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Host jumping onto close relatives and across kingdoms by *Tyrannicordyceps* (Clavicipitaceae) gen. nov. and *Ustilaginoidea* (Clavicipitaceae)[1]

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- *Premise of study:* This research seeks to advance understanding of conditions allowing movement of fungal pathogens among hosts. The family Clavicipitaceae contains fungal pathogens exploiting hosts across three kingdoms of life in a pattern that features multiple interkingdom host shifts among plants, animals, and fungi. The tribe Ustilaginoideae potentially represents a third origin of plant pathogenesis, although these species remain understudied. Fungal pathogens that cause ergot are linked morphologically with Clavicipitaceae, but are not yet included in phylogenetic studies. The placement of Ustilaginoideae and ergot pathogens will allow differentiation between the host habitat and host relatedness hypotheses as mechanisms of phylogenetic diversification of Clavicipitaceae.
- *Methods:* A multigene data set was assembled for Clavicipitaceae to test phylogenetic placement and ancestral character-state reconstructions for *Ustilaginoidea virens* and *U. dichromonae* as well as the ergot mycoparasite *Cordyceps fratricida*. Microscopic morphological observations of sexual and asexual states were also performed.
- *Key results:* Phylogenetic placement of *U. virens* and *U. dichromonae* represents a third acquisition of the plant pathogenic lifestyle in Clavicipitaceae. *Cordyceps fratricida* was also placed in Clavicipitaceae and recognized as a new genus *Tyrannicordyceps*. Ancestral character state reconstructions indicate initially infecting hemipteran insect hosts facilitates subsequent changes to a plant pathogenic lifestyle. The ancestor of *T. fratricida* is inferred to have jumped from grasses to pathogens of grasses.
- *Conclusions:* The host habitat hypothesis best explains the dynamic evolution of host affiliations seen in Clavicipitaceae and throughout Hypocreales. Co-occurrence in the same habitat has allowed for host shifts from animals to plants, and from plants to fungi.

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

The tendency of fungal pathogens to switch hosts remains an important field of study as humans facilitate the intentional and unintentional dissemination of biological materials across the globe. Of particular interest are the fungi traditionally classified in Clavicipitaceae (Ascomycota: Hypocreales), whose members play important roles in biological control efforts, and also represent important pathogens of food crops (Scholte et al., 2005; Kanzok and Jacobs-Lorena, 2006; Haarmann et al., 2009). The use of molecular phylogenetics has greatly advanced our understanding of relationships and the evolution of nutritional modes for these fungi. Previously considered a single large and diverse family, Clavicipitaceae sensu Rogerson (1970) is now recognized as encompassing three families, Clavicipitaceae s.s., Cordycipitaceae, and Ophiocordycipitaceae (Sung et al., 2007). Pathogens of plants are unique to Clavicipitaceae s.s. and are hypothesized to have evolved from animal pathogenic ancestors at least twice. Species infecting other fungi are known from all three families and are represented in Clavicipitaceae by a single asexually reproducing species, *Verticillium epiphytum* (Spatafora et al., 2007). In spite of advances in our understanding of phylogenetic relationships and evolution of nutritional modes, many species of clavicipitoid fungi remain inadequately sampled and their ecology unknown.

Phylogenetic analyses and ancestral character state reconstructions support an origin of arthropod pathogens near the Jurassic–Cretaceous boundary, essentially concurrent with the rise of angiosperms and the modern orders of phytophagous insects (Sung et al., 2008). The subsequent acquisition of plant and fungal hosts are supported as shifts from arthropod hosts (Spatafora et al., 2007). Theoretical explanations of how such divergent host ranges evolved among closely related species have been limited to specific cases and have involved two possible hypotheses. The host habitat hypothesis posits that new hosts are acquired due to their proximity in the environment, whereas under the host relatedness hypothesis, pathogens are more likely to jump to new species closely related to the original host. The host habitat hypothesis was found to explain host jumping between cicada nymphs and false truffles owing to their co-occurrence near plant roots (Nikoh and Fukatsu, 2000; Spatafora et al., 2007). Shifts from animal to plant hosts have not been explicitly tested, although pathogens of hemipteran scale insect hosts (families Coccidae, Diaspididae) have been postulated as the ancestral state (Hywel-Jones and Samuels, 1998; Bischoff et al., 2005).

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The majority of plant-associated species in Clavicipitaceae form a clade including the genus *Claviceps*, known to cause ergotism in wheat and other cereals. *Shimizuomyces*, which infects seeds of genus *Smilax* (Liliales, Smilacaceae), represents an independent evolution of the plant pathogenic lifestyle (Sung et al., 2007). The tribe Ustilaginoideae represents a third possible origin of plant pathogenesis among clavicipitaceous fungi (Diehl, 1950; Bischoff et al., 2004). The placement of Ustilaginoideae within Hypocreales has remained elusive due to the autapomorphic asexual reproduction of its species, a lack of extensive knowledge regarding its sexual reproductive states, and the ambiguity of results from molecular data evaluated thus far. Ustilaginoideae includes fungi mostly known from their asexual states. *Ustilaginoidea virens* (Cooke) Takah. is the only member of this tribe with a known sexual state, which is rarely observed in nature, and originally classified as *Claviceps virens* Sakurai ex Nakata. Subsequent work cast doubt on this association, and it was determined to be morphologically distinct from *Claviceps* (Tanaka et al., 2008). *Ustilaginoidea virens* is the causal agent of rice false smut, an agriculturally important disease that reduces rice yields (Ikegami, 1963). Bischoff et al. (2004) provided phylogenetic evidence on the monophyly of the tribe Ustilaginodeae using data from the large subunit of rDNA (LSU), but its phylogenetic placement among the clavicipitaceous fungi was not resolved. In an analysis of acetaldehyde dehydrogenase sequence data, *U. virens* was resolved as monophyletic with the grass endophyte genera of Clavicipeae and Balansieae, but the taxon sampling was insufficient to test alternative hypotheses of the phylogenetic placement among the families of Hypocreales (Tanaka and Tanaka, 2008).

Pathogenesis or parasitism of other fungi is present among all three clavicipitoid families, including asexually reproducing *Simplicillium* (Cordycipitaceae), which infects mushrooms (Zare and Gams, 2001), and *V. epiphytum* (Clavicipitaceae), which is a parasite of rusts (Zare et al., 2001; Spatafora et al., 2007; Sung et al., 2008). *Elaphocordyceps* (Ophiocordycipitaceae) is the only mycopathogenic lineage with known sexually reproductive states and infects false truffles of the genus *Elaphomyces*. Pathogens of ergot are a group of mycoparasites unrepresented in previous phylogenetic analyses and considered residual species of *Cordyceps*. Morphological characteristics support association with the clavicipitoid fungi, but are insufficient to place them within the current taxonomic framework. Five species infecting the sclerotia of *Claviceps* spp. have been described, with a significant representation of diversity in Japan (Örtegren, 1916; Imai, 1936; Kobayasi, 1980; Tanda and Kobayasi, 1984).

Multigene sequence data were obtained from new material for members of tribe Ustilaginoideae and the ergot pathogens to resolve their phylogenetic relationships among representative taxa from the major clades of Clavicipitaceae. Ancestral character state reconstructions were performed with insect hosts coded to order to test the hypothesis that arthropod pathogens, specifically of hemipteran hosts, gave rise to plant pathogens, and to distinguish between the explanatory power of the host habitat vs. host relatedness hypotheses with respect to interkingdom host-jumping of the family.

MATERIALS AND METHODS

***Specimen collection*—**Fresh material for *Ustilaginoidea virens* was isolated from rice false smut balls that were collected from rice paddy field IPU010 in Kanazawa, Ishikawa, Japan on 5 September 2007. A reference culture was deposited at the Ministry of Agriculture, Forestry and Fisheries (MAFF). Specimens of *T. fratricida* were collected from Mt. Iwakisan, Hirosaki-shi, Aomori Pref. on sclerotia of *Claviceps* sp. infecting *Phragmites* sp. in 2007. Sclerotia were taken back to the laboratory and incubated over winter. Stromata emerged the following spring. Cultures were obtained from single ascospore isolates. Additional analysis was performed for *U. virens* ATCC 16180, *U. dichromonae* MRL 1B9228, and *Dussiella tuberiformis* (Berk. & Ravenel) Pat. collected by J. F. White in North Carolina from *Arundinaria tecta* in 2000.

***DNA extraction, PCR, and sequencing*—**Genomic DNA of *U. virens* was extracted using QuickGene DNA extraction kit (Fujifilm, Japan). Samples of *T. fratricida* were prepared for downstream molecular work by grinding stromatal tissue with a plastic pestle attached to a power drill in 50 μL hexadecyltrimethylammonium bromide (CTAB) buffer. Ground tissue was transferred to a FastDNA lysing matrix A tube (MP Biomedical, Salon, Ohio, USA), combined with an additional 400 μL CTAB buffer and further macerated with the fast prep machine for two rounds, 20 s each. Tubes were then placed in a water bath at 60°C for 20 min to further facilitate cell lysis. Coarse fungal tissue was then separated from the supernatant by centrifugation for 10 min at 14 000 rpm. The supernatant was cleaned further by transferring 400 μL to 1.5 mL tubes Eppendorf tubes with 500 μL chloroform–isoamyl alcohol (24 : 1) and another centrifugation at 14 000 rpm for 20 min. Then 300 μL of supernatant was removed, and DNA in the supernatant was then concentrated using the GeneCleanIII Kit (MP Biomedical) following the recommended protocol and eluting with 30 μL of water.

Following DNA extraction, amplification of multiple loci was achieved by polymerase chain reaction (PCR). The complete span of the internal transcribed spacer (ITS) region of ribosomal DNA, including ITS1, 5.8S, and ITS2, was sequenced as a quality control measure and to serve as a template for later barcoding efforts, but this locus was not used in phylogenetic analyses. Attempts were made to amplify fragments of five nuclear loci for phylogenetic analysis: nuclear ribosomal small subunit (SSU) and nuclear ribosomal large subunit (LSU) DNA, elongation factor 1α (TEF), and the largest and second largest subunits of RNA polymerase II (RPB1 and RPB2, respectively) with a total read length nearing 5000 bp. Primer information is given in Table 1. PCR reactions were performed in either an iCycler or MyCycler thermocycler (BioRad, Hercules, California, USA) using MasterAmp 2× PCR premix E (Epicenter, Madison, Wisconsin, USA) and Novagen *Taq* polymerase (Darmstadt, Germany). Reaction conditions were the same as those used in Johnson et al. (2009). PCR products were cleaned using the GeneClean III kit following the manufacturer's instructions and sequenced using the Macrogen (Seoul, South Korea) sequencing service with the primers used for the initial amplifications.

TABLE 1. Information for primers used to amplify sequences used in this study.

Gene	Primer	5′-Sequence-3′	Source
TEF	983F	GCYCCYGGHCAYCGTGAYTTYAT	Carbone and Kohn, 1999
TEF	2218R	ATGACACCRACRGCRACRGTYTG	Rehner and Buckley, 2005
LSU	LR5	ATCCTGAGGGAAACTTC	Vilgalys and Sun, 1994
LSU	LR0R	GTACCCGCTGAACTTAAGC	Vilgalys and Sun, 1994
SSU	SR7	CTTCCGTCAATTCCTTTAAG	White et al., 1990
SSU	NS4	CTTCCGTCAATTCCTTTAAG	White et al., 1990
SSU	NS3	GCAAGTCTGGTGCCAGCAGCC	White et al., 1990
SSU	NS1	GTAGTCATATGCTTGTCTC	White et al., 1990
RPB1	RPB1Cr	CCNGCDATNTCRTTRTCCATRTA	Castlebury et al., 2004
RPB1	CRPB1A	CAYCCWGGYTTYATCAAGAA	Castlebury et al., 2004
RPB2	fRPB2-7cR	CCCATRGCTTGTYYRCCCAT	Liu et al., 1999
RPB2	fRPB2-5F	GAYGAYMGWGATCAYTTYGG	Liu et al., 1999

***Phylogenetic analyses*—**Raw sequence reads were edited using CodonCode Aligner, version 2.0.6 (Dedham, Massachusetts, USA). Sequences generated in this study were combined with previously published data for species in Clavicipitaceae, as well as outgroup taxa from Hypocreaceae, Cordycipitaceae, and Ophiocordycipitaceae. GenBank and specimen voucher information is provided in Table 2. Individual gene alignments were generated using the program MAFFT version 6 (Katoh et al., 2002; Katoh and Toh, 2008) and improved by direct examination in the program BioEdit version 7.05. Ambiguously aligned regions were excluded from phylogenetic analyses and gaps were treated as missing data. Analysis of neighbor joining trees between individual genes with the program compat.py (Kauff and Lutzoni, 2002) did not reveal any significant levels of conflict among the sequences. Maximum likelihood (ML) analysis was performed with the program RAxML version 7.2.6 (Stamatakis, 2006) on a concatenated data set containing all five genes. The data set consisted of 11 data partitions, one each for SSU and LSU plus nine for each of the three codon positions for the protein-coding genes *TEF*, *RPB1*, and *RPB2*. The CAT-GAMMA model of evolution was employed during the generation of 500 bootstrap replicates, and the GTR-GAMMA model of evolution was specified for the final likelihood tree. Bayesian analyses were conducted with the program Mr. Bayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Two runs were executed simultaneously, each with one hot and three cold chains for 10 million generations. The data set was partitioned as in the maximum likelihood run with the GTR+G+I model specified for each partition. After completion of the analysis both runs were inspected with the program Tracer v1.4 (Drummond and Rambaut 2007) to determine whether they had converged on a stationary phase and to configure the burnin. Post burnin trees were then used to construct a strict consensus tree in the program Mr.Bayes. All phylogenetic analyses were conducted using the Genome Cluster at the Center for Genome Research and Biocomputing, Oregon State University.

***Ancestral character-state reconstruction*—**Two character-coding schemes were employed to examine host shifts in Clavicipitaceae. A four-state scheme (fungi, plant, animal, and soil), with hosts coded to kingdom, was used for comparisons with previous studies (Spatafora et al., 2007; Sung et al., 2008). Animal hosts included the insect orders Coleoptera, Hemiptera, and Lepidoptera, as well as mollusks and rotifers. A six-state scheme was also used, with insect hosts coded to order. It is often difficult to diagnose insect cadavers to finer taxonomic levels because of the extreme morphological disfiguration that accompanies infection and the relatively character-poor morphologies of some of the life stages infected. Species attacking the following insect orders were represented in the taxon sampling: Coleoptera, Hemiptera, and Lepidoptera. Species attacking plants or fungi were scored simply as "plant" or "fungi", respectively. A category for hosts outside of the class Insecta (e.g., mollusks or nematodes) and specimens obtained from soil samples was also included.

Results of the Bayesian analysis were used to reconstruct ancestral character states (Ekman et al., 2008). The final 200 trees saved during one run of the Bayesian analysis were sampled for use in Bayesian posterior mapping of ancestral characters (Huelsenbeck and Bollback, 2001; Huelsenbeck et al., 2003) with the program SIMMAP (Bollback, 2006). An empirical prior was used for the bias parameter. Values for the gamma prior were determined by the "configure mcmc" function in SIMMAP followed by analysis of the output in the R statistical package with the sumprmcmc.r script distributed with the program. For the kingdom level coding, the selected values for the gamma prior were $\alpha = 5.512$, $\beta = 2.461$, and $k = 10$. For coding of insects to order the selected values for the gamma prior were $\alpha = 13.508$, $\beta = 2.22$, and $k = 10$. Branch lengths for the trees analyzed were rescaled to one. The analysis was run with 100 samples and 100 draws from the prior distribution. For both coding schemes, maximum likelihood reconstructions were performed on the Bayesian consensus tree in the program Mesquite 2.74 (Maddison and Maddison, 2011). The Mk1 model of state changes was used for the reconstructions. Best states were determined by a difference of two log-likelihoods from the next best state (Pagel, 1999) and the likelihood decision threshold (LDT) was set to 2.

***Morphological examination*—**To observe conidial masses of *U. virens*, we processed tissues using a paraffin-embedding technique. Briefly, conidia were fixed in FAA solution (4% formaldehyde, 5% glacial acetic acid, and 50% ethanol [v/v]), dehydrated, embedded in paraffin and sectioned at a thickness of 10 μm. For observing conidiogenesis in culture, 50 mL of a simple liquid medium (0.1% yeast extract, 0.1% tryptone, 1% glucose [w/v]) was inoculated with *U. virens* conidia and incubated at 25°C on rotary shaker (180 rpm) in 300-mL conical flask for several days, then mounted on a slide with a cover slip. Conidiogenesis of the fungus was observed after incubating the mounted slide for a few hours in a humid chamber.

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

RESULTS

***Gene sampling and phylogenetic analyses*—**Amplification and sequencing efforts for *U. virens* were successful for LSU, *TEF*, and *RPB2*. Repeated efforts failed to obtain quality sequences for SSU and *RPB1*. For *T. fratricida*, amplification and sequencing was successful for SSU, LSU, *RPB1*, and *RPB2*. After removing ambiguously aligned positions the combined alignment contained 4717 bp. GenBank information for all sequences used in this analysis is provided in Table 2.

Topologies resulting from Bayesian and ML tree searches largely agreed with one another, although some branches receiving support in Bayesian analyses were unsupported (<70% BP) under ML. The inclusion of taxa considered in this analysis did not alter the overall topology of hypocrealean fungi observed in previous works (Sung et al., 2007; Johnson et al., 2009). We identified two major divisions within the family: The *Metacordyceps* clade, containing pathogens that primarily infect hosts in Coleoptera and Lepidoptera (Kepler et al., 2011) and a well-supported plant-hemipteran pathogen clade. The genus *Ustilaginoidea* was found to diverge at the base of the plant-Hemiptera clade, independent of the previously identified clades of plant-infecting species. We find strong support for the placement of *T. fratricida* as a sister taxa to the asexual *V. epiphytum* nested within a clade of grass-associated species. The sexually reproducing taxa most closely related to *T. fratricida* in our phylogeny are species of the genera *Balansia* and *Myriogenospora*.

***Ancestral character-state reconstructions*—**The values for the proportional likelihoods and posterior probabilities obtained from ML and Bayesian ancestral character-state reconstructions (ACSR), respectively, are given for both coding schemes in Table 3. Values are only reported for nodes of the plant-Hemiptera clade subtending interkingdom host shifts with Bayesian posterior probabilities of 0.95 or higher (Fig. 1). For the four-state coding scheme, node A, at the base of the plant-hemiptera clade, and node B receive strong support for an animal pathogenic ancestor in ML analyses. However, in Bayesian analyses, an animal pathogenic ancestor is only moderately supported for node A, and node B is ambiguous between a plant and animal pathogenic ancestor. An animal pathogenic ancestor for nodes C and D is strongly supported by both methods. Nodes E and F are strongly supported as having a plant pathogenic ancestor by both methods.

For the six-state coding scheme, maximum likelihood ACSR analyses fail to adequately resolve a single best state for nodes A, B, C, and D using a threshold of two log-likelihood differences. Node A, the most recent common ancestor (MRCA) to the plant-hemipteran clade, is resolved equivocally as plant (0.345) or hemipteran (0.443). However, a hemipteran host was always the highest scoring for nodes B (0.678), C (0.769), and D (0.753), with a plant pathogenic ancestor as the next best state (0.303, 0.223, 0.232, respectively). Nodes E (0.911) and F (0.891) were resolved as plant pathogen ancestors in support of the *Tyrranicordyceps*-*Verticillium* clade as being derived from a plant pathogenic ancestor.

TABLE 2. Taxa, hosts, specimen vouchers, and sequence information for specimens used in this study.

Species	Voucher_info	Host	GenBank accession numbers					
			ITS	SSU	LSU	TEF	RPB1	RPB2
Aschersonia cf *.badia*	BCC 7016	Hemiptera	JN049839	DQ372091	DQ384941	DQ384969	DQ385009	DQ452460
Aschersonia confluence	BCC 7961	Hemiptera	JN049841	DQ372100	DQ384947	DQ384976	DQ384998	DQ452465
Aschersonia placenta	BCC 7869	Hemiptera	JN049842	EF469121	EF469074	EF469056	EF469085	EF469104
Balansia epichloë	AEG 96-15a	Plant	JN049848	EF468949		EF468743	EF468851	EF468908
Balansia henningsiana	GAM 16112	Plant	JN049815	AY545723	AY545727	AY489610	AY489643	DQ522413
Balansia pilulaeformis	AEG 94-2	Plant	JN049816	AF543764	AF543788	DQ522319	DQ522365	DQ522414
Claviceps fusiformis	ATCC 26019	Plant	JN049817	DQ522539	U17402	DQ522320	DQ522366	
Claviceps paspali	ATCC 13892	Plant	JN049818	U32401	U47826	DQ522321	DQ522367	DQ522416
Claviceps purpurea	SA cp11	Plant		EF469122	EF469075	EF469058	EF469087	EF469105
Claviceps purpurea	GAM 12885	Plant	U57669	AF543765	AF543789	AF543778	AY489648	DQ522417
Conoideocrella luteorostrata	NHJ 12516	Hemiptera	JN049860	EF468994	EF468849	EF468800	EF468905	EF468946
Conoideocrella luteorostrata	NHJ 11343	Hemiptera	JN049859	EF468995	EF468850	EF468801	EF468906	
Conoideocrella tenuis	NHJ 345.01	Hemiptera		EU369111	EU369045	EU369030		EU369088
Conoideocrella tenuis	NHJ 6293	Hemiptera	JN049862	EU369112	EU369044	EU369029	EU369068	EU369087
Conoideocrella tenuis	NHJ 6791	Hemiptera	JN049863	EU369113	EU369046	EU369028	EU369069	EU369089
Cordyceps brongniartii	BCC 16585	Lepidoptera	JN049867	JF415951	JF415967	JF416009	JN049885	JF415991
Cordyceps gunnii	OSC 76404	Lepidoptera	JN049822	AF339572	AF339522	AY489616	AY489650	DQ522426
Cordyceps militaris	OSC 93623	Lepidoptera	JN049825	AY184977	AY184966	DQ522332	DQ522377	AY545732
Dussiella tuberiformis	J.F. White, Scale on *Arundinaria tecta*, North Carolina, 2000	Hemiptera				JQ257027	JQ257015	JQ257020
Elaphocordyceps ophioglossoides	OSC 106405	Fungi		AY489691	AY489723	AY489618	AY489652	DQ522429
Epichloë typhina	ATCC 56429	Plant	JN049832	U32405	U17396	AF543777	AY489653	DQ522440
Hypocrea rufa	CBS 114374	Plant		AY489694	AY489726	AY489621	AY489656	EF692510
Hypocrella discoidea	BCC 8237	Hemiptera	JN049840		DQ384937	DQ384977	DQ385000	DQ452461
Metacordyceps atrovirens	TNM F10184	Coleoptera	JN049882	JF415950	JF415966		JN049884	
Metacordyceps chlamydosporia	CBS 101244	Nematode/Rotifer	JN049821	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
Metacordyceps indigotica	TNS F18553	Lepidoptera	JN049874	JF415953	JF415968	JF416010	JN049886	JF415992
Metacordyceps indigotica	TNS F18554	Lepidoptera	JN049875	JF415952	JF415969	JF416011	JN049887	JF415993
Metacordyceps khaoyaiensis	BCC 14290	Lepidoptera	JN049868		JF415971	JF416013	JN049889	
Metacordyceps khaoyaiensis	BCC 12687	Lepidoptera	JN049869		JF415970	JF416012	JN049888	
Metacordyceps kusanagiensis	TNS F18494	Coleoptera	JN049873	JF415954	JF415972	JF416014	JN049890	
Metacordyceps liangshanensis	EFCC 1523	Lepidoptera		EF468961	EF468814	EF468755		EF468918
Metacordyceps liangshanensis	EFCC 1452	Lepidoptera		EF468962	EF468815	EF468756		
Metacordyceps martialis	EFCC 6863	Lepidoptera			JF415974	JF416015		JF415994
Metacordyceps martialis	TTZ070716-04	Lepidoptera	JN049871	JF415955	JF415973		JN049891	
Metacordyceps martialis	HMAS 197472(S)	Lepidoptera	JN049881	JF415956	JF415975	JF416016	JN049892	JF415995
Metacordyceps owariensis	NBRC 33258	Hemiptera	JN049883		JF415976	JF416017		JF415996
Metacordyceps pseudoatrovirens	TNSF 16380	Coleoptera	JN049870		JF415977		JN049893	JF415997
Metacordyceps taii	ARSEF 5714	Lepidoptera	JN049829	AF543763	AF543787	AF543775	DQ522383	DQ522434
Metacordyceps yongmunensis	EFCC 2135	Lepidoptera		EF468979	EF468834	EF468769	EF468877	
Metacordyceps yongmunensis	EFCC 2131	Lepidoptera	JN049856	EF468977	EF468833	EF468770	EF468876	
Metarhizium album	ARSEF 2082	Hemiptera	AY375446	DQ522560	DQ518775	DQ522352	DQ522398	DQ522452
Metarhizium anisopliae	ARSEF 3145	Coleoptera	JN049834	AF339579	AF339530	AF543774	DQ522399	DQ522453
Metarhizium flavoviride	ARSEF 2037	Hemiptera	AF138271	AF339580	AF339531	DQ522353	DQ522400	DQ522454
Metarhizium sp.	HMAS 199590	Coleoptera	JN049876	JF415960	JF415983	JF416023	JN049898	JF416002
Metarhizium sp.	HMAS 199592	Coleoptera	JN049877	JF415961	JF415984	JF416024	JN049899	JF416003
Metarhizium sp.	HMAS 199596	Coleoptera	JN049878	JF415962	JF415985	JF416025	JN049900	JF416004
Metarhizium sp.	HMAS 199603	Coleoptera	JN049880	JF415963	JF415986	JF416026	JN049901	JF416005
Moelleriella mollii	BCC 7963	Hemiptera		DQ372087		DQ384964	DQ385004	DQ452466
Moelleriella schizostachyi	BCC 1985	Hemiptera		DQ372105	DQ384939	DQ384959	DQ385012	DQ452471
Myriogenospora atramentosa	AEG 96-32	Plant	JN049835	AY489701	AY489733	AY489628	AY489665	DQ522455
Nomuraea cylindrospora	TNS 16371	Hemiptera		JF415964	JF415987	JF416027	JN049902	
Nomuraea cylindrospora	RCEF 3632	Hemiptera	JN049872	JF415959	JF415982	JF416022		
Nomuraea rileyi	CBS 806.71	Lepidoptera	AY624205	AY624205	AY624250	EF468787	EF468893	EF468937
Ophiocordyceps sinensis	EFCC 7287	Lepidoptera	JN049854	EF468971	EF468827	EF468767	EF468874	EF468924
Orbiocrella petchii	NHJ 6209	Hemiptera	JN049861	EU369104	EU369039	EU369023	EU369061	EU369081
Orbiocrella petchii	NHJ 5318	Hemiptera		EU369105	EU369040	EU369021	EU369062	EU369080
Paecilomyces carneus	CBS 239.32	Soil	AY624171	EF468988	EF468843	EF468789	EF468894	EF468938
Paecilomyces marquandii	CBS 182.27	Soil	AY624193	EF468990	EF468845	EF468793	EF468899	EF468942
Pochonia bulbillosa	CBS 145.70	Nematode/Rotifer		AF339591	AF339542	EF468796	EF468902	EF468943
Pochonia chlamydosporia	CBS 504.66	Nematode/Rotifer	AJ292398	AF339593	AF339544	EF469069	EF469098	EF469120
Pochonia gonioides	CBS 891.72	Nematode/Rotifer	AJ292409	AF339599	AF339550	DQ522354	DQ522401	DQ522458
Pochonia rubescens	CBS 464.88	Nematode/Rotifer		AF339615	AF339566	EF468797	EF468903	EF468944
Regiocrella camerunensis	ARSEF 7682	Hemiptera			DQ118735	DQ118743	DQ127234	
Samuelsia rufobrunnea	P.C. 613	Hemiptera			AY986918	AY986944	DQ000345	
Shimizuomyces paradoxus	EFCC 6279	Plant	JN049847	EF469131	EF469084	EF469071	EF469100	EF469117

TABLE 2. Continued.

Species	Voucher_info	Host	GenBank accession numbers					
			ITS	SSU	LSU	TEF	RPB1	RPB2
Shimizuomyces paradoxus	EFCC 6564	Plant		EF469130	EF469083	EF469072	EF469101	EF469118
Tyrannicordyceps fratricida	TNS 19011	Fungi		JQ257022	JQ257023	JQ257028	JQ257016	JQ257021
Ustilaginoidea dichromonae	MRL IB9228	Plant				JQ257025	JQ257013	JQ257018
Ustilaginoidea virens	ATCC 16180	Plant				JQ257026	JQ257014	JQ257019
Ustilaginoidea virens	MAFF 240421	Plant	JQ349068		JQ257011	JQ257026		JQ257017
Verticillium epiphytum	CBS 154.61	Fungi	AJ292404	AF339596	AF339547	EF468802		EF468947
Verticillium epiphytum	CBS 384.81	Fungi		AF339596	AF339547	DQ522361	DQ522409	DQ522469

Bayesian posterior probabilities provided comparable levels of support for the resolution of ancestral character states with some exceptions. Ancestral character states for node A (MRCA of the plant-hemipteran clade), node D (MRCA to *Regiocrella* [scale insect pathogen] and *Shimizuomyces*), and node F (MRCA to *Tyrannicordyceps* and *Balansia* clades) were weakly resolved in Bayesian reconstructions (0.754, 0.749, and 0.714, respectively). Bayesian reconstructions did result in stronger support for hemipteran pathogenic ancestors for node B (0.934), the plant-hemipteran clade excluding *Ustilaginodea*, and node C (0.977), the common ancestor to *Shimizuomyces* and the larger scale pathogen clade. SIMMAP analyses also strongly supported a plant-associated ancestor for node E, the common ancestor of the grass symbiont and *Tyrannicordyceps* clades.

Morphological examinations—*Ustilaginoidea virens* is characterized by producing a mycelial ball (= pseudosclerotium) on the spikelet of rice (Fig. 2C, G). Its conidia are produced in the outer layer of the mycelial ball that is comprised of spore-bearing hyphae, which produces conidia pleurogenously

TABLE 3. Likelihood scores and posterior probabilities from maximum likelihood (ML) and Bayesian ancestral character-state reconstructions

Clade	Kingdom-level character-coding			Insect-order character-coding		
	Character state	ML proportional likelihood [a]	Bayesian posterior probability [b]	Character state	ML proportional likelihood [a]	Bayesian posterior probability [b]
A	Fungi	8.11762216E-5	0.000008	Fungi	0.00545249	0.002655
	Plant	0.00735799	0.171603	Plant	0.34578026*	0.211416
	Animal	0.99248548*	**0.828389**	Hemiptera	0.44345763*	**0.754572**
	Soil	7.53599662E-5	0.000001	Coleoptera	0.00592862	0.004312
				Lepidoptera	0.11271432*	0.002067
				Other	0.08666668*	0.024978
B	Fungi	1.50912004E-5	0.000025	Fungi	0.00138345	0.000529
	Plant	0.00639613	0.401514	Plant	0.30252438*	0.063773
	Animal	0.993582397*	**0.59846**	Hemiptera	0.67885695*	**0.935054**
	Soil	6.38541092E-6	0	Coleoptera	0.000680	0.000215
				Lepidoptera	0.00933281	0.000215
				Other	0.00722223	0.000215
C	Fungi	3.20683272E-5	0.000013	Fungi	0.00100245	0.001720
	Plant	0.00489343	0.01925	Plant	0.22288895*	0.016959
	Animal	0.995044299*	**0.980732**	Hemiptera	0.76920216*	**0.977012**
	Soil	3.02029611E-5	0.000001	Coleoptera	0.0007928	0.001436
				Lepidoptera	0.00337124	0.001436
				Other	0.00274232	0.001436
D	Fungi	0.00106606	0.000047	Fungi	0.00304001	0.020291
	Plant	0.01887501	0.034174	Plant	0.23177899*	0.169898
	Animal	0.97899414*	**0.965773**	Hemiptera	0.75345494*	**0.749403**
	Soil	0.00106479	0.000006	Coleoptera	0.00289995	0.020136
				Lepidoptera	0.00462323	0.020136
				Other	0.00420288	0.020136
E	Fungi	0.03776503	0.001359	Fungi	0.03824035	0.040382
	Plant	0.91490396*	**0.963192**	Plant	0.91199291*	**0.941525**
	Animal	0.04649292	0.035448	Hemiptera	0.04538849	0.012216
	Soil	8.38081973E-4	0.000001	Coleoptera	0.00123826	0.001959
				Lepidoptera	0.00161607	0.001959
				Other	0.00152391	0.001959
F	Fungi	0.07156496	0.032226	Fungi	0.00152391	0.274489
	Plant	0.902798599*	**0.936584**	Plant	0.89188251*	**0.714014**
	Animal	0.02479434	0.031188	Hemiptera	0.02478018	0.002874
	Soil	8.42097812E-4	0.000002	Coleoptera	0.00137277	0.002874
				Lepidoptera	0.00157308	0.002874
				Other	0.00152422	0.002874

[a] Asterisks indicate best states in ML character-state reconstructions with likelihood decision threshold = 2.
[b] Highest posterior probability values for each node are in boldface

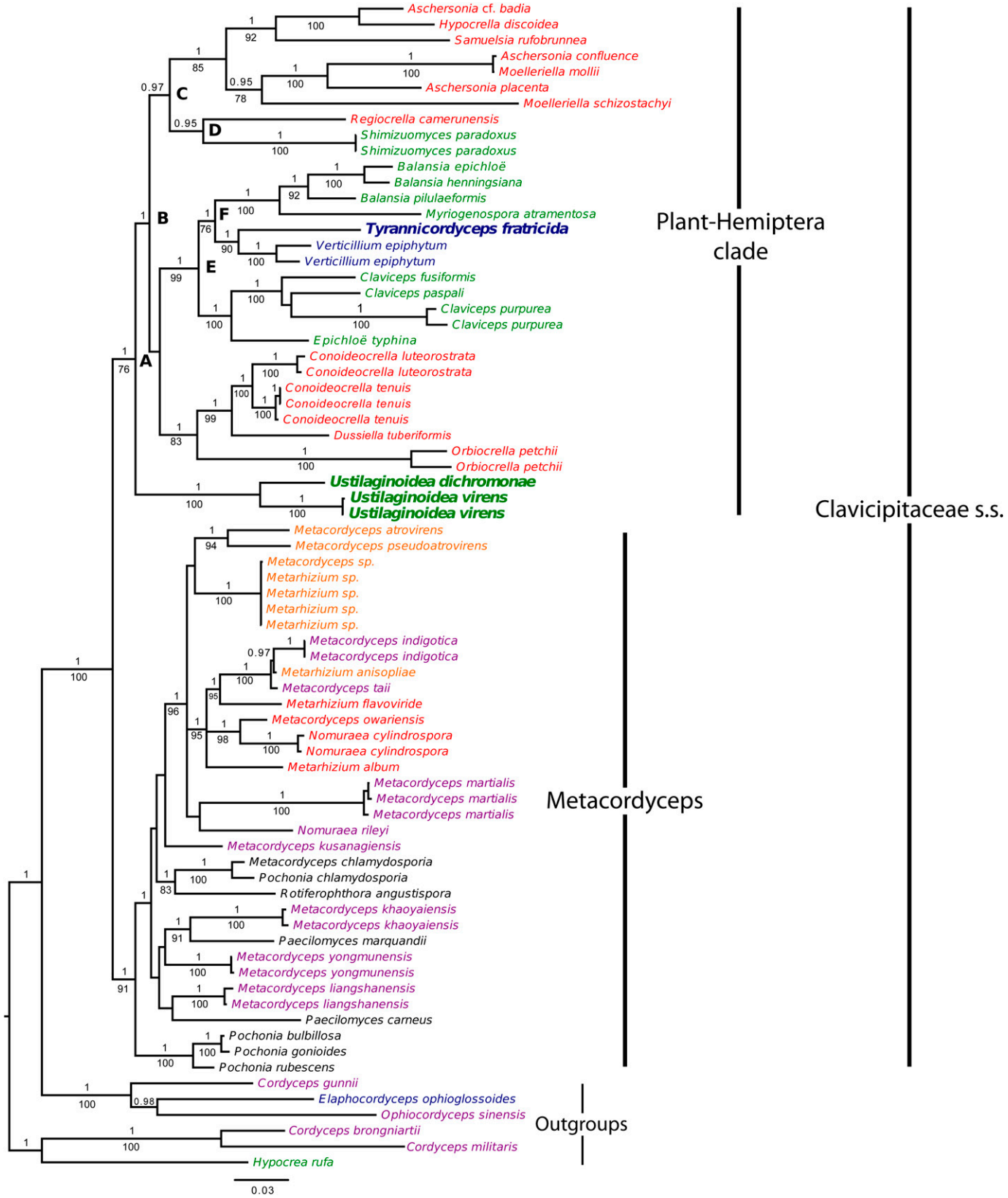

Fig. 1. Strict consensus tree produced by Bayesian analysis of a concatenated data set of five genes (SSU, LSU, TEF, *RPB1*, *RPB2*) showing the placement of *Tyrannicordyceps fratricida* and *Ustilaginoidea virens*. Numbers above branches represent Bayesian posterior probabilities. Maximum likelihood bootstrap proportions greater than 70% are given below branches. Letters indicate nodes of interest in ancestral character-state reconstructions. Species are colored by host association: red = Hemiptera, green = plant, blue = fungi, orange = Coleoptera, purple = Lepidoptera, black = other.

and holoblastically on short sterigmata (Bischoff et al., 2004; Takahashi, 1896) (Fig. 2A). Its conidia are verrucose, thick-walled, and dark-green and can germinate to produce secondary conidia, which are hyaline and globose to subglobose (Fig. 2B, F). Conidiogenesis of secondary conidia involves conidiogenous cells that are typically simple and hyaline and conidia are produced holoblastically and sympodially at the apex of each conidiophore (Fig. 2D). These holoblastic conidia appeared to be produced in whorls at the apex of conidiogeneous cells (Fig. 2E).

Tyrannicordyceps fratricida produces a stipe that emerges from the ergot sclerotium after overwintering (Fig. 2H). The stipe is pale to ochraceous with perithecia clustered apically in a loosely defined clava, and partially pseudoimmersed. The ascus is cylindrical with a prominent apical cap, typical of Clavicipitaceae (Fig. 2I, K). Ascospores are filiform and form part-spores at maturity (Fig. 2J). Cultures obtained from germinated ascospores produced a *Verticillium*-like anamorph (Fig. 2L) (Zare et al., 2001). Hyphae and conidia are hyaline (Fig. 2M, N). Conidia were produced in a droplet at the tips of the phialides (Fig. 2N). The ITS locus was amplified from DNA extracted from stromata and culture as a quality control step and found to be 100% identical for both sources.

TAXONOMY

Tyrannicordyceps Kepler & J. W. Spatafora genus novum— Stroma pallidum vel interdum cinnabarinum, sclerotia specierum in Clavicipibus exortum. Perithecia super stipite dispersa vel ad apicem fasciculata. Asci cylindrici operculo apicali. Ascosporae filiformes, septa formantes in sporis-partibus fortasse disarticulantibus.

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

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

Cordyceps fratricida (Tanda & Kobayasi)—Mycobank number: 563726

Tyrannicordyceps clavicipiticola (Tokun. & S. Imai) Kepler & Spatafora comb. nov—Mycobank number: 563728

Basionym: *Cordyceps clavicipiticola* Tokun. & S. Imai, Trans. Sapporo Nat. Hist. Soc. 14: 104 (1935).

Tyrannicordyceps clavicipitis (Örtegren) Kepler & Spatafora comb. nov—Mycobank number: 563727

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

Tyrannicordyceps ergoticola (Tanda & Kawat.) Kepler & Spatafora comb. nov—Mycobank number: 563729

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

Tyrannicordyceps fratricida (Tanda & Kobayasi) Kepler & Spatafora comb. nov—Mycobank number: 563731

Basionym: *Cordyceps fratricida* Tanda & Kobayasi, J. Agric. Sci., Tokyo Univ. Agric. 29:36 (1984).

Tyrannicordyceps sclerotium (Kobayasi) Kepler & Spatafora comb. nov—Mycobank number: 563732

Basionym: *Cordyceps sclerotium* Kobayasi, Journ. Jap. Bot., Volume 55:89 (1980).

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

DISCUSSION

The array of host–pathogen relationships found in the Clavicipitaceae are the most diverse among hypocrealean fungi and are marked by repeated interkingdom host jumps, a fact highlighted by the inclusion of *U. virens*, *U. dichromonae*, and *T. fratricida*. The plant-hemipteran clade of Clavicipitaceae illustrates this diversity, and molecular tools have fostered a recent burst of activity describing the taxa associated with this group (Chaverri et al., 2005a, b, 2008; Johnson et al., 2009). Plant-associated fungi, such as the *Claviceps-Balansia* clade and *Shimizuomyces* are hypothesized to have independently evolved from lineages of animal pathogens (Spatafora et al., 2007; Sung et al., 2008). The placement of *Ustilaginoidea* represents a third lineage of plant pathogenic fungi in Clavicipitaceae that is not monophyletic with either of the two established plant-associated clades. With the placement of *T. fratricida*, we see that the plant pathogenic lifestyle in turn gave way to one that utilizes other fungi for nutrition (Fig. 1). The ancestral character state reconstruction for *Ustilaginoidea* is equivocal between hemipteran and plant ancestral hosts. The ancestral host affiliation for the Clavicipitaceae, however, is resolved as an animal (arthropod) pathogen (Spatafora et al., 2007; Sung et al., 2008), and all scale insect pathogens and plant-associated fungi of the family are confined to the plant-hemipteran subclade of the family (Fig. 1).

Scale insects and the origin of plant-associated Clavicipitaceae— Previous studies have suggested interkingdom host shifts onto plants are facilitated by ancestors infecting scale insects (Hywel-Jones and Samuels, 1998; Bischoff et al., 2004, 2005; Koroch et al., 2004). Maximum likelihood and Bayesian ACSR analyses find good support for an animal pathogen ancestor when characters states were coded as animal, plant, fungi, and other (Table 3; Sung et al., 2007, 2008; Spatafora et al., 2007). ACSR analyses with a larger number of character states based on coding animal host to the ordinal level resulted in less significant reconstructions, however (Table 3). This finding of a diminished ability to reconstruct ACS under more complex character coding has been observed in other systems (Hibbett, 2004; Kondo et al., 2007) and is a limitation of these analyses. The overall picture emerging from these results is a close phylogenetic relationship between the plant-associated fungi of Clavicipitaceae and pathogens of scale insects, which we have informally termed the plant-hemipteran clade (Fig. 1).

Scale insects have a sessile lifestyle where their stylet is inserted directly into plant tissue. Pathogens that infect these insects are therefore in close proximity to a consistent source of nutrition greatly exceeding the biomass of the original host.

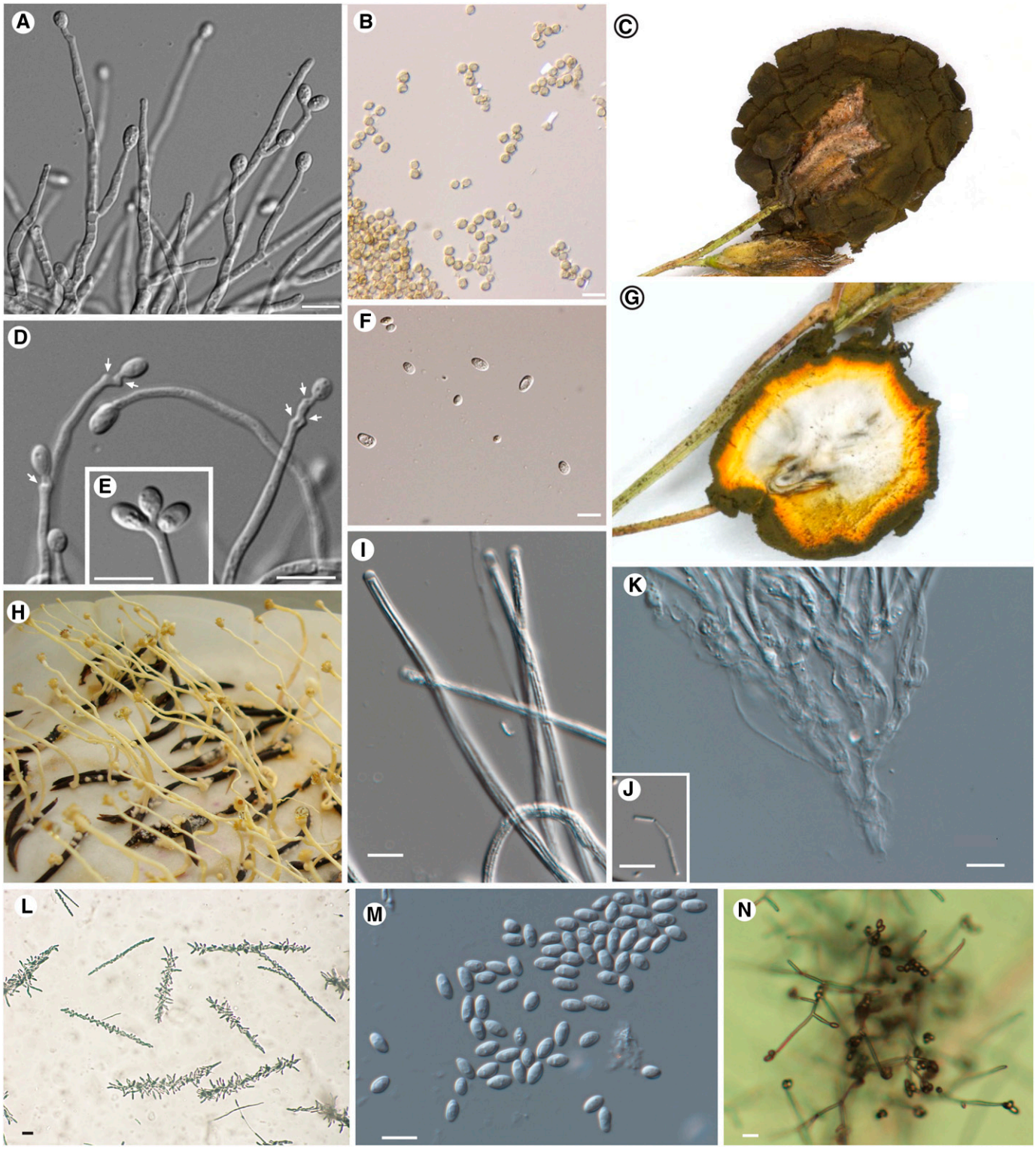

Fig. 2. Macroscopic and microscopic features of *Ustilaginoidea virens* and *Tyrannicordyceps fratricida*. (A–G) *U. virens*. (A) Conidia developing as swelling of the entire tip of hyphal conidiogenous cells. Scale bar = 5 µm. (B) Dark-colored, verrucose thick-walled conidia. Scale bar = 10 µm. (C) Rice false smut ball with remnants of the glumes. (D) Sympodial branching of conidiophore in succession of short lateral proliferation. Detached points (arrows) of conidia. Scale bar = 5 µm. (E) Conidia appear to occur in whorls at the apex of conidiogenous cell. (F) Secondarily produced spherical conidia. (G) Cross section of false smut ball, which contains remnants of the ovule. (H–N) *T. fratricida*. (H) *T. fratricida* emerging from sclerotia of ergot after overwintering. (I) Ascus. Scale bar = 10 µm. (J) Part-spores. Scale bar = 10 µm. (K) Foot of asci. Scale bar = 10 µm. (L) Germinating ascospores. Scale bar = 50 µm. (M) Conidia produced by culture from germinated ascospores. Scale bar = 10 µm. (N) Phialides of *Verticillium*-like anamorph, showing conida in droplet at tips. Scale bar = 10 µm.

Dussiella tuberiformis (Berk. & Ravenel) Pat., another species in the Clavicipitaceae, infects scale insects and then indirectly absorbs plant nutrients. By absorbing the sap leaking from the stylet of the insect cadaver, *D. tuberiformis* can grow to be much larger than its insect host (Koroch et al., 2004). Notably, scale insect hosts of this fungus are herbivores of grasses and may indicate a transitional lifestyle toward plant pathogenesis similar to that experienced by grass symbionts such as *Claviceps*. In an analysis of nutrient utilization, *D. tuberiformis* was suspected to be reliant on living plants for the supply of nutrients essential for growth and reproduction obtained from phloem sap, particularly reduced forms of nitrogen (Koroch et al., 2004). Additionally, several species of *Hypocrella* that infect scale insects make use of exuded plant juices, attaining sizes much larger than their scale insect hosts and were not at first recognized as insect pathogens (Hywel-Jones and Samuels, 1998).

Utilization of scale insects for access to plant nutrients is also assumed to be operating outside of Clavicipitaceae s.s. The genera *Ascopolyporous* and *Hyperdermium* produce large fruit bodies originating from a scale insect host, and molecular evidence places these genera within the Cordycipitaceae (Sullivan et al., 2000; Bischoff et al., 2005), although these fungi show no close phylogenetic affinity with any obligate plant pathogens. Further development of hypotheses on interkingdom host jumps from scale insect to plant among clavicipitaceous fungi will rely on incorporation of unexamined plant-associated species (e.g., *Munkia* and *Neomunkia*) and increased taxon sampling in a multigene phylogenetic framework across associated hypocrealean families.

Multigene phylogenetic analyses of Clavicipitaceae inferred two main subclades for the family: *Metacordyceps* and the plant-hemipteran clade. All scale insect pathogens and plant-associated fungi are members of the plant-hemipteran clade, but neither the scale pathogens nor the plant-associated fungi formed monophyletic groups. Multiple interkingdom host shifts must be inferred to explain this distribution, although ACSR with more complex character state coding proved to be of little utility in definitively resolving the polarity of character states transformations. Given the weight of the evidence of the phylogenetic distribution of host affiliations in the Clavicipitaceae, we propose the hypothesis that the common ancestor to the plant-hemipteran clade was a pathogen of animals and that multiple interkingdom host jumps occurred between plants and scale insects. We cannot discount the possibility of dynamic host jumps occurring bidirectionally between plant and scale insect hosts, particularly for node B, where Bayesian reconstructions are equivocal between plant and animal host usage (Table 3).

Tyrannicordyceps and the evolution of fungal pathogenesis—Ancestral character-state reconstructions of Clavicipitaceae suggest that the clade containing *V. epiphytum* and *T. fratricida* was derived from a plant pathogenic ancestor. Based on the reconstructions we hypothesize the switch from plant-based nutrition to mycoparasitism was facilitated by the repeated coinfection of host plants by the ancestor of *T. fratricida* and a species of *Claviceps*, placing them in close physical proximity and setting the stage for resource competition. This change in nutritional mode is notable because the host for *T. fratricida* is a closely related species in the grass pathogen in the genus *Claviceps*. The phenomenon of parasites being closely related to their hosts is seen across the tree of life. In attacking closely related species, *T. fratricida* also functions as an adelphoparasite, similar to species of parasitic red algae or mistletoe (Goff et al., 1997).

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

The multiple shifts between hemiptera and plant hosts were likely facilitated—at least in some cases—by the close and intimate contact between scale insects and their plant hosts. An animal pathogenic ancestor provides an explanatory mechanism for not only the origin of the fungal–plant interaction, but also the origin of their novel secondary metabolites (e.g., ergot alkaloids), which are toxic to animals and not plants. The recent genome sequencing of *Metarhizium*, an arthropod pathogen of Clavicipitaceae (Fig. 1), revealed that it possessed the complete pathway for ergot biosynthesis (Gao et al., 2011), further supporting the animal pathogen origin hypothesis for the plant-associated fungi.

Conclusion—Clavicipitaceae in particular, and the hypocrealean fungi in general, exhibit a remarkable range of host associations that provides a tractable system in which to study the phenomenon of host jumping. Establishing the placement of *T. fratricida* within Clavicipitaceae and sister to *V. ephiphytum* is yet another important piece in the development of our understanding of the evolution of host affiliation in fungi by linking both reproductive and nutritional modes. This placement is remarkable in that the host switch for *Tyrannicordyceps* involves movement onto closely related species, possibly as a result of competition after coinfection of host plants. These analyses also provide additional support for the close phylogenetic relationship between scale pathogens and plant-associated fungi of Clavicipitacaeae and more fully develop the hypothesis that scale insects played an important role in interkingdom host shifts.

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