

## *Ophiocordyceps pulvinata* sp. nov., a pathogen of ants with a reduced stroma

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**Abstract** *Ophiocordyceps pulvinata*, a pathogen of ants, is formally described as a new species. Genus level designation of this species is difficult due to several apparently conflicting morphological and ecological characters. Affinity with *Ophiocordyceps* is suggested by the dark color stroma and ascospore morphology. However, the species was included in a book of entomopathogenic fungi of Japan as *Torrubiella* sp. due to the production of perithecia on an astipitate stroma. Phylogenetic analyses of molecular data support a close relationship with *O. unilateralis*, a finding consistent with morphological characteristics of the color, asci and ascospores and ecological traits of host affiliation. Thus, *O. pulvinata* represents another example of the loss of stipe for the hypocrealean arthropod pathogenic fungi and highlights the utility of asci and ascospore morphology as taxonomically informative characters of closely related taxa.

**Keywords** Astipitate · Multigene phylogeny · Morphological plasticity · Ophiocordycipitaceae · Taxonomy

### Introduction

The majority of arthropod pathogenic fungi were historically classified in the genera *Cordyceps* and *Torrubiella* of the family Clavicipitaceae sensu Diehl (1950) (Rogerson 1970; Kobayasi 1981a, b, 1982). These fungi display a staggering variety of morphologies, colors and host affiliations that formed the basis for past classification schemes (Masse 1895; Kobayasi 1941; Mains 1958; reviewed in Sung et al. 2007b). Sung et al. (2007a, b) determined *Cordyceps* to be polyphyletic with molecular phylogenies not supporting most of the previously proposed subgeneric taxa. Characters that were used to delineate genera and subgenera, such as disarticulated/intact ascospores and superficial/embedded presentation of perithecia, were found to be distributed throughout all clades and in most cases do not reflect monophyletic groups. Based on the results of a five gene phylogeny, three well-supported clades were designated as families comprising four genera of species formerly classified in *Cordyceps*: Clavicipitaceae (*Metacordyceps*), Cordycipitaceae (*Cordyceps* s.s.) and Ophiocordycipitaceae (*Elaphocordyceps* and *Ophiocordyceps*) (Sung et al. 2007b).

*Torrubiella* has been recognized as closely related to *Cordyceps*, with the main distinguishing feature being the lack of a stipe (Kobayasi 1941, 1982). Perithecia are typically produced superficially on a subiculum, although perithecia may be surrounded by loosely connected hyphal strands (Kobayasi and Shimizu 1982). The form of the subiculum may range from nearly nonexistent to a thick fleshy mass. Stromatal color is typically pallid, with many

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forms white or yellowish, although brightly colored species have been described. Host affiliations for *Torrubiella* are predominantly with spiders, and, to a lesser degree, scale insects, although other hosts including ants are known (Kobayasi and Shimizu 1982; Samson et al. 1989). Nine anamorph genera have been linked to *Torrubiella* with the genera *Gibellula*, *Pseudogibellula* and *Granulomanus* known only from *Torrubiella* (Hodge 2003).

Just as the traditional characters used to describe diversity in the genus *Cordyceps* were found to be phylogenetically uninformative, the characters traditionally used to distinguish *Torrubiella* from other genera were also not supported by phylogenetic analyses. Johnson et al. (2009) conducted a multigene phylogenetic analysis of *Torrubiella* species representing multiple host affiliations and morphologies for the genus. Species occurring on spiders and producing *Gibellula* anamorphs form a well-supported clade in the Cordycipitaceae, and the name *Torrubiella* was provisionally retained for this clade. *Torrubiella* spp., known to occur on scale insects and producing anamorphic types other than *Gibellula*, were placed outside of the core *Torrubiella* clade and in most cases outside of the Cordycipitaceae. For example, *Torrubiella pruinosa* Petch [= *Ophiocordyceps pruinosa* (Petch) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora] was shown to belong to the Ophiocordycipitaceae, consistent with the morphology of its *Hirsutella* anamorph. Within the Clavicipitaceae scale pathogens classified in *Torrubiella* comprised two distinct clades; the genus *Conoideocrella* was erected for *T. tenuis* Petch and *T. luteo-rostrata* Zimm. and the genus *Orbiocrella* was proposed for *T. petchii* Hywel-Jones (Johnson et al. 2009).

Recently, an undescribed, astipitate ant pathogen was found that is not readily placed in either *Cordyceps s.l.* or *Torrubiella*. Similar specimens were first collected by Yoshitaka Kaitsu, and although officially undescribed, Shimizu (1997) illustrated them in a book of Japanese entomopathogens as *Torrubiella* sp. and gave it the Japanese common name Kobugata-aritake (the bump-neck ant fungus). Through microscopic observations and phylogenetic analyses, we confirm this is a new species, *Ophiocordyceps pulvinata* sp. nov. We demonstrate that it is closely related to the ant pathogen, *O. unilateralis* (Tul. & C. Tul.) Petch, and that *O. pulvinata* represents another independent loss of the stipe in the evolution of arthropod pathogenic fungi and another integration point of torrubielloid and cordycepioid morphologies.

## Materials and methods

### Field collections

During September of 2007 a large epizootic event of Kobugata-aritake occurred on *Camponotus obscuripes*

Mayr (Hymenoptera: Formicidae) in Fukushima Prefecture, Japan. Numerous specimens were collected by members of the Japan Society for Vegetable Wasps and Plant Worms (Tohchukaso No Kai), and three specimens were kindly provided to us by Dr. Okuzawa and Mr. and Mrs. Kajiyama for microscopic examination and molecular phylogenetic analyses. Specimens were air-dried and placed in sealed glass bottles until examination. All specimens are deposited at the National Museum of Science and Nature mycological herbarium (TNS), Tsukuba City, Ibaraki, Japan.

### Morphological examinations

Specimens were prepared for microscopic observation by rehydration in sterile water. After tissue became pliable, the material was mounted in lactic acid. Observations of microscopic features were made using Nomarski DIC on an Olympus BX51 (Olympus Corp., Tokyo, Japan) fitted with a Nikon Digital Sight DS-5M imaging system (Nikon Corp., Tokyo, Japan). All measurements were made with the ruler tool in Photoshop CS3 extended (Adobe Systems Inc., San Jose, CA). Macroscopic images were obtained with the BK Plus Lab System (Visionary Digital, Palmyra, VA) and the composite image assembled using Helicon Focus (Helicon Soft Ltd., Kharkov, Ukraine).

### DNA extraction, PCR and sequencing

DNA extractions were performed by grinding tissue with a drill equipped with a plastic Eppendorf pestle in 50  $\mu$ l CTAB buffer, then transferred to a FastDNA lysing matrix A tube (MP Biomedical, Salon, OH) and combined with an additional 400- $\mu$ l CTAB buffer. The lysing matrix tube was then placed in a fast prep machine and masticated twice for 20 s. Afterwards mastication tubes were placed in a water bath at 60°C for 20 min and then centrifuged for 10 min at 14,000 rpm. After centrifugation, the 400  $\mu$ l of supernatant was transferred to 1.5-ml tubes and mixed with 500  $\mu$ l chloroform:isoamyl alcohol (24:1). The mixture was then centrifuged at 14,000 rpm for 20 min, after which 300  $\mu$ l of supernatant was removed. DNA in the supernatant was then further cleaned using the GeneCleanIII Kit, following the recommended protocol.

To confidently place species in the context of previous studies (e.g., Sung et al. 2007b; Johnson et al. 2009), attempts were made to amplify five nuclear loci: nu-rSSU and nu-rLSU, elongation factor 1 $\alpha$  (TEF), and the largest and second largest subunits of RNA polymerase II (RPB1 and RPB2 respectively) with a total read length nearing 5,000 bp. The amplification of nu-rSSU was performed using two overlapping sets of primers, NS1/SR7 and NS3/NS4 (White et al. 1990). The nu-rLSU was amplified with the primers LR5/LROR (Vilgalys and Sun 1994). Amplification of TEF was

**Table 1** Taxon, specimen voucher and sequence information for specimens used in this study

Taxon	Specimen voucher <sup>a</sup>	Host/substratum	GenBank accession nos				
			nu-rSSU	nu-rLSU	TEF	RPB1	RPB2
<i>Akanthomyces arachnophilus</i>	NHJ 10469	Spider (Arachnida)	EU369090	EU369031	EU369008	EU369047	
<i>A. cinereus</i>	NHJ 3510	Spider (Arachnida)	EU369091		EU369009	EU369048	EU369070
<i>A. novoguineensis</i>	NHJ 13117	Spider (Arachnida)	EU369092		EU369010	EU369049	EU369073
<i>A. novoguineensis</i>	NHJ 13161	Spider (Arachnida)	EU369093		EU369011	EU369050	
<i>A. novoguineensis</i>	NHJ 4314	Spider (Arachnida)	EU369094		EU369012	EU369051	EU369071
<i>A. novoguineensis</i>	NHJ 11923	Spider (Arachnida)	EU369095	EU369032	EU369013	EU369052	EU369072
<i>Akanthomyces</i> sp.	NHJ 3582	Stink bug (Hemiptera)	EU369097	EU369034	EU369015		
<i>Aschersonia badia</i>	BCC 8105	Scale insect (Hemiptera)	DQ522573	DQ518752	DQ522317	DQ522363	DQ522411
<i>A. placenta</i>	BCC 7869	Scale insect (Hemiptera)	EF469121	EF469074	EF469056	EF469085	EF469104
<i>Balansia henningsiana</i>	GAM 16112	<i>Panicum</i> sp. (Poaceae)	AY545723	AY545727	AY489610	AY489643	DQ522413
<i>B. pilulaeformis</i>	AEG 94-2	Poaceae	AF543764	AF543788	DQ522319	DQ522365	DQ522414
<i>Bionectria ochroleuca</i>	CBS 114056	On bark	AY489684	AY489716	AY489611	DQ842031	DQ522415
<i>Claviceps fusiformis</i>	ATCC 26019	Poaceae	DQ522539	U17402	DQ522320	DQ522366	
<i>C. paspali</i>	ATCC 13892	Poaceae	U32401	U47826	DQ522321	DQ522367	DQ522416
<i>C. purpurea</i>	GAM 12885	<i>Dactylis glomerata</i> (Poaceae)	AF543765	AF543789	AF543778	AY489648	DQ522417
<i>Conoideocrella luteorostrata</i>	NHJ 11343	Scale insect (Hemiptera)	EF468995	EF468850	EF468801	EF468906	
<i>C. luteorostrata</i>	NHJ 12516	Scale insect (Hemiptera)	EF468994	EF468849	EF468800	EF468905	EF468946
<i>C. tenuis</i>	NHJ 345.01	Scale insect (Hemiptera)	EU369111	EU369045	EU369030		EU369088
<i>C. tenuis</i>	NHJ 6293	Scale insect (Hemiptera)	EU369112	EU369044	EU369029	EU369068	EU369087
<i>C. tenuis</i>	NHJ 6791	Scale insect (Hemiptera)	EU369113	EU369046	EU369028	EU369069	EU369089
<i>Cordyceps cardinalis</i>	OSC 93609	Lepidopteran larva	AY184973	AY184962	DQ522325	DQ522370	DQ522422
<i>C. confragosa</i>	CBS 101247	<i>Coccus viridis</i> (Hemiptera)	AF339604	AF339555	DQ522359	DQ522407	DQ522466
<i>C. gunnii</i>	OSC 76404	Lepidopteran larva	AF339572	AF339522	AY489616	AY489650	DQ522426
<i>C. militaris</i>	OSC 93623	Lepidopteran pupa	AY184977	AY184966	DQ522332	DQ522377	AY545732
<i>C. piperis</i>	CBS 116719	Scale insect (Hemiptera)		AY466442	DQ118749	DQ127240	EU369083
<i>C. scarabaeicola</i>	ARSEF 5689	Scarabaeid adult (Coleoptera)	AF339574	AF339524	DQ522335	DQ522380	DQ522431
<i>C. tuberculata</i>	OSC 111002	Lepidoptera	DQ522553	DQ518767	DQ522338	DQ522384	DQ522435
<i>Cosmospora coccinea</i>	CBS 114050	<i>Inonotus nodulosus</i> (Hymenomycetes)	AY489702	AY489734	AY489629	AY489667	DQ522438
<i>Elaphocordyceps capitata</i>	OSC 71233	<i>Elaphomyces</i> sp. (Euascomycetes)	AY489689	AY489721	AY489615	AY489649	DQ522421
<i>E. fracta</i>	OSC 110990	<i>Elaphomyces</i> sp. (Euascomycetes)	DQ522545	DQ518759	DQ522328	DQ522373	DQ522425
<i>E. ophioglossoides</i>	OSC 106405	<i>Elaphomyces</i> sp. (Euascomycetes)	AY489691	AY489723	AY489618	AY489652	DQ522429
<i>Engyodontium aranearum</i>	CBS 309.85	Spider (Arachnida)	AF339576	AF339526	DQ522341	DQ522387	DQ522439
<i>Epichloë typhina</i>	ATCC 56429	<i>Festuca rubra</i> (Poaceae)	U32405	U17396	AF543777	AY489653	DQ522440
<i>Gibellula</i> cf. <i>alba</i>	NHJ 11679	Spider (Arachnida)			EU369016	EU369054	
<i>G. longispora</i>	NHJ 12014	Spider (Arachnida)	EU369098		EU369017	EU369055	EU369075
<i>G. pulchra</i>	NHJ 10808	Spider (Arachnida)	EU369099	EU369035	EU369018	EU369056	EU369076
<i>Gibellula</i> sp.	NHJ 10788	Spider (Arachnida)	EU369101	EU369036	EU369019	EU369058	EU369078
<i>Gibellula</i> sp.	NHJ 13158	Spider (Arachnida)	EU369100	EU369037	EU369020	EU369057	EU369077
<i>Gibellula</i> sp.	NHJ 5401	Spider (Arachnida)	EU369102			EU369059	EU369079
<i>Glomerella cingulata</i>	CBS 114054	<i>Fragaria</i> sp. (Rosaceae)	AF543762	AF543786	AF543773	AY489659	DQ522441
<i>Haptocillium balanoides</i>	CBS 250.82	Nematode	AF339588	AF339539	DQ522342	DQ522388	DQ522442
<i>H. sinense</i>	CBS 567.95	Nematode	AF339594	AF339545	DQ522343	DQ522389	DQ522443
<i>Hirsutella</i> sp.	OSC 128575	Hemipteran adult	EF469126	EF469079	EF469064	EF469093	EF469110
<i>Hydropisphaera erubescens</i>	ATCC 36093	<i>Cordyline banksii</i> (Laxmanniaceae)	AY545722	AY545726	DQ522344	DQ522390	AY545731

Table 1 continued

Taxon	Specimen voucher <sup>a</sup>	Host/substratum	GenBank accession nos				
			nu-rSSU	nu-rLSU	TEF	RPB1	RPB2
<i>H. peziza</i>	CBS 102038	On bark	AY489698	AY489730	AY489625	AY489661	DQ522444
<i>Hypocrea lutea</i>	ATCC 208838	On decorticated conifer wood	AF543768	AF543791	AF543781	AY489662	DQ522446
<i>H. schizostachyi</i>	BCC 14123	Scale insect (Hemiptera)	DQ522557	DQ518771	DQ522346	DQ522392	DQ522447
<i>Hypocrella</i> sp.	GJS 89-104	Scale insect (Hemiptera)	U32409	U47832	DQ522347	DQ522393	DQ522448
<i>Hypomyces polyporinus</i>	ATCC 76479	<i>Trametes versicolor</i> (Hymenomycetes)	AF543771	AF543793	AF543784	AY489663	
<i>Isaria farinosa</i>	OSC 111005	Lepidopteran pupa	DQ522558	DQ518772	DQ522348	DQ522394	
<i>I. takamizusanensis</i>	NHJ 3497	Stink bug (Hemiptera)	EU369096	EU369033	EU369014	EU369053	EU369074
<i>I. tenuipes</i>	OSC 111007	Lepidopteran pupa	DQ522559	DQ518773	DQ522349	DQ522395	DQ522449
<i>Lecanicillium antillanum</i>	CBS 350.85	Agaric (Hymenomycetes)	AF339585	AF339536	DQ522350	DQ522396	DQ522450
<i>L. psalliotae</i>	CBS 532.81	Soil	AF339609	AF339560	EF469067	EF469096	EF469112
<i>L. psalliotae</i>	CBS 101270	Soil	EF469128	EF469081	EF469066	EF469095	EF469113
<i>Mariannaea pruinosa</i>	ARSEF 5413	<i>Iragoides fasciata</i> (Lepidoptera)	AY184979	AY184968	DQ522351	DQ522397	DQ522451
<i>Metacordyceps chlamydosporia</i>	CBS 101244	Egg of slug (Diplopoda)	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
<i>M. taii</i>	ARSEF 5714	Lepidoptera	AF543763	AF543787	AF543775	DQ522383	DQ522434
<i>M. album</i>	ARSEF 2082	<i>Cofana spectra</i> (Hemiptera)	DQ522560	DQ518775	DQ522352	DQ522398	DQ522452
<i>M. anisopliae</i>	ARSEF 3145	<i>Oryctes rhinoceros</i> (Coleoptera)	AF339579	AF339530	AF543774	DQ522399	DQ522453
<i>M. flavoviride</i>	ARSEF 2037	<i>Nilaparvata lugens</i> (Hemiptera)	AF339580	AF339531	DQ522353	DQ522400	DQ522454
<i>Myriogenospora atramentosa</i>	AEG 96-32	<i>Andropogon virginicus</i> (Poaceae)	AY489701	AY489733	AY489628	AY489665	DQ522455
<i>Nectria cinnabarina</i>	CBS 114055	<i>Betula</i> sp. (Betulaceae)	U32412	U00748	AF543785	AY489666	DQ522456
<i>Nomuraea atypicola</i>	CBS 744.73	Spider (Arachnida)	EF468987	EF468841	EF468786	EF468892	
<i>Ophiocordyceps acicularis</i>	OSC 128580	Coleoptera	DQ522543	DQ518757	DQ522326	DQ522371	DQ522423
<i>O. agriotidis</i>	ARSEF 5692	Coleoptera	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
<i>O. aphodii</i>	ARSEF 5498	<i>Aphodius hewitti</i> (Coleoptera)	DQ522541	DQ518755	DQ522323		DQ522419
<i>O. brunneipunctata</i>	OSC 128576	Coleoptera	DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
<i>O. heteropoda</i>	OSC 106404	Nymph of cicada (Hemiptera)	AY489690	AY489722	AY489617	AY489651	
<i>O. pulvinata</i>	TNS-F 30044	<i>Camponotus obscuripes</i> (Hymenoptera)	GU904208		GU904209	GU904210	
<i>O. melolonthae</i>	OSC 110993	Scarabaeid larva (Coleoptera)	DQ522548	DQ518762	DQ522331	DQ522376	
<i>O. pruinosa</i>	NHJ 12994	Scale insect (Hemiptera)	EU369106	EU369041	EU369024	EU369063	EU369084
<i>O. ravenelii</i>	OSC 110995	Coleopteran larva	DQ522550	DQ518764	DQ522334	DQ522379	DQ522430
<i>O. stylophora</i>	OSC 111000	Elaterid larva (Coleoptera)	DQ522552	DQ518766	DQ522337	DQ522382	DQ522433
<i>O. unilateralis</i>	OSC 128574	Ant (Hymenoptera)	DQ522554	DQ518768	DQ522339	DQ522385	DQ522436
<i>O. variabilis</i>	ARSEF 5365	Dipteran larva	DQ522555	DQ518769	DQ522340	DQ522386	DQ522437
<i>Ophionectria trichospora</i>	CBS 109876	On liana	AF543766	AF543790	AF543779	AY489669	DQ522457
<i>Orbiocrella petchii</i>	NHJ 5318	Scale insect (Hemiptera)	EU369105	EU369040	EU369021	EU369062	EU369080
<i>O. petchii</i>	NHJ 6209	Scale insect (Hemiptera)	EU369104	EU369039	EU369023	EU369061	EU369081
<i>O. petchii</i>	NHJ 6240	Scale insect (Hemiptera)	EU369103	EU369038	EU369022	EU369060	EU369082
<i>Paecilomyces lilacinus</i>	CBS 431.87	<i>Meloidogyne</i> sp. (Nematoda)	AY624188	EF468844	EF468791	EF468897	EF468940
<i>P. lilacinus</i>	CBS 284.36	Soil	AY624189	AY624227	EF468792	EF468898	EF468941
<i>Pochonia gonioides</i>	CBS 891.72	Nematode	AF339599	AF339550	DQ522354	DQ522401	DQ522458
<i>Pseudonectria rousseliana</i>	CBS 114049	<i>Buxus sempervirens</i> (Buxaceae)	AF543767	U17416	AF543780	AY489670	DQ522459
<i>Regiocrella camerunensis</i>	ARSEF 7682	Scale insect (Hemiptera)		DQ118735	DQ118743	DQ127234	
<i>Rotiferophthora angustispora</i>	CBS 101437	Rotifer (Rotifera)	AF339584	AF339535	AF543776	DQ522402	DQ522460

**Table 1** continued

Taxon	Specimen voucher <sup>a</sup>	Host/substratum	GenBank accession nos				
			nu-rSSU	nu-rLSU	TEF	RPB1	RPB2
<i>Roumegueriella rufula</i>	CBS 346.85	<i>Globodera rostochiensis</i> (Nematoda)	DQ522561	DQ518776	DQ522355	DQ522403	DQ522461
<i>Shimizuomyces paradoxus</i>	EFCC 6279	<i>Smilax sieboldi</i> (Smilacaceae)	EF469131	EF469084	EF469071	EF469100	EF469117
<i>Simplicillium lamellicola</i>	CBS 116.25	<i>Agaricus bisporus</i> (Hymenomycetes)	AF339601	AF339552	DQ522356	DQ522404	DQ522462
<i>S. lanosoniveum</i>	CBS 101267	<i>Hemileia vastatrix</i> (Urediales)	AF339603	AF339554	DQ522357	DQ522405	DQ522463
<i>S. lanosoniveum</i>	CBS 704.86	<i>Hemileia vastatrix</i> (Urediales)	AF339602	AF339553	DQ522358	DQ522406	DQ522464
<i>Sphaerostilbella berkeleyana</i>	CBS 102308	Polypore (Hymenomycetes)	AF543770	U00756	AF543783	AY489671	DQ522465
<i>Torrubiella ratticaudata</i>	ARSEF 1915	Spider (Arachnida)	DQ522562	DQ518777	DQ522360	DQ522408	DQ522467
<i>Torrubiella</i> sp.	DJ 29	Spider (Arachnida)	EU369108		EU369027	EU369065	
<i>Torrubiella</i> sp.	NHJ 5112	Spider (Arachnida)	EU369109	EU369043	EU369026	EU369066	
<i>Torrubiella</i> sp.	NHJ 6709	Spider (Arachnida)	EU369110	EU369042	EU369025	EU369067	EU369086
<i>Torrubiella</i> sp.	NHJ 7859	Spider (Arachnida)	EU369107			EU369064	EU369085
<i>T. wallacei</i>	CBS 101237	Lepidoptera	AY184978	AY184967	EF469073	EF469102	EF469119
<i>Verticillium dahliae</i>	ATCC 16535	<i>Crataegus crus-galli</i> (Rosaceae)	AY489705	AY489737	AY489632	AY489673	DQ522468
<i>V. epiphytum</i>	CBS 384.81	<i>Hemileia vastatrix</i> (Uredinales)	AF339596	AF339547	DQ522361	DQ522409	DQ522469
<i>V. incurvum</i>	CBS 460.88	<i>Ganoderma lipsiense</i> (Hymenomycetes)	AF339600	AF339551	DQ522362	DQ522410	DQ522470
<i>Viridispora diparietispora</i>	CBS 102797	<i>Crataegus crus-galli</i> (Rosaceae)	AY489703	AY489735	AY489630	AY489668	DQ522471

<sup>a</sup> Herbarium abbreviations follow those of Johnson et al. (2009) plus TNS, National Museum of Nature and Science

achieved with the primers 983F/2218R (Stephen Rehner personal communication). RPB1 was amplified using the primers CRPB1A and RPB1CR (Castlebury et al. 2004). RPB2 was amplified using the primers fRPB2-5F and fRPB2-7cR (Liu et al. 1999). PCR reactions were performed in either an iCycler or MyCycler (BioRad, Hercules, CA) using MasterAmp 2× PCR premix E (Epicenter, Madison WI) and Novagen Taq polymerase. Reaction conditions were the same as those used in Johnson et al. (2009). PCR products were cleaned using the GeneClean III kit following the manufacturer's instructions and sequenced using the MacroGen (Seoul, South Korea) sequencing service with the primers used for the initial amplifications.

#### Phylogenetic analyses

Raw sequence reads returned from MacroGen were edited using the CodonCode Aligner, version 2.0.6 (Dedham, MA). Sequences generated for this study were combined with the dataset used in Johnson et al. (2009) to compare the new species with a broad sampling of other torrubiellid morphologies. Genbank and specimen voucher information is provided in Table 1. An initial alignment of edited sequence data was obtained for each individual gene using MAFFT version 6 (Katoh et al. 2002; Katoh and Toh 2008), and minor adjustments were made manually using

the program BioEdit version 7.05 (Hall 1999). Ambiguously aligned regions were excluded from phylogenetic analyses, and gaps were treated as missing data. Maximum likelihood analyses were conducted using RAxML version 7.0.4 (Stamatakis 2006) with 500 rapid bootstrap replicates by multithreading across four 2× AMD Opteron 285 Dual Core Processors on a concatenated dataset containing all five genes. The CAT-GAMMA model of evolution was employed during the rapid bootstrapping phase, and the GTR-GAMMA model of evolution was specified for the final likelihood tree. The dataset consisted of 11 data partitions, one each for nu-rSSU and nu-rLSU plus nine for each of the three codon positions for the protein coding genes *TEF*, *RPB1* and *RPB2*.

## Results

### DNA sequencing

Of the five genes sought for *O. pulvinata*, it was not possible to obtain sequence data for nu-rLSU and RPB2. As a quality control check, all sequenced product was subjected to a BLAST of the Genbank nucleotide database. During this check it was discovered that the top blast hit for nu-rLSU of *O. pulvinata* was not in place in the Hypocreales.

Parsimony analyses of the nu-rLSU sequences used in this analysis confirm that the sequence occupies a long branch basal to the rest of the tree (data not shown). Based upon these results, this sequence was interpreted to be from a contaminant and excluded from further analysis. Several attempts at amplifying RPB2 failed; thus, data from this gene were unavailable for analysis. All other sequences passed the above quality control checks and were retained for analysis. The resulting alignment contained approximately 3,000 bp (nu-rSSU, TEF, RPB1) for *O. pulvinata*.

#### Phylogenetic relationships

Phylogenetic analyses supported the family level classification of Sung et al. (2007b) with bootstrap proportions (BP) of 100%; for Cordycipitaceae, Ophiocordycipitaceae and Clavicipitaceae the overall log-likelihood for the tree was  $-85,177.8$ . Missing data for *O. pulvinata* and other taxa were not considered detrimental since over 50% of the genes sought were present in the final alignment, and no disruption of strongly supported nodes in Johnson et al. (2009) was observed. Total evidence or supermatrix methods of molecular phylogenetics are capable of providing robust, well-supported trees in spite of missing data (de Queiroz and Gatesy 2007). *Ophiocordyceps pulvinata* was resolved as a sister species to *O. unilateralis* (Fig. 1). Although this species lacks a stipe, several other morphological characteristics unite these two taxa including color of the perithecia and fertile region, location of ascoma production on the host, morphology of the ascospores and an ant pathogenic lifestyle where the host exhibits summit disease (Fig. 2). Examination of perithecium, ascus, ascospore and host characters combined with phylogenetic analyses of molecular data revealed that Kobugataaritake is distinct from *O. unilateralis* and other similar described species. Therefore, a new species, *O. pulvinata*, is proposed.

#### Taxonomy

*Ophiocordyceps pulvinata* Kepler, Kaitsu & Spatafora, sp. nov.

*Mycobank* no.: MB 516675

Stroma astipitata, inter capitulumque thoracem in aucto colloideo brunneo vel brunneolo hospitem quasi omnino cingenti emergens. Perithecia in massa densa hyphali inclusa,  $400\text{--}600 \times 150\text{--}250 \mu\text{m}$ , ostiolis expositis atrob-runneis. Asci 8-spori, hyalini, clavati,  $220\text{--}300 \times 9\text{--}19 \mu\text{m}$ , pileo apicali distincto  $4\text{--}5.4 \times 6\text{--}9 \mu\text{m}$ . Ascosporae filiformes, apicibus contractis,  $160\text{--}220 \times 3\text{--}5 \mu\text{m}$ , septa formantes, segmenta  $10\text{--}14 \times 3\text{--}5 \mu\text{m}$ , non in sporis partitis secedentes.

*Type*: Japan, Fukushima Prefecture, on *Camponotus obscuripes* Mayr (Hymenoptera: Formicidae) clinging to twigs, September, 2007, Collector: anonymous (TNS-F-30044, holotype; TNS-F-30045 and TNS-F-30046, paratypes).

*Genbank sequence information*: nu-rSSU GU904208; TEF GU904209; RPB1 GU904210

*Etymology*: The species epithet is derived from the cushion-like shape of the stroma.

*Anamorph*: unknown

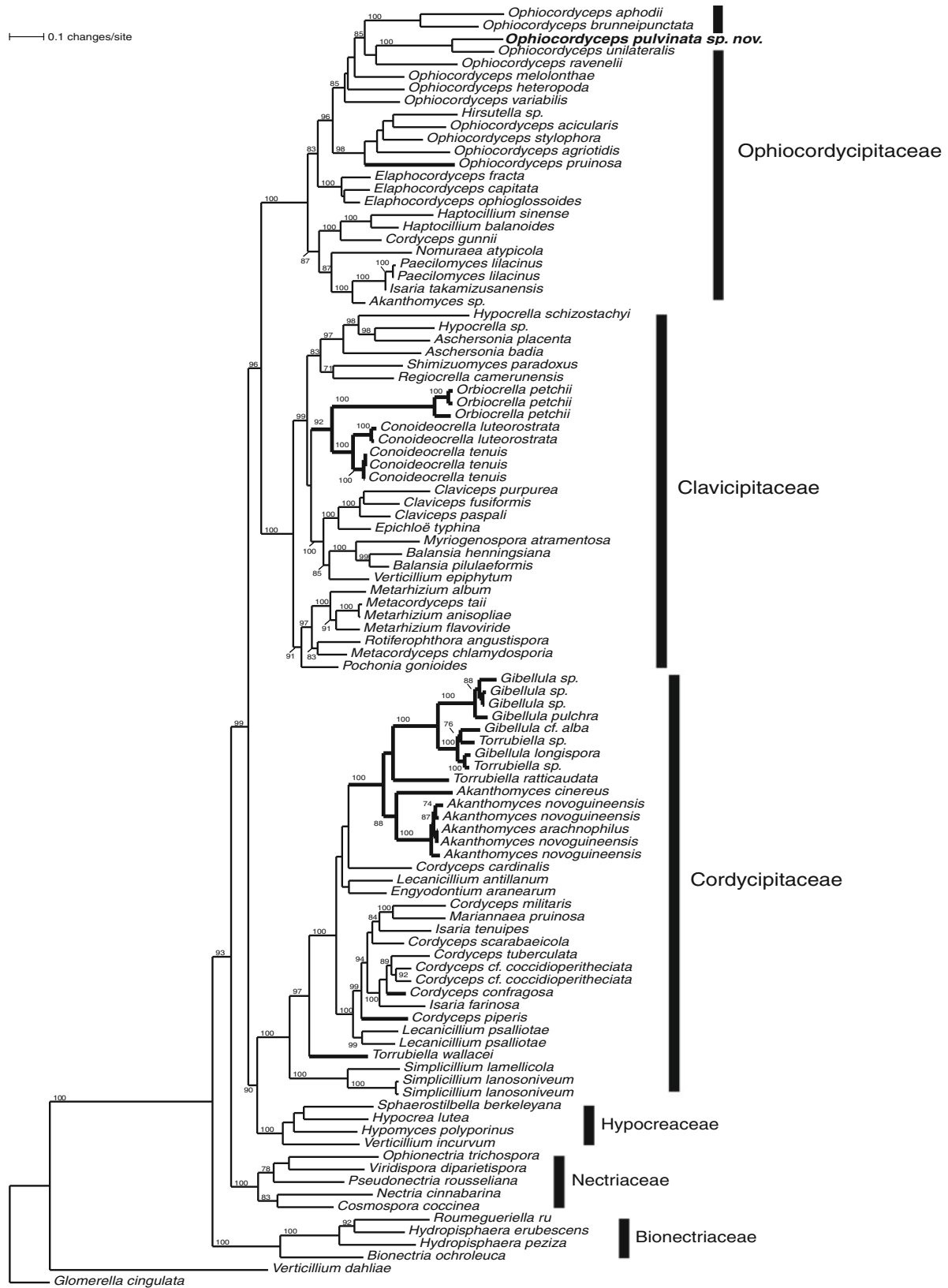
Infected ant occurring on elevated plant material with jaws clamped on the substrate. Host affixed to substrate by brown hyphae emerging from the mouth and segmental fissures of the legs. Stroma astipitate, emerging between the head and thorax in a collar-like growth almost completely encircling the host, brown to tan. Perithecia embedded in dense hyphal mass,  $400\text{--}600 \times 150\text{--}250 \mu\text{m}$ , ostioles exposed dark brown. Asci 8-spored, hyaline, clavate  $220\text{--}300 \times 9\text{--}19 \mu\text{m}$ , with distinct apical cap  $4\text{--}5.4 \times 6\text{--}9 \mu\text{m}$ . Ascospores filiform, tips tapering,  $160\text{--}220 \times 3\text{--}5 \mu\text{m}$ , forming septae, segments  $10\text{--}14 \times 3\text{--}5 \mu\text{m}$  not disarticulating into partspores.

#### Discussion

*Ophiocordyceps pulvinata* is a species currently known only from Japan. The specimens examined for this description were collected during an epizootic event with hundreds of infected ants found in a localized area. The cadavers were found affixed to twigs and blades of grass elevated off of the ground. Similar species include *O. unilateralis* and *O. ridleyi* (Masse) Kobayasi. *Ophiocordyceps unilateralis* is a morphologically variable species, and several species that differ slightly in the formation of the perithecial pad have since been recognized as synonyms (e.g., *C. subunilateralis* Henn.). However, the perithecial pad always occurs on a stipe that may vary from slender-filiform to robust-clavate (as in *var. clavata*). The species newly described here can be distinguished from *O. unilateralis* by the complete lack of a stipe as well as larger perithecia and ascospores. *Ophiocordyceps ridleyi* and *O. formicivora* also produce their perithecia on stipes (Kobayasi 1939). Reports exist of *O. formicivora* specimens lacking or with much reduced stipes (Kobayasi 1939). However, the perithecia are described as semi-immersed, while those of *O. pulvinata* are completely immersed, with only the tip of the ostiole exposed at the surface.

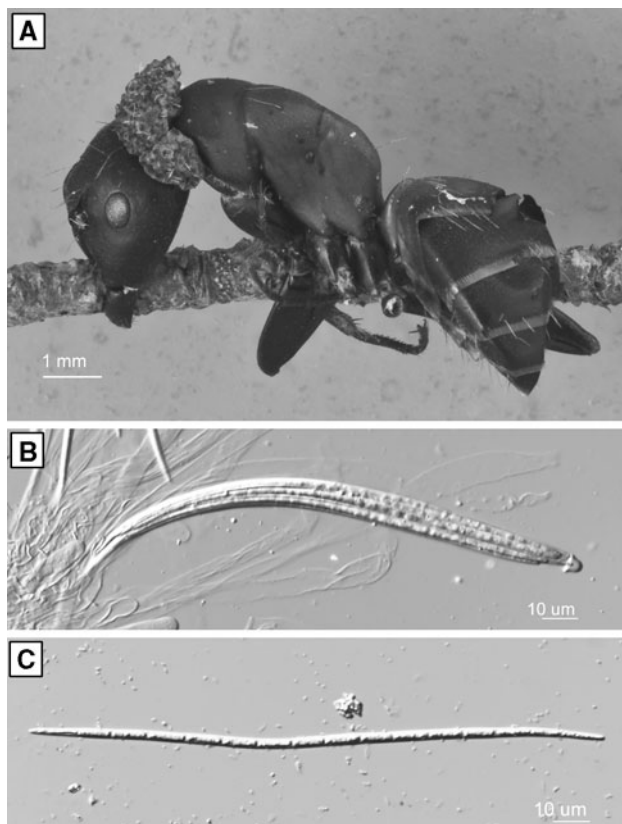
Although lacking a stipe, *O. pulvinata* is similar to *O. unilateralis* in several respects, and microscopic similarities provide strong support for the relationship observed in the phylogenetic reconstruction. The shape of the ascus





**Fig. 1** Maximum likelihood tree obtained from a concatenated dataset of five genes (nu-rSSU, nu-rLSU, TEF, RPB1, RPB2) showing the placement of *O. pulvinata* relative to other taxa with a torribellid

morphology (species subtended by bold branches). Numbers above the branches indicate BS proportions  $\geq 70$



**Fig. 2** *Ophiocordyceps pulvinata* (TNS-F-30045). **a** Growth habit on adult *Camponotus obscuripes*. Note the mandibles clamped around the twig. **b** Ascus. **c** Ascospore

in both species is more clavate (Fig. 2b) than cylindrical as is typical for many species of cordycepioid fungi. Likewise, in both species the ascospores are septate but do not disarticulate at maturity, and they taper slightly at the end to form fusiform tips (Fig. 2c). The asci and spores of *O. pulvinata* are consistent with the generic concept of *Ophiocordyceps* as proposed by Petch (1931), which included *O. unilateralis*. Overall, the asci and ascospores of *O. pulvinata* are longer, but there is some overlap in the dimensions of width (Table 2). Macroscopically, the brown coloration of both species is also similar. Sung et al. (2007b) found color to be a relatively informative characteristic at the level of family, with darkly pigmented species or species bruising dark upon handling generally found in the Ophiocordycipitaceae. Species of *Torrubiella* are often pallid, cream colored or whitish (Kobayasi and

Shimizu 1982). Sung et al. (2007b) also proposed that texture is another informative character useful in determination of family level relationships. The Ophiocordycipitaceae typically produce stromata that range from fibrous to wiry. The extremely reduced external morphology makes assessing this trait difficult, further compounded by examination of dried specimens. However, both the coloration and texture of the perithecial pads are similar for both *O. unilateralis* and *O. pulvinata* in dried specimens.

The diversity contained within the *O. unilateralis* clade represents a complex of species, including the newly described *O. pulvinata*. Kobayasi (1939) described *O. unilateralis* var. *clavata* (as *C. unilateralis* var. *clavata*) to accommodate specimens found in Japan possessing a much more robust stipe than specimens found in tropical habitats. Evans and Samson (1982) described *O. kniphofiodes* (H.C. Evans & Samson) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora as an ant pathogen similar to *O. unilateralis* with regards to the asci and spores but producing a brightly colored, apical clava on a long filiform stipe, rather than a darkly colored, lateral pad with a comparatively shorter, more robust stipe. *Ophiocordyceps cucumispora* (H.C. Evans & Samson) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora has a similar macromorphology to *O. unilateralis*; however, the spores of this species disarticulate at maturity, forming partspores with a shape and bumpy surface similar to a cucumber (hence, the species epithet) and may not be a part of this complex (Evans and Samson 1982). Where the anamorph is known for species in the *O. unilateralis* complex, they are all *Hirsutella*, but no anamorph was observed for *O. pulvinata*, and a culture was not established from the field collected material.

Species of *Torrubiella* typically produce superficial perithecia on a subiculum loosely surrounded by hyphae, although some forms may deviate from this pattern. *Torrubiella globosostipitata* Kobayasi & Shimizu produces its perithecia on a pulvinate pad (Kobayasi and Shimizu 1982), and *T. formicarum* Samson, Reenen & H.C. Evans and *T. pseudogibbellulae* Samson, Reenen & H.C. Evans produce perithecia on elevated tufts of loose hyphae and a diffuse stroma, respectively. Notably, *T. pseudogibbellulae* and *T. formicarum* were described from ant hosts, a rare condition for species of *Torrubiella* (Samson et al. 1989). Both species are similar to *O. pulvinata* in that the color is brownish, and *T. pseudogibbellulae* has ascospores that do

**Table 2** Comparison of *Ophiocordyceps pulvinata* with similar species

Species	Host in Japan	Presentation of perithecia	Size of asci ( $\mu\text{m}$ )	Size of ascospores ( $\mu\text{m}$ )	Source
<i>O. pulvinata</i>	<i>Camponotus obscuripes</i>	Immersed	220–300 $\times$ 9–19	160–220 $\times$ 3–5	This study
<i>O. unilateralis</i>	<i>Polyrhachis lamellidens</i>	Immersed	105–157 $\times$ 8–13	100–145 $\times$ 3–4	Kobayasi (1939)
<i>O. formicivora</i>	Not known	Semi-immersed	Not known	Not known	Kobayasi (1939)



not break into partspores, although septations were also not observed. *Torrubiella formicarum* is distinguished from *O. pulvinata* by ascospores that disarticulate into partspores and an arrangement of perithecia singly or in clumps covering the entire body of the host. The embedded perithecia of *O. pulvinata* are confined to a mass of stroma forming a collar-like growth between the head and thorax of the host.

The diversity of clavicipitaceous teleomorphs sampled to date does not place any ant pathogen outside of the Ophiocordycipitaceae (Sung et al. 2007b), a finding consistent with the results of this study. The astipitate morphology of *O. pulvinata* suggests a relationship to torrubielloid fungi; however, the perithecia are embedded in a stroma that is morphologically similar to the lateral stromatal pad of perithecia in *O. unilateralis*. The phylogenetic placement of *O. pulvinata* as a sister taxon to *O. unilateralis* highlights the morphological diversity present throughout all clades of clavicipitaceous fungi, especially in the macroscopic features. Additionally, *O. pulvinata* represents a novel evolution of the astipitate morphology relative to the currently known astipitate taxa. Microscopic features of the ascus and ascospore proved to be reliable characters for taxonomic determination of this species in the original sense of the genus sensu Petch (1931).

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