

Torrubiella piperis sp. nov. (Clavicipitaceae, Hypocreales), a new teleomorph of the *Lecanicillium* complex

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Abstract: Recent publications have distinguished clavicipitaceous *Verticillium sensu lato* anamorphs into six genera. One genus, *Lecanicillium*, includes the majority of these taxa, including the anamorphs of *Cordyceps militaris* and *Torrubiella confragosa*. *Torrubiella confragosa* has been linked to *Lecanicillium lecanii* (= *Verticillium lecanii*). During a recent collection trip to Panama a *Torrubiella* sp. that produced a *Verticillium*-like anamorph was found on the corpses of scale insects attached to *Piperaceae*. It could be distinguished from *Torrubiella confragosa* based on presence of a true stroma, immersed perithecia, conidial size (3–5 × 1–2 µm), and phialide size (10–30 × 1–2 µm). Thus we describe this new collection as *Torrubiella piperis* sp. nov. In addition, based on the morphological observations and phylogenetic analyses using partial LSU rDNA data we determined it to belong to the *Lecanicillium* group (100 % posterior probability).

Taxonomic novelty: *Torrubiella piperis* J.F. Bischoff & J.F. White sp. nov.

Key words: Barro Colorado Island, Coccoidea, Cordyceps, Lecanicillium, Piperaceae, scale insect, Verticillium.

INTRODUCTION

The entomogenous fungal genus *Torrubiella* Boudier was erected to accommodate *Cordyceps*-like species that do not produce stromatic clavae (Boudier 1885, Petch 1923). Kobayasi (1982) included 59 species in *Torrubiella*, most of which are pathogens of the arthropod orders *Araneida* and *Homoptera*. However, based on phylogenetic analyses of the 18S and 28S ribosomal DNA (rDNA), *Torrubiella* appears to be a polyphyletic grouping (Artjariyasriping et al. 2001). These findings are supported by the ubiquity of its anamorphic connections (Hodge 2003). One such anamorph connection was with the form genus *Verticillium* Nees.

Verticillium sensu lato was also a polyphyletic genus and found to be connected to various orders in *Ascomycota* (Gams 1971), when clavicipitaleans with connections to *Verticillium s.l.* were included in *Verticillium* sect. *Prostrata* W. Gams. However, recent work based on morphological and molecular data has segregated this grouping into distinct genera (Gams & Zare 2001). One of these genera, *Lecanicillium* W. Gams & Zare, is typified by *L. lecanii* (Zimmerm.) Zare & W. Gams (= *Verticillium lecanii*) and was linked to the scale insect (*Coccoidea*, *Homoptera*) pathogen *Torrubiella confragosa* Mains (Evans & Samson 1982). *Lecanicillium* is distinguished from other genera formerly classified in

Verticillium sect. *Prostrata* by the frequent formation of octahedral crystals in culture, entomogenous or fungicolous habit, phialides discrete and aculeate, conidia accumulating at phialide tips, mostly single-celled conidia (2-celled conidia occur rarely in *L. attenuatum* Zare & W. Gams, *L. longisporum* (Petch) Zare & W. Gams, and *L. psalliotae* (Treschow) Zare & W. Gams), and lack of dictyochlamydospores or chlamydospores. The anamorph of *Cordyceps militaris* (L.) Fr., the type species of the genus, is also of the *Lecanicillium* type (Zare & Gams 2001a, b).

During a recent collection trip to Barro Colorado Island (BCI) in Panama, a stromatic scale insect pathogen was found on the stems, petioles, and primary vein of leaves of two species of *Piperaceae*. These collections represent a new species that was determined to be congeneric with the type species, *Torrubiella aranicida* Boudier, as the genus is currently accepted. In addition, it was found to produce an anamorph of the *Lecanicillium* type. In this study we describe a new species of *Torrubiella*.

MATERIALS AND METHODS

Isolates

Collections of this new fungus were made on Barro Colorado Island (BCI), Panama in August of 2003.

Table 1. Large Subunit Ribosomal DNA sequences included in analyses.

Anamorph	Teleomorph	Isolates	Location	Substrate	GenBank No.
<i>Beauveria bassiana</i> (Bals.-Criv.) Vuill.	<i>Cordyceps bassiana</i>	GB4605	Veracruz, Mexico	Decaying needles of <i>Pinus</i> sp.	AF245300
<i>Beauveria brongniartii</i> (Sacc.) Petch	<i>Cordyceps brongniartii</i>	IFO 5299	Japan	NA	AB027381
<i>Cladobotryum mycophilum</i> (Oudem.) W. Gams & Hooz.	<i>Hypomyces odoratus</i> G.R.W. Arnold	TFC 98-53	Estonia	<i>Tricholoma</i> sp.	AF160240
<i>Cladobotryum verticillatum</i> (Link) S. Hughes	<i>Hypomyces armeniacus</i> Tul. et. C. Tul.	TFC 95-154	Estonia	<i>Lactarius helvus</i>	AF160239
<i>Gliocladium</i> sp.	<i>Hypocrea lutea</i> (Tode) Petch	IFO9061	NA	NA	AB027384
<i>Haptocillium sinense</i> (K.Q. Zhang, L. Cao & Z.Q. Liang) Zare & W. Gams	Unknown	CBS 131.95	China	<i>Actinidia deliciosa</i>	AF339546
<i>Haptocillium sphaerosporum</i> (J.B. Goodey) Zare & W. Gams	Unknown	CBS 522.80	Münster, Germany	<i>Ditylenchus trififormis</i>	AF339541
<i>Haptocillium zeosporum</i> (Drechsler) Zare & W. Gams	Unknown	CBS 335.80	Iraq	Nematoda	AF339540
<i>Lecanicillium antillanum</i> (R.F. Castañeda & G.R.W. Arnold) Zare & W. Gams	Unknown	CBS 350.85	Santiago de las Vegas, Cuba	<i>Agaricus</i> sp.	AF339536
<i>Lecanicillium aranearum</i> (Petch) Zare & W. Gams	<i>Torrubiella alba</i> Petch	CBS 726.73a	Begoro, Ghana	<i>Araneida</i> sp.	AF339537
<i>Lecanicillium fuisporum</i> (W. Gams) Zare & W. Gams	Unknown	CBS 164.70	Deeler Woud, Netherlands	<i>Coltricia perennis</i>	AF339549
<i>Lecanicillium lecanii</i> (Zimmermann) Zare & W. Gams	<i>Torrubiella confragosa</i> Mains	ATCC 58909	Wisconsin, U.S.A.	<i>Acer saccharum</i> leaf litter	U17414
		ATCC 46578	NA ¹	NA	U17421
		IMI 304807	West Indies	<i>Coccoidea</i> on coffee	AF339555
<i>Lecanicillium psalliotae</i> (Treschow) Zare & W. Gams	Unknown	CBS 363.86	Kunmin, Prov. Yunnan, China	<i>Agaricus bisporus</i>	AF339559
<i>Lecanicillium</i> sp. JB209	<i>Torrubiella piperis</i>	CBS 116719	Barro Colorado Island, Panama	Scale insect on <i>Piper</i> sp.	AY466442
<i>Lecanicillium</i> sp. CBS 639.85	Unknown	CBS 639.85	Germany	Rhizosphere of <i>Pseudotsuga menziesii</i>	AF339561
<i>Lecanicillium</i> sp. GJS 93-51	<i>Cordyceps militaris</i> (L.) Link	GJS 93-51	Maryland, U.S.A.	Insect larvae	AF043135
<i>Pochonia chlamydosporia</i> var. <i>catenulata</i> (Kamyschko ex G.L. Barron & Onions) Zare & W. Gams	<i>Cordyceps</i> sp.	CBS 504.66	Canada	Soil	AF339544
<i>Pochonia gonioides</i> (Drechsler) Zare & W. Gams	Unknown	CBS 891.72	Germany	<i>Pulcherriium caeruleum</i>	AF339550
<i>Pochonia rubescens</i> Zare, W. Gams & López-Llorca	Unknown	CBS 464.88	Scotland, U.K.	Eggs of <i>Heterodera avenae</i>	AF339566
<i>Simplicillium lamellicola</i> (F.E.V. Sm.) Zare & W. Gams	Unknown	CBS 116.25	U.K.	<i>Agaricus bisporus</i>	AF339552
<i>Simplicillium lanosoniveum</i> (J.F.H. Beyma) Zare & W. Gams	<i>Torrubiella</i> sp.	IMI 317442	Jamaica	<i>Hemileia vastatrix</i>	AF339554
	<i>Torrubiella</i> sp.	CBS 704.86	Venezuela	<i>Hemileia vastatrix</i>	AF339553
<i>Simplicillium obclavatum</i> (W. Gams) Zare & W. Gams	Unknown	CBS 311.74	Kamal, India	Air above surgarcane field	AF339517
<i>Verticillium epiphytum</i> Hansf.	Unknown	CBS 384.81	Thailand	<i>Helminthosporium triumfettae</i>	AF339547
<i>Verticillium incurvum</i> Helfer	Unknown	CBS 460.88	Naturschutzgebiet Mittelsteighütte, Germany	<i>Ganoderma lipsiense</i>	AF339551
<i>Verticillium pseudohemipterigenum</i> H.C. Evans & Jun	Unknown	ARSEF 5687	Parrylands, Siparia, Trinidad	<i>Coccoidea</i>	AF339562

¹NA = Information is not available.

The material was brought to the field station and isolated onto 2 % potato-dextrose agar (PDA; Difco) with antibiotics (gentamycin 40 mg/L; streptomycin 40 mg/L; penicillin 20 mg/L). Stromata were then placed in vials of 90 % ethanol. Voucher material was deposited in the herbarium at the New York Botanical Gardens (NY), and the Rutgers Mycological Herbarium (RUTPP). Upon return to Rutgers University, subcultures were made onto potato-carrot agar (PCA) to enhance conidial development. Cultures were maintained at approximately 26 °C in a light environment (fluorescent lights). Conidial development occurred within 10 d of subculturing. Cultures were submitted to the Centraalbureau voor Schimmelcultures (CBS 116719).

Morphology

Microscopic observations were made from squash mounts and sections. Sections of fresh material were fixed and prepared as described by Sullivan *et al.* (2000). Squash mounts were made from the material stored in ethanol and from the culture isolations. Microscopic evaluations were made with a Zeiss Axioskop microscope. Macroscopic evaluations were made with a Zeiss Stemi SV8 dissecting scope. Photographs were taken with a Nikon® Coolpix 880 digital camera.

DNA isolation, amplification, and phylogeny

Fresh mycelium was taken from cellulose acetate sheets overlaid on PDA and ground with liquid nitrogen. Genomic DNA was extracted using the DNA-Easy™ kit. Internal transcribed spacer (ITS 1 and ITS 2) regions, the 5.8S gene, and the 5' end of the LSU rDNA were amplified from 4 µL of genomic DNA using primers ITS5 (Vilgalys & Hester 1990) and LR7SM (Sullivan *et al.* 2000) in a 50 µL PCR reaction set to 95 °C for 10 s, 54 °C for 30 s, and 72 °C for 10 min. Sequencing reactions to amplify domains 1–3 of the LSU region, and reaction analysis were performed as described by Sullivan *et al.* (2000). LSU sequence of this new species was submitted to GenBank (Accession number AY466442). Sampled taxa were chosen based on their inclusion and placement in previous work regarding *Verticillium* sect. *Prostrata* (Sung *et al.* 2001). Members of *Hypocreaceae* were used as outgroup taxa due to their close relationship to the *Clavicipitaceae* (Spatafora & Blackwell 1993) and their use as outgroup taxa in the work of Sung *et al.* (2001). Sequences obtained from GenBank are listed in Table 1 along with information pertaining to the isolates included in the sequence analysis.

Sequencher (Genecodes, Ann Arbor, MI) was used to analyze and determine the consensus sequence from sequence products. Matrix alignment was performed in Microsoft Word (Microsoft, Seattle, WA). Alignment decisions were based on the

ribosomal secondary structure and annotated as described by Kjer (1995). The alignment is available at TreeBase.

Modeltest 3.06 (Posada & Crandall 1998) was used to select the model of evolution that best fits the data. This model was used as input into PAUP v. 4.0b10 Alivitec (Swofford 2002). A maximum likelihood analysis was performed with model parameters: invariable sites (I), gamma distribution (G), base frequencies, and the R matrix as determined by Modeltest. Taxa were added randomly in 100 replicates with a random starting seed. One tree was held at each step during stepwise addition using the TBR algorithm. Branches were collapsed if branch length was less than or equal to 1^{-10} .

MrBayes 3.0, a Bayesian phylogenetic inference program (Huelsenbeck & Ronquist 2001), was used to determine branch support (posterior probabilities). Bayesian analysis was run with 4 mcmc chains (3 cold; 1 heated) for 2 000 000 generations, sampling every 100 generations (including the first generation) which yielded 20 001 trees. These trees were graphed to determine at which point the trees being recovered were asymptotic. The trees that were not asymptotic were discarded (burn in; Huelsenbeck 2000). Bayesian analysis was performed five times. These trees were imported into PAUP and a majority-rule consensus tree was produced to determine posterior probabilities and are reported on the maximum likelihood tree (Fig. 2).

RESULTS

Morphology

Torrubiella piperis J.F. Bischoff & J.F. White, sp. nov. MycoBank MB500023. Fig. 1A–L.

Stromata epibiotica, 3–10 × 3–6 mm, alba vel flava; Stromata pulvinata, flava vel aurea, 0.5–2 mm diametro. Perithecia immersa, 175–290 × 40–80 µm lata, obpyriformia vel cymbiformia, conferta; asci cylindrici, apice incrassato, 120–170 × 3–5 µm; ascospores filiformes, multiseptatae, partes 4–9 × 1–2 µm disarticulantes. Conidiophora verticillata vel solitaria, 150–400 µm longa. Phialides cylindricae, 10–30 × 1–2 µm. Conidia subcylindrica vel subglobosa, hyalina, aseptata, 3–5 × 1–2 µm, congregata. Cristalla polyhedrica copiosa.

Stromata sessile, covering the insect and extending onto substratum, white to yellow, 3–10 × 3–6 mm, subglobose to cylindrical. *Perithecial stromata* pulvinate, yellow to orange, 0.5–2 mm in diameter. *Perithecia* immersed, 175–290 × 40–80 µm, obpyriform to cymbiform but mostly variable due to crowding in ascomata. *Asci* cylindrical, 120–170 × 3–5 µm, with an apical perforated cap. *Ascospores* fili-

form, disarticulating into part-spores; part-spores rectangular, $4\text{--}9 \times 1\text{--}2 \mu\text{m}$. *Conidiophores* upright, verticillate, along stroma surface. In culture conidiophores mostly upright, verticillate, $150\text{--}400 \mu\text{m}$ long. *Phialides* cylindrical but slightly attenuated towards the apex, $10\text{--}30 \times 1\text{--}2 \mu\text{m}$. *Conidia* aseptate, hyaline, subcylindrical, rarely subglobose, $3\text{--}5 \times 1\text{--}2 \mu\text{m}$, aggregating into heads (frequently globose) at phialide tips. Polyhedral crystals, that were some-

times conjoined, developed in the medium beneath mycelia.

Holotype: Panama, Barro Colorado Island, Lutz Creek, scale insect (*Coccoidea*, *Homoptera*) on *Piper carrilloanum* (*Piperaceae*); August, 2003, J.F. Bischoff & J.F. White, Jr., New York Botanical Garden (NY), culture ex-type CBS 116719.

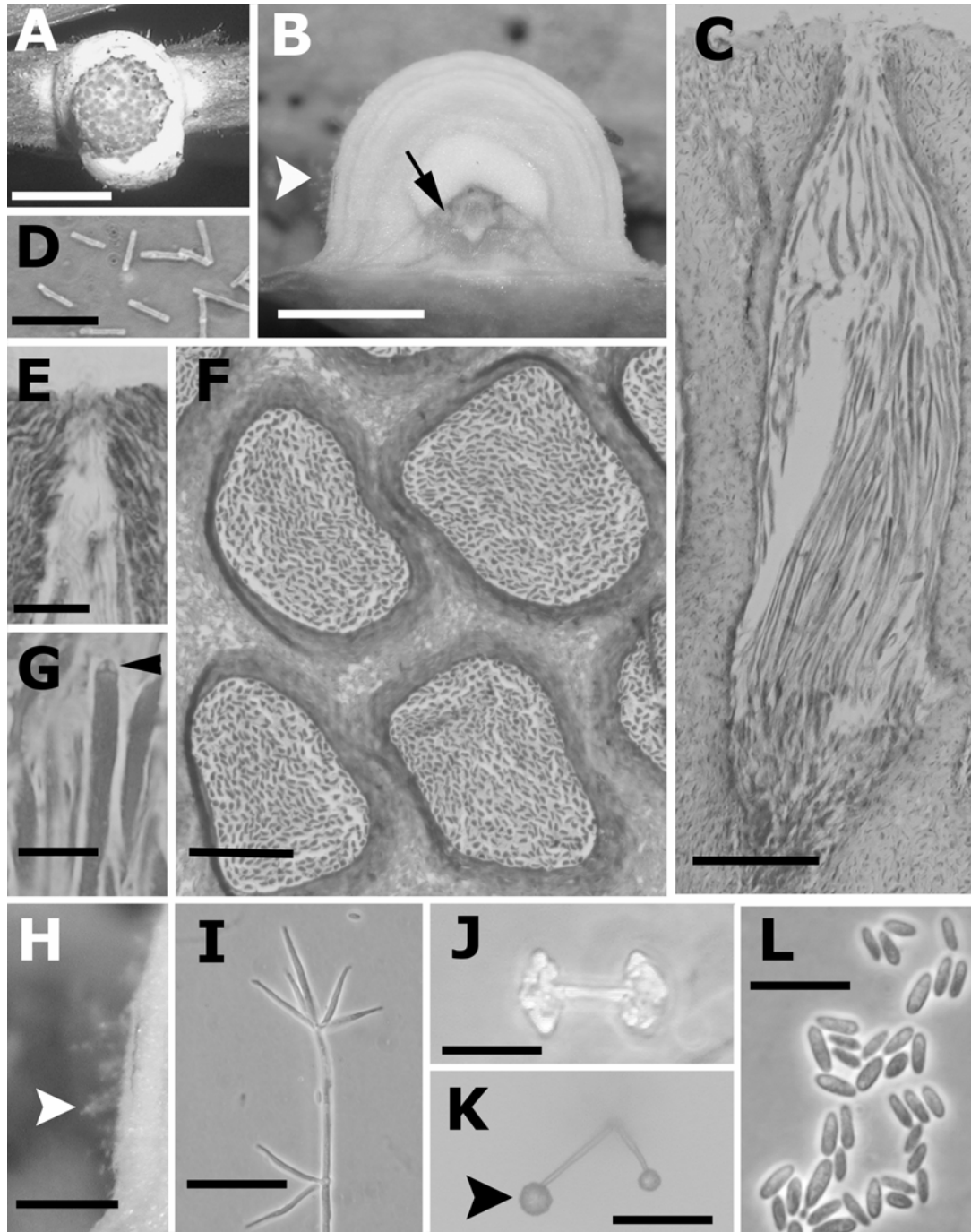


Fig 1. *Torrubiella piperis*. A. Stroma with ascomata. B. Cross-section of stroma with remnants of scale insect (arrow) and erect conidiophores on stroma surface (arrowhead). C. Perithecium. D. Part ascospores. E. Ostiole canal with no periphyses. F. Cross-section of stroma showing the cross-sections of multiple immersed perithecia with amorphous shape due to crowding. G. Portion of ascus with thickened apical cap (arrowhead). Bar = $10 \mu\text{m}$. H. Stroma surface with erect conidiophores (arrowhead). I. Conidiophore. J. Dumb-bell-shaped crystal produced in culture (PCA). K. Phialide tips with round conidial heads (arrowhead). L. Conidia. Scale bars: A = 2 mm ; B = 2 mm ; C = $30 \mu\text{m}$; D = $15 \mu\text{m}$; E = $10 \mu\text{m}$; F = $30 \mu\text{m}$; G = $10 \mu\text{m}$; H = $600 \mu\text{m}$; I = $25 \mu\text{m}$; J = $6 \mu\text{m}$; K = $25 \mu\text{m}$; L = $10 \mu\text{m}$.

Notes: Based on the descriptions and key provided by Zare & Gams (2001a) regarding species of *Lecanicillium*, the anamorph of *Torrubiella piperis* correlates closely with *L. muscarium*. However, when the ITS and 5.8S regions of the rDNA of *T. piperis* were compared with the equivalent sequences of *L. muscarium* they were shown to be distinct (Tree not shown).

Torrubiella confragosa and *T. piperis* can be distinguished based on stromatic and perithecial characteristics. *Torrubiella confragosa* lacks a true stroma and has perithecia that are superficial and larger ($360\text{--}510 \times 175\text{--}240\ \mu\text{m}$) than observed in *T. piperis*. In addition, the specimen of *T. confragosa* observed by Evans & Samson (1982) was collected from a coffee plant (*Coffea arabica*).

Phylogenetic analysis

Sequencing of *Torrubiella piperis* yielded 1360 nucleotides. This sequence was trimmed to accommodate the GenBank sequences obtained for this study. GenBank sequences ranged from 691 to 873 base pairs (without gaps). When aligned, the matrix included 875 unambiguously aligned characters.

The Akaike Information Criterion (AIC; Akaike 1974) in Modeltest selected TrN including the proportion of invariable sites (I) and gamma distribution shape parameter (G) as the model that best fit the data (TrN + I + G; Tamura & Nei 1993). The parameters include: base frequencies of A = 0.2558, C = 0.2276, G = 0.3219, T = 0.1947; rate matrix of A \leftrightarrow C = 1.0000, A \leftrightarrow G = 2.1429, A \leftrightarrow T = 1.0000, C \leftrightarrow G = 1.000, C \leftrightarrow T = 11.4128, G \leftrightarrow T 1.000; I = 0.5968; G = 0.5799. With this information PAUP found the most likely tree (–ln 2860.99766; Fig. 2) 28 times out of the 100 replications.

Of the 100 005 trees produced through bayesian analysis 98 820 were kept after the 'burn in' trees were discarded. Clade A contains taxa with *Lecanicillium* anamorphs, including *Torrubiella piperis*, and *Beauveria* species (100 % posterior probability). The representative taxa of the genus *Simplicillium* form a clade ancestral to clade A and are supported by 100 % posterior probability. In the generic grouping, *Pochonia* species were placed ancestrally to clade A and *Simplicillium* (53 % posterior probability) but were not fully resolved. *Verticillium incurvum*, *V. pseudohemipterigenum*, and *V. epiphytum* each bifurcate individually from within the ingroup (70 %, 79 %, and 57 % posterior probabilities, respectively). *Haptocillium* was supported as a monophyletic group with a 100 % posterior probability. The outgroup supported the inclusion of all ingroup taxa (*Clavicipitaceae*) in a single clade (100 % posterior probability).

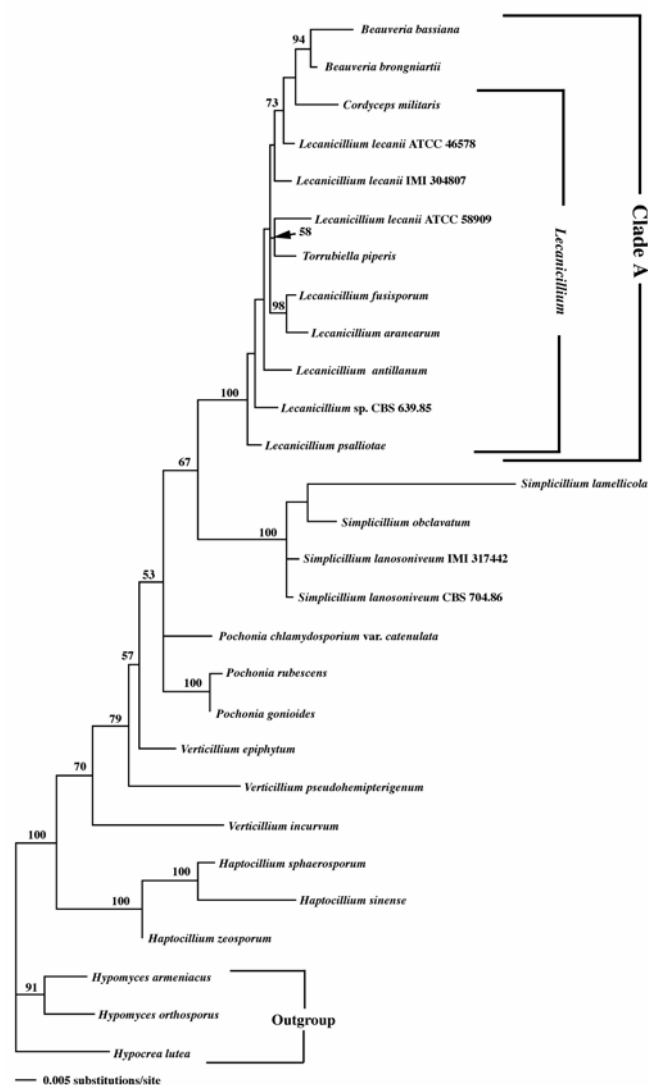


Fig. 2. The most likely tree (–ln 2860.99766) based on partial rDNA LSU sequence data analysis as determined by PAUP Altvect 4.0b10 using the TrN + I + G model of evolution. The numbers on the branches indicate the posterior probabilities expressed as a percentage for the node they precede.

DISCUSSION

Our hypothesized phylogeny is in agreement with the results of Sung *et al.* (2001) in their revision of *Verticillium* sect. *Prostrata* with the exception of *V. incurvum*. Sung *et al.* placed *V. incurvum* in the *Hypocreaceae* while our study finds the taxon nested and well supported in the *Clavicipitaceae* (100 % posterior probability; Fig. 2). Like Zare *et al.* (2001), we were unable to group *V. incurvum*, *V. epiphytum*, and *V. pseudohemipterigenum* with distinct lineages.

The morphological and molecular data support the inclusion of *Torrubiella piperis* together with other taxa that express a *Lecanicillium* anamorph (Clade A, Fig. 2). The studies of Zare *et al.* (2000, 2001), and Zare & Gams (2001a, b) provided detailed morpho-

logical descriptions of distinct species formally classified in the *Verticillium* sect. *Prostrata* and helped us to develop a phylogenetic hypothesis based on the anamorph morphology of *Torrubiella piperis*. However, the morphological characters that distinguish *Lecanicillium* from other *Verticillium*-like groups do not represent synapomorphic characters for clade A. These are conflicts that Gams & Zare (2001a) were willing to accept. Considering the vast improvements their work has made in the taxonomy of *Verticillium* s.l. we are also willing to accept the paraphyly of *Lecanicillium*.

Nutrient acquisition

The size of the *Torrubiella piperis* stroma is much larger than that of its scale insect host (Fig. 1B). The disparity in fungal stroma size to scale insect is evident in other clavicipitalean genera (e.g. *Dussiella*, *Hyperdermium*, *Ascopolyporus*). In their study of *Hyperdermium*, Sullivan *et al.* (2000) proposed that the fungus first parasitises the scale insect, consumes it and then uses the insect's remaining stylet to acquire carbohydrates from the plant. If that hypothesis is indeed true then *Torrubiella piperis* may also receive additional nutrients in this fashion.

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