Sequences produced for this study. Other sequences were obtained from GenBank.

[1] This specimen has been temporarily placed in *Torrubiella*; however it is possible that it belongs in a new genus. *Torrubiella sensu stricto* comprises species parasitic on spiders. Many other morphologically similar taxa have been placed in *Torrubiella*, but probably belong in other genera. The genus *Torrubiella* is in need of critical revision.

[2] Source: Castlebury et al 2004.

Inc., Valencia, California). Because CUP CH-264 did not grow, DNA was extracted from the herbarium specimen. The stroma was rehydrated briefly with sterile distilled water, several centri were removed from the perithecium with a fine needle under the dissecting scope and placed in an Eppendorf tube. The DNA was extracted with Ultra Clean™ Forensic DNA Kit. Three partial gene regions were amplified (i.e., large subunit nuclear ribosomal DNA [LSU], translation elongation factor 1-α [TEF], and RNA polymerase II subunit [RPB1]). The primers used were LSU: LRORf (5′-GTACCCGCTGAACTTAAGC- 3′ and LR5r (5′- ATCCTGAGGGAAACTTC- 3′) (Vilgalys and Hester 1990); TEF: 983f (5′-GCYCCYGGHCAYCGTGAYTTYAT-3′) (Carbone and Kohn 1999) and 2218r (5′-ATGACACCRACRGCRACRGTYTG-3′) (Rehner 2001); RPB1: cRPB1Af (5′-CAYCCWGGYTTYATCAAGAA-3′) and RPB1Cr (5′-CCNGCDATNTCRTTRTCCATRTA-3′) (Castlebury et al 2004). Each 50 μL PCR reaction contained 25 μL of Promega 2× PCR Master Mix (Promega Corp., Madison, Wisconsin), 2.5 μL of each forward and reverse primers (10 mM), 1 μL DMSO (dimethyl sulfoxide), ca. 25 ng of genomic DNA and sterile distilled water. The PCR reactions were placed in an Eppendorf Mastercycler thermocycler (Eppendorf, Westbury, New York) under these conditions: for LSU (i) 5 min at 94 C; (ii) 35 cycles of denaturation at 94 C for 30 s, annealing at 50 C for 45 s, and extension at 72 C for 1 min; (iii) and 7 min at 72 C; for TEF (i) 10 min at 95 C; (ii) 40 cycles of denaturation at 94 C for 30 s, annealing at 55 C for 30 s, and extension at 72 C for 1 min; (iii) and 72 C for 10 min; and for RPB1 (i) 5 min at 95 C; (ii) 40 cycles of denaturation at 95 C for 1 min, annealing at 50 C for 2 min and extension at 72 C for 2 min; (iii) and 72 C for 10 min. The resulting PCR products were purified with the QIAquick™ PCR Purification Kit (QIAGEN, Inc.). Sequencing of forward and reverse strands was performed at the DNA Sequencing Facility (Center for Agricultural Biotechnology, University of Maryland, College Park, Maryland). Sequences were assembled and edited with Sequencher 4.2 (Gene Codes, Madison, Wisconsin). Sequences have been deposited in GenBank (TABLE I) and the alignment in TreeBase (study number S1315, http://treebase.bio.buffalo.edu/treebase/).

Phylogenetic analyses.—The sequences were aligned with Clustal X 1.81 (Thompson et al 1997), and the alignment was refined by hand with GeneDoc 2.6.002 (Nicholas et al 1997). Maximum parsimony (MP) and Bayesian inference (BI) analyses were carried out with all sequences. The MP analysis was done in PAUP* version b10 (Swofford 2002) using a heuristic search, with a starting tree obtained via 1000 random stepwise addition sequences, tree-bisection-reconnection as the branch-swapping algorithm, and MULTREES on. Bootstrap values from 500 replicates were calculated with full heuristic search. MrBayes 3.0 b4 (Huelsenbeck 2000, Huelsenbeck et al 2001) was used to reconstruct phylogenetic trees based on the Bayesian approach (Mau et al 1999, Rannala and Yang 1996). The Bayesian analysis used a different model of evolution for each of the three loci (LSU, TEF, RPB1). The models of DNA substitution were estimated with Modeltest 3.6 (Posada and Crandall 1998). In addition, partitions were defined based on codon positions (1st, 2nd, 3rd) for TEF and RPB1. A total of five partitions were used in the analyses. Four chains and 5 000 000 Markov chain Monte Carlo generations were run, and the current tree was saved to a file every 100 generations. Stability of likelihood scores was confirmed with the software TRACER version 1.2.1 (Rambaut and Drummond 2004), which traces the parameter against the generation number. Once stability was reached both in terms of likelihood scores and parameter estimation, the first 5 000 trees were discarded (as burn-in). The remaining trees (''post-burn-in'') were pooled and a 50% majority-rule consensus tree was obtained with PAUP*. Members of the Hypocreaceae, *Hypocrea lutea* (Tode) Petch and *H. rufa* (Pers. : Fr.) Fr., were used as outgroup species. Castlebury et al (2004, FIG. 2) showed that the family Hypocreaceae is sister of the Clavicipitaceae.

Topological incongruence was examined with a reciprocal 70% bootstrap (BP) or a 95% posterior probability (PP) threshold (Mason-Gamer and Kellogg 1996, Reeb et al 2004) to determine whether the sequences from the three genes should be combined in a single analysis. Bootstrap values were generated with neighbor joining (NJ) with 1000 replicates and a maximum likelihood distance. Posterior probabilities were calculated with Bayesian analysis in MrBayes. A conflict was assumed to be significant if two relationships for the same taxa, one being monophyletic and the other nonmonophyletic, both with BP = 70% and PP = 95%, were observed on each LSU, TEF and RPB1 majority-rule consensus trees. The three partitions could be combined if no significant conflicts were detected. SIMMAP (Bollback 2004) was used to reconstruct ancestral states and trace phenotypic characters (type of anamorph and host) over the phylogenetic tree. SIMMAP implements a Bayesian method called ''posterior mapping'' (Nielsen 2002) for mapping characters with stochastic substitution models. SIMMAP summarizes character maps and uses sampling from posterior probabilities (PP) for evaluating statistical significance through the use of *P*-values. In this study the morphological characters were mapped over the Bayesian tree constructed for the phylogenetic analyses. Type of anamorph was coded as: 1 = with pycnidial-acervular conidiomata, 0 = other. The type of host of the fungal genus was coded as: 1 = on scale insects or white flies, 2 = on living plants, 3 = on other insects, 0 = on none of the above. An ancestral state at a given node was considered significant and desirable over the other if its posterior probability was ≥95%. To trace the morphological characters over the phylogenetic tree, the option ''simulate mappings'' was used, with the number of realizations for each tree/each site set to 1000 repetitions.

RESULTS

Morphological analysis.—The new genus, named *Regiocrella* below and comprising two species, has several morphological characteristics that distinguish it from similar genera (TABLE II). Even though the anamorph of *Regiocrella* is comparable to other sphacelial anamorphs, the branched conidiophores, enteroblastic conidiogenesis and unicellular conidia distinguish it. *Claviceps, Epichlöe, Hypocrella* and *Regiocrella* have enteroblastic phialidic conidiogenesis and ameroconidia, while *Atkinsonella, Balansia, Myriogenospora* and *Neoclaviceps* have holoblastic sympodial conidiogenesis and septate conidia. In addition clavicipitaceous genera with sphacelial or ephelidial conidiomata are associated with plants; in contrast *Regiocrella* is associated with scale insects.

Other unique characteristics of *Regiocrella* are the asci and ascospores. The asci are not capitate, and the ascospores are unicellular and short fusiform. The majority of the genera in the Clavicipitaceae have capitate asci and filiform multiseptate ascospores. The perithecia of *Regiocrella* are orange, obpyriform, laterally collapsing, becoming purple when 3% KOH is added and are partly immersed in an orange subiculum that covers the scale insect. The available anamorph of *Regiocrella* (*R. camerunensis* CUP 67512, culture ARSEF 7682) resembles *Aschersonia* in culture. Its colonies are slow growing, orange, compact and elevated. As in many species of *Aschersonia*, the conidia of *Regiocrella* are released in slimy orange cirrhi. However the conidia in *Aschersonia* are generally fusiform whereas *Regiocrella* has ellipsoidal conidia.

Because the anamorph of *Regiocrella sinensis* CUP CH-264 is not available, the two species can be compared only in the characteristics of the teleomorph. The two specimens are recognized here as two new species: *R. camerunensis* (CUP 67512) and *R. sinensis* (CUP CH-264). *Regiocrella camerunensis* has slightly shorter asci and larger ascospores than *R. sinensis*. The mature asci of *R. camerunensis* and *R. sinensis* are respectively 70–75 µm and 74–80 µm long. The ascospores of *R. camerunensis* are 8.0–

TABLE II. Clavicipitaceous genera with pycnidial-acervular conidiomata

Genus	Host	Generalized anamorph	Conidiogenesis and conidiophores	Conidia	Synanamorph	Bibliographic source
Epichlöe	Plant	*Neotyphodium*	Phialidic, generally not branched	Unicellular	*Sphacelia*-like	Kuldau et al 1997, Rykard et al 1984
Claviceps	Plant	*Sphacelia*	Phialidic, rarely branched	Unicellular	Micro- and macroconidia	Pažoutová et al 2004, Rykard et al 1984
Regiocrella	Insect	Not named; *Sphacelia*-like	Phialidic, on branched conidiophores	Unicellular	None seen	Personal observations
Hypocrella	Insect	*Aschersonia*	Phialidic, generally not branched	Unicellular	When present, *Hirsutella*-like	Personal observations
Atkinsonella	Plant	*Ephelis*-like	Holoblastic, sympodial and often form whorls consisting of 3–8 spores.	Multiseptate	*Neotyphodium*-like; sometimes *Sphacelia*-like	Kuldau et al 1997, Morgan-Jones and White 1992, Rykard et al 1984
Balansia	Plant	*Ephelis*	*Idem*	Sometimes septate	Sometimes *Neotyphodium*-like	Diehl 1950, Kuldau et al 1997, Rykard et al 1984
Myriogenospora	Plant	*Ephelis*-like	*Idem*	Multiseptate		Rykard et al 1982
Neoclaviceps	Plant	*Ephelis*-like	*Idem*	Multiseptate		Sullivan et al 2001

9.3 µm long vs. 7.0–7.5 µm long in *R. sinensis*. In addition the subiculum of *R. sinensis* is thinner than that of *R. camerunensis*, and it is therefore possible to distinguish the body of the scale insect.

Phylogenetic analyses.—Sequence alignment of three gene loci included a total of 2519 characters in the analyses (802 for LSU, 934 for TEF, and 783 for RPB1), including insertions and deletions. Ambiguously aligned regions were excluded from the analyses. RPB1 provided the majority of the phylogenetically informative characters (44%), followed by TEF (37%) and LSU (19%). In the maximum parsimony analyses, the consistency and homoplasy indices for the combined dataset were respectively 0.418 and 0.582. The models of DNA substitution calculated by Modeltest were used to analyze incongruence among loci. Modeltest suggested general time reversible (GTR + G + I, nst = 6) models with gamma distributions and invariable sites for all three loci. The parameters selected for the LSU model were: base frequencies = 0.2283, 0.2716, 0.3201; rates (Rmat) = 0.4772, 2.7390, 0.5495, 0.7659, 6.3667; gamma shape = 0.4985; proportion invariable sites (pinvar) = 0.6212. The parameters for the TEF model were: base frequencies = 0.1952, 0.3442, 0.2443; rates (Rmat) = 0.6044, 1.0873, 0.7875, 0.5494, 4.6887; gamma shape = 1.1425; proportion invariable sites (pinvar) = 0.551. The parameters for the RPB1 model were: base frequencies = 0.2335, 0.2826, 0.2611; rates (Rmat) = 1.8006, 3.6967, 0.8486, 0.9245, 6.9621; gamma shape = 0.8631; proportion invariable sites (pinvar) = 0.3752. The reciprocal 70% bootstrap and 95% posterior probability thresholds for individual loci show that the topologies of the three genes are congruent (results not shown) and therefore the partitions were combined. BI and MP analyses show that the two species of *Regiocrella* form a monophyletic group supported by 93% bootstrap (BP) and 100% posterior probability (PP) (FIG. 1) distinct from other genera represented in the analyses. *Regiocrella camerunensis* and *R. sinensis* differ by 149 base pairs (26 bp in LSU, 50 bp in TEF and 73 bp in RPB1). In the MP combined analyses, BP does not support the phylogenetic position of *Regiocrella*. On the other hand BI analyses as well as neighbor joining support the relationship among *Regiocrella, Hypocrella* and the plant parasitic genera (100% PP).

The genera that produce pycnidial-acervular conidiomata form a monophyletic group supported by 100% PP (group A in FIG. 1). This monophyletic group

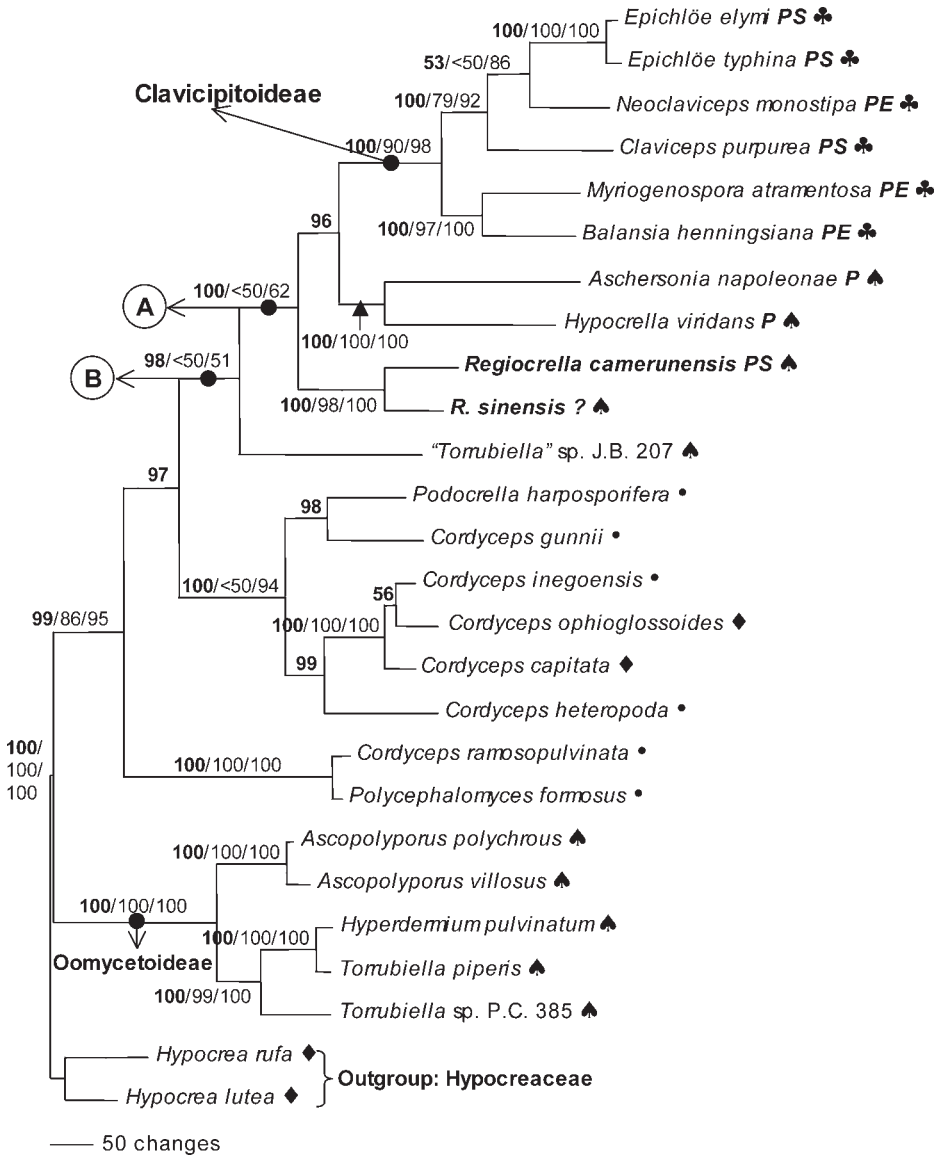


FIG. 1. Relationships of representative clavicipitaceous genera based on Bayesian inference (50% majority rule consensus tree of the combined LSU, TEF, and RPB1 data sets). Symbols: ***P*** = species with pycnidial-acervular conidiomata; ***S*** = species with sphacelial conidiomata; ***E*** = species with ephelidial conidiomata; **?** = anamorph not known; ♣ = plant associated; ♠ = parasitic on scale insects; ● other insects; ◆ = other hosts, not insects. Clade A includes clavicipitaceous genera known to have pycnidial-acervular anamorphs; clade B includes an unidentified species of ''*Torrubiella*'' that is basal to clade A. Branch lengths were calculated and averaged in MrBayes. Numbers at branches indicate posterior probability (%)/MP bootstrap value/NJ bootstrap value.

includes genera of Clavicipitaceae subfamily Clavicipitoideae (i.e. *Balansia, Claviceps, Epichlöe, Myriogenospora* and *Neoclaviceps*) and the scale insect parasites *Hypocrella* and *Regiocrella*. On the other hand genera with sphacelial conidiomata (i.e. *Claviceps, Epichlöe,* and *Regiocrella*) and ephelidial conidiomata (i.e. *Balansia, Myriogenospora* and *Neoclaviceps*) are polyphyletic within the Clavicipitaceae subfamily Clavicipitoideae. Only *Balansia* and *Myriogenospora* form a monophyletic group supported by 100% PP and 97% BP. The subfamily Clavicipitoideae is supported by 100% PP and 90% BP. The genera that are parasitic on scale insects are polyphyletic within the Clavicipitaceae (FIG. 1). One group, which is correlated to the Clavicipitaceae subfamily Oomycetoideae, contains the scale insect parasites *Ascopolyporus, Hyperdermium* and *Torrubiella*; this group is supported by 100% PP and 100% BP. The other clade (group B in FIG. 1) contains the other scale insect parasites *Hypocrella, Regiocrella* and an unidentified *Torrubiella*-like species (J.B. 207).

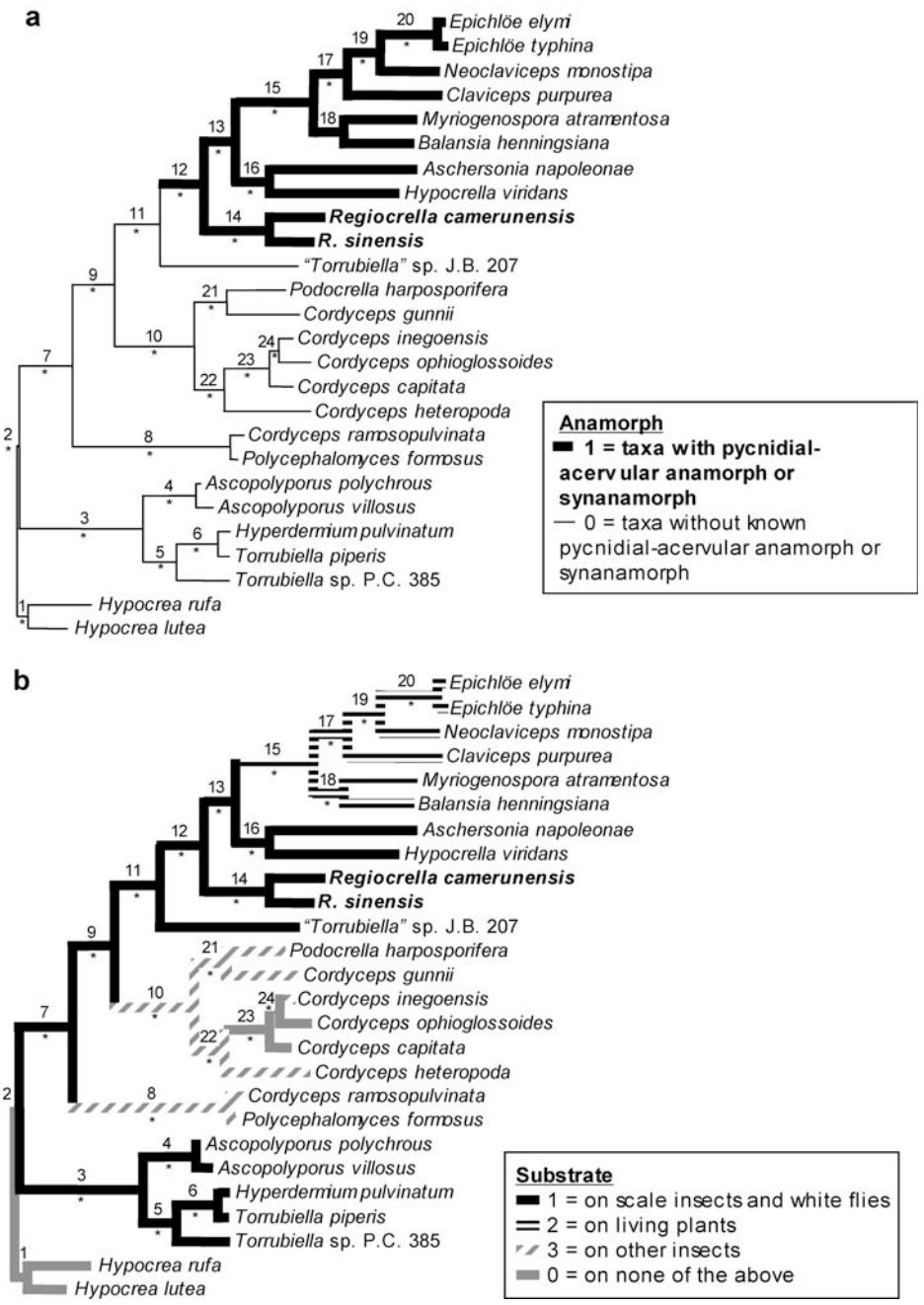


FIG. 2. Reconstruction of ancestral characters and tracing of morphological characters. The characters were mapped over the same Bayesian tree shown (FIG. 1). a. Map of pycnidial-acervular conidiomata character within the Clavicipitaceae (1 = with pycnidial-acervular conidiomata, 0 = other). b. Map of type of substrate within the Clavicipitaceae (1 = on scale insects or white flies, 2 = on living plants, 3 = on other insects, 0 = on none of the above). Node numbers are indicated at branches. Asterisk (*) at nodes represent those with posterior probabilities >95%, thus, the character state that is more likely at that node.

The reconstruction of ancestral states and mapping of morphological characters over the phylogenetic tree is provided (FIG. 2). The cladogram shows that the ancestral state for scale-insect parasitism (FIG. 2b) has a significantly higher PP on 11 nodes, including the most basal clade Oomycetoideae (node 3). The ancestral state for plant association has a significantly higher PP at node 15, which is the Clavicipitoideae. With respect to the ancestral state for pycnidial-acervular conidiomata (FIG. 2a), only node 12 has a significantly higher PP, possibly indicating the single evolutionary origin of the state.

TAXONOMY

Based on our morphological and molecular results we conclude that it is appropriate to describe a new genus comprising two new species, as follows.

Regiocrella Chaverri et K.T. Hodge, gen. nov.

Anamorph: *Sphacelia*-like.

Type species: *Regiocrella camerunensis* Chaverri & H.C. Evans.

Subiculum effusum, subaurantium, Coccideas (Homoptera, Insecta) parasitans. Perithecia aggregata, semiimmersa, aurantia, obpyriformia, a latere collapsa, 3% KOH ope purpurea. Asci cylindrici, nec capitata. Ascosporae unicellulares, hyalina, fusiformes, glabrae. Anamorphosis *Sphaceliae* similis, conidiomata pycnidialia vel acervularia. Conidiophora ramosa. Phialides lageniformes. Conidia ellipsoidea, hyalina, glabra. Holotypus: *Regiocrella camerunensis* Chaverri & H.C. Evans.

Subiculum or stroma restricted to areas that cover scale insects, pale orange, formed of loosely intertwined hyphae; subicular hyphae hyaline, smooth-walled, becoming purple in 3% KOH. Perithecia partly immersed in subiculum, almost naked, obpyriform, collapsing laterally when dry, smooth, a deeper orange than subiculum, KOH− over lower half of perithecium, deep purple in KOH over upper half and papilla. Asci cylindrical, not capitate, 8-spored. Ascospores unicellular, hyaline, smooth, fusiform, sometimes allantoid. Anamorph, where known, forming pale orange colonies in vitro, restricted, elevated, compact, becoming deep purple in KOH. Conidiomata *Sphacelia*-like, somewhat pycnidial to acervular, cupulate to involute. Conidiophores highly aggregated in a hymenium, short, irregularly branched. Phialides flask-shaped. Conidia hyaline, smooth, unicellular, ellipsoidal. On scale insects (Coccidae, Homoptera).

Notes. At first glance, *Regiocrella* resembles the genus *Hypocrella* in the color of the stromata, the anamorph, and the fact that it occurs on scale insects. It also resembles *Torrubiella s. l.* in its almost naked perithecia seated in a subiculum. *Regiocrella* can be distinguished from *Hypocrella*, *Torrubiella* and other genera in the Clavicipitaceae by its unicellular and short fusiform ascospores and its *Sphacelia*-like conidiomata that are unknown in any other genus of entomopathogenic Clavicipitaceae.

Regiocrella camerunensis Chaverri et H.C. Evans, sp. nov. FIGS. 3–17

Anamorph: *Sphacelia*-like.

Subiculum effusum, subaurantium, Coccideas (Homoptera, Insecta) parasitans. Perithecia aggregata, semiimmersa, aurantia, obpyriformia, a latere collapsa, 3% KOH ope purpurea, (184–)192–212(–226) × (115–)124–146(–162). Asci cylindrici, nec capitata, (67–)70–75(–78) × (3.2–)3.5–3.8(–4.0) µm. Ascosporae unicellulares, fusiformes, (6.5–)8.0–9.3(–12.2) × (1.8–)2.0–2.2(–2.5) µm. Anamorphosis *Sphaceliae* similis, conidiomata pycnidialia vel acervularia. Conidiophora ramosa. Phialides lageniformes, (8.0–10.2)4.0–4.5(–6.0) × (1.7–)2.0–2.2(–2.7) µm. Holotypus CUP 67512 (cultura viva ARSEF 7682).

Subiculum restricted to scale-insect hosts, pale orange, formed of loosely intertwined hyphae; subicular hyphae hyaline, smooth-walled, 2–3 µm diam, becoming purple in 3% KOH. Perithecia obpyriform, collapsing laterally when dry, a deeper orange than subiculum, KOH− over lower half of perithecium, deep purple in KOH over upper half and papilla, (184–)192–212(–226) × (115–)124–146(–162) µm. Asci cylindrical, not capitate, (67–)70–75(–78) × (3.2–)3.5–3.8(–4.0) µm. Ascospores unicellular, hyaline, smooth, fusiform, sometimes allantoid, (6.5–)8.0–9.3(–12.2) × (1.8–)2.0–2.2(–2.5) µm. Colonies on PDA at 25 C after ca. 4 wk of growth, pale orange, restricted, elevated, compact, becoming deep purple in KOH. Conidiomata *Sphacelia*-like, forming irregular pycnidial-acervular concave depressions or cavities in colony and lacking a differentiated wall; conidial masses oozing from conidiomata in deep orange, slimy cirrhi. Conidiophores highly aggregated into a compact hymenium lining cavities, short, irregularly branched, sometimes unbranched, once monochasial, monoverticillate, or 2-level monochasial. Phialides flask-shaped, (8.0–)10.2–14.0(–16.5) × 2.0–2.5(–3.0) µm. Conidia hyaline, ellipsoidal, unicellular, (2.5–)4.0–4.5(–6.0) × (1.7–)2.0–2.2(–2.7) µm.

Habitat. On scale insects (Coccidae, Homoptera).

Known distribution. Cameroon (type locality).

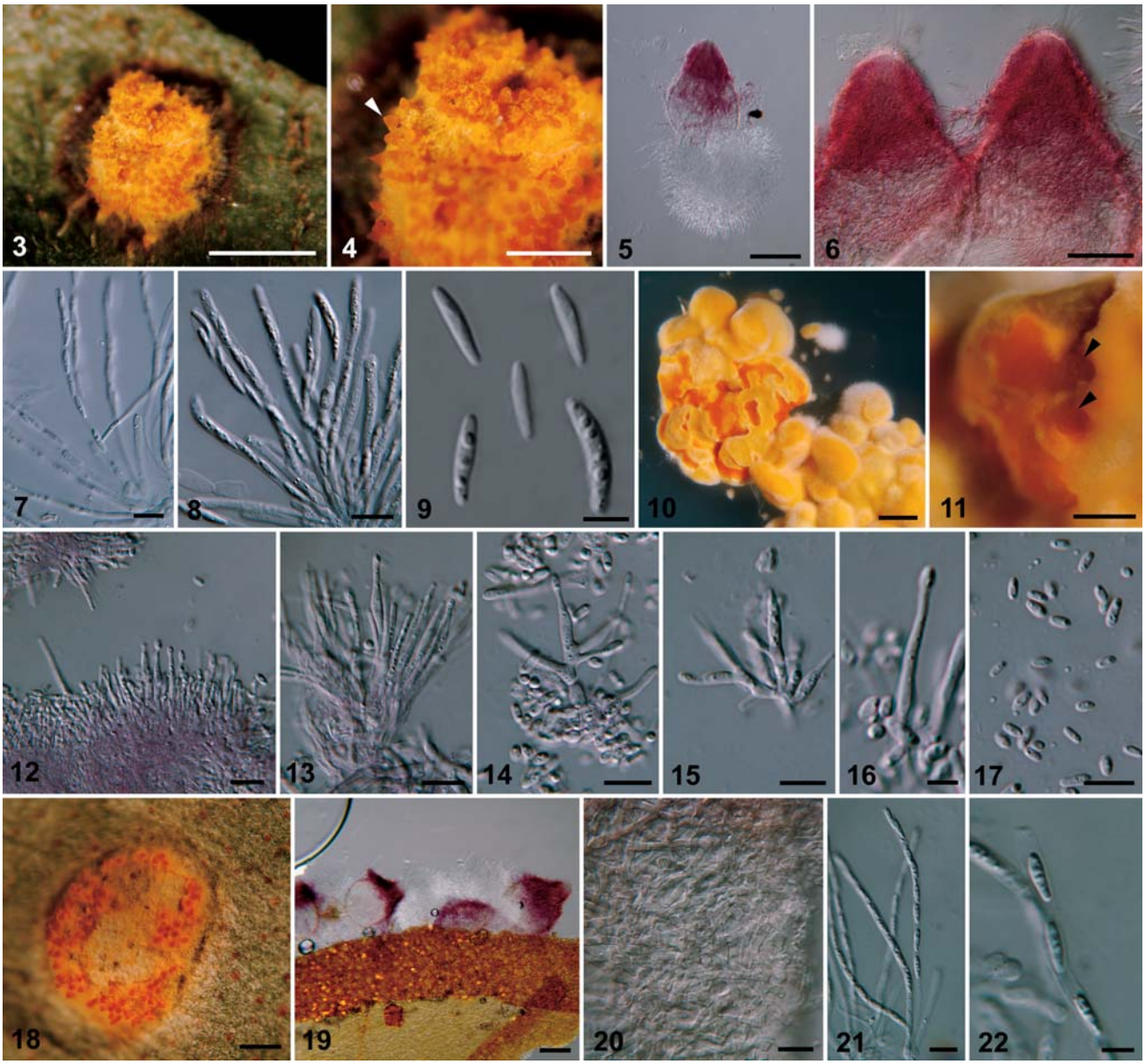
Holotype. CAMEROON. SOUTHWEST PROVINCE: Korup National Park, on scale insects on living fern leaves, Oct 2003, *H.C. Evans I03-1242* (P.C. 725 = CUP 67512; culture ex type ARSEF 7682).

Notes. *Regiocrella camerunensis* can be distinguished from *R. sinensis* by the smaller ascospores and perithecia of the latter. In addition the subiculum of *R. camerunensis* is somewhat thicker than that of *R. sinensis*.

Regiocrella sinensis Chaverri et K.T. Hodge, sp. nov. FIGS. 18–22

Anamorph: Not known.

Subiculum effusum, subaurantium, Coccideas (Homoptera, Insecta) parasitans. Perithecia aggregata, semiimmersa, aurantia, obpyriformia, a latere collapsa, 3% KOH ope purpurea, (162–)172–198(–206) × (120–)129–155



FIGS. 3–22. *Regiocrella*. 3-17. *Regiocrella camerunensis* HOLOTYPE. 3. One stroma. 4. Stroma close-up showing partly naked perithecia (arrow). 5. One perithecium mounted in 3% KOH (notice the darker pigmentation on the upper half of the perithecium). 6. Perithecia, also mounted on 3% KOH. 7, 8. Asci and ascospores. 9. Ascospores. 10, 11. Colony on PDA at 25 C after ca. 4 wk, showing slimy conidial masses and cirrhi (arrows) oozing from pycnidial-acervular conidiomata. 12. Hymenial layer of conidiophores. 13–16. Conidiophores and phialides. 17. Conidia. 18–22. *Regiocrella sinensis* HOLOTYPE. 18. One stroma. 19. Perithecia on host. 20. Tissue of stroma or subiculum. 21. Asci and ascospores. 22. Ascospores. Bars: 3, 10 = 1 mm; 4, 11, 18 = 500 µm, 5, 19 = 100 µm, 6 = 50 µm, 7–9, 12–15, 17, 20, 21 = 10 µm, 16, 22 = 5 µm.

(–162) µm. Asci cylindrici, nec capitata, (72–)74–79(–86) × (3.0–)3.2–3.7(–4.0) µm. Ascosporae unicellulares, fusiformes, (6.0–)7.0–7.5(–8.5) × (1.7–)2.0–2.2(–2.5) µm. Holotypus CUP CH-264.

Subiculum restricted to scale-insect hosts, pale orange, formed of loosely intertwined hyphae; subicular hyphae hyaline, smooth-walled, 2–3 µm diam, becoming purple in 3% KOH. Perithecia obpyriform, collapsing laterally when dry, a deeper orange than subiculum, KOH− over lower half of perithecium, deep purple in KOH over upper half and papilla, (162–)172–198(–206) × (120–)129–155(–162) µm. Asci cylindrical, not capitate, (72–)74–79(–86) × (3.0–)3.2–3.7(–4.0) µm. Ascospores unicellular, hyaline, smooth, fusiform, sometimes allantoid, (6.0–)7.0–7.5(–8.5) × (1.7–)2.0–2.2(–2.5) µm.

Habitat. On scale insects (Coccidae, Homoptera).

Known distribution. China (type locality).

Holotype. CHINA. GUANGDONG PROVINCE: Dinghushan Biosphere Reserve, on scale insects on living dicotyledonous leaves, 10 Aug 2004, *B. Huang DHS 040810-26* (CUP CH-264).

Notes. *Regiocrella sinensis* can be distinguished from *R. camerunensis* by the larger ascospores and perithecia of the latter. In addition the subiculum of *R. sinensis* is somewhat thinner than that of *R. camerunensis*.

DISCUSSION

Phylogenetics of Regiocrella.—*Regiocrella* has pycnidial-acervular conidiomata that resemble the *Aschersonia* anamorphs of *Hypocrella*. Morphological and molecular evidence reveal that the new genus *Regiocrella* is closely related to *Hypocrella* and other genera with similar anamorphs. The *Regiocrella* anamorph is also similar to the *Sphacelia* anamorphs of *Claviceps* and the *Neotyphodium* anamorphs of *Epichlöe*. Phylogenetic analyses place *Regiocrella* and *Hypocrella* closely related to the Clavicipitaceae subfamily Clavicipitoideae, which includes *Balansia, Claviceps, Epichlöe, Myriogenospora* and *Neoclaviceps*. Members of the subf. Clavicipitoideae have anamorphs or synanamorphs that are pycnidial-acervular as well as *Regiocrella* and *Hypocrella*.

The most parsimonious explanation for the distribution of scale insect-associated taxa in clade A on our tree (FIGS. 1, 2b) is that scale parasitism is a plesiomorphic character within this group, as seen by the basal position of an unidentified species of ''*Torrubiella*'' in group B. Tracing and reconstruction of ancestral characters (FIG. 2) also supports the hypothesis that *Regiocrella* might have evolved from scale parasitic ancestors. It also is possible that *Hypocrella/Aschersonia* and the clavicipitaceous plant associates evolved from a *Regiocrella*-like ancestor, given that *Regiocrella* is basal in the clade that includes the plant associates, *Hypocrella/Aschersonia* and *Regiocrella* (clade A in FIG. 1, node 12 FIG. 2). However this conclusion could be further supported by the addition of other genera in the Clavicipitaceae.

Evolution of pycnidial-acervular conidiomata within the Clavicipitaceae.—Within the Clavicipitaceae, pycnidial to acervular anamorphic forms have been assigned to one of three genera: *Ephelis* Fr., *Sphacelia* or *Aschersonia*. These anamorphs are known only for plant-associated genera *Atkinsonella, Balansia, Claviceps, Epichlöe, Myriogenospora* and *Neoclaviceps* and the scale insect parasites *Hypocrella* and *Regiocrella*. The results of this study suggest that this anamorph form has a single evolutionary origin in the Clavicipitaceae (clade A in FIG. 1, node 12 FIG. 2a). This conclusion is supported by previous studies (Kuldau et al 1997, Sullivan et al 2001). Within the group of genera with pycnidial-acervular conidiomata, *Hypocrella/Aschersonia* is easily distinguished by its unicellular fusiform conidia; unpublished data confirm that *Hypocrella/Aschersonia* is a monophyletic group. In contrast several other genera have *Sphacelia*- and *Ephelis*-like anamorphs but do not form monophyletic groups. Sphacelial and ephelidial forms seem to have arisen or have been lost many times within the evolution of the Clavicipitaceae subf. Clavicipitoideae. In conclusion Diehl's (1950) tribes, Clavicipiteae and Balansiae, are not monophyletic. In the Hypocreales, in addition to the clavicipitaceous genera mentioned above, a few species in the Nectriaceae have pycnidial anamorphs (i.e. *Nectria* spp.: anam. *Zythiostroma* Höhnel and *Gyrostroma* Namouv and *Cosmospora kurdica* [Petrak] Rossman & Samuels: anam. pycnidial *Fusarium*) (Rossman et al 1999, Samuels and Seifert 1987).

We speculate that the evolution of pycnidial-acervular anamorphs and glioconidia (conidia borne in a moist substance) is a mere adaptation for spore dispersal. In *Sphacelia*, *Ephelis* and *Neotyphodium* the extrusion of conidia in slime appears to aid dispersal by insects (Butler et al 2001, Hodge 2003, Loveless 1964, Mower et al 1973, Mower and Hancock 1975, Samways 1983). Because scale insects and white flies often secrete sticky honeydew that is attractive to wasps and ants, it also is possible that *Hypocrella/Aschersonia*, as well as related genera, evolved to produce slimy conidia to disperse the spores more efficiently through insects seeking the honeydew. Because the slimy masses of conidia are hygroscopic when water is added, it also might be an adaptation for water dispersal (Chaverri and Samuels 2003, Hodge 2003, Parkin 1906). Insects, such as wasps and ants, could disperse the spores across long distances; water, such as rain splash and run-off, could disperse across short distances.

Evolution of scale insect parasitism and plant associations within the Clavicipitaceae.—The Clavicipitaceae includes a majority of the entomopathogenic fungi. It also includes many taxa that are associated with plants as endophytes or pathogens and fewer taxa that are hyperparasites of other fungi. Previous studies suggested that entomopathogenic genera are polyphyletic within the

Clavicipitaceae (Bischoff et al 2005, Gams and Zare 2001, Sung et al 2001); results of the present study support this assumption. On the other hand previous studies demonstrated that clavicipitaceous plant associates (i.e. *Neoclaviceps, Claviceps, Myriogenospora, Balansia, Atkinsonella, Epichlöe* and *Echinodothis* Atk.) formed a monophyletic group (Bischoff et al 2005, Gams and Zare 2001, Kuldau et al 1997, Sullivan et al 2001, Sung et al 2001). Our study also supports the plant-associated genera as a monophyletic group that is correlated with Diehl's Clavicipitaceae subf. Clavicipitoideae. The monophyly of the tribe Ustilaginoideae (i.e. *Neomunkia* and *Ustilaginoidea*) was proven in Bischoff et al (2004) with phylogenetic analyses of LSU; however its relationship to the other plant-associated genera was unresolved. A more recent study that included more taxa showed that the tribe Ustilaginoideae is closely related and in the same clade as the plant-associated genera (FIG. 3 in Bischoff et al 2005). Additional unpublished data based on additional gene loci support the hypothesis that the tribe Ustilaginoideae is closely related or within the clade of plant-associated genera (J.F. Bischoff pers comm).

We also conclude that scale insect parasitism is polyphyletic and probably an ancestral trait that has been lost or gained many times in the evolution of the family (FIGS. 1, 2). We did not include other taxa that are known to be parasites of scale insects such as *Cordyceps clavulata* (Schw.) Ellis & Everh. (anamorph *Hirsutella*); *Cordyceps coccidiicola* Kobayasi & D. Shimizu (anamorph *Hirsutella*- like); *Cordyceps novae-zealandiae* Dingley (anamorph *Akanthomyces*-like); *Cordyceps yahagiana* Kobayasi & D. Shimizu (anamorph *Hirsutella*-like); *Torrubiella confragosa* Mains (anamorph similar to *Lecanicillium lecanii* [Zimm.] Zare & W. Gams); *Torrubiella iriomoteana* Kobayasi & D. Shimizu (anamorph *Hirsutella*); *Torrubiella lecanii* Johnston (anamorph similar to *L. lecanii*); *Torrubiella luteorostrata* Zimm. (anamorph *Paecilomyces*); *Torrubiella petchii* Hywel-Jones (anamorph *Hirsutella*); *Torrubiella rubra* Patouillard & Lagerh. (anamorph maybe *Engyodontium*); *Torrubiella sphaerospora* Samson, van Reenan & Evans (unknown anamorph); *Torrubiella superficialis* Kobayasi & D. Shimizu (unknown anamorph); *Torrubiella tenuis* Petch (unknown anamorph); and *Torrubiella tomentosa* Patouillard (anamorph *Akanthomyces*-like). However we consider that, because of the great variation in anamorphs and teleomorphs and some preliminary data that include some of the species listed above (Chaverri et al 2005, FIG. 1; Gams and Zare 2001; Sung et al 2001), scale insect parasitism is most likely polyphyletic.

Phylogenetic analyses show the close relationship of a few scale insect parasites and the plant-associated species. Scale-insect parasitism seems to be a plesiomorphic character in the Clavicipitaceae based on the basal position of the Oomycetoideae, which includes mostly scale insect parasites. Therefore we hypothesize that plant-associated species might have evolved from a scale insect pathogen or vice versa, the former being more likely based on the results of the ancestral state reconstruction and tracing of phenotypic characters (FIG. 2b). However to prove this hypothesis further studies that include more genera in the Clavicipitaceae are needed. Several papers have discussed secondary parasitism or ''parasitism by proxy'' in clavicipitaceous genera parasitic on scale insects (i.e. *Hyperdermium*, *Hypocrella/Aschersonia* and *Ascopolyporus* [Hywel-Jones and Samuels 1998, Sullivan et al 2001]); in these genera the stromatal mass greatly exceeds that of the scale insect host. Once the fungus has consumed the scale insect body, the fungus may continue to access plant nutrients through the insect's stylet or the mechanism of nutrient acquisition is through the living scale insect that forms a bridge between the fungus and the plant. Experimental evidence to distinguish between these hypotheses is lacking. We hypothesize that there was a jump from ancestral scale parasitism to plant association or vice versa, due to hyphal growth through the scale insect's stylet to the plant or from the plant to the scale insect. Future studies could help resolve this interesting hypothesis.

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