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ORIGINAL ARTICLE

Cordyceps cuncunae (Ascomycota, Hypocreales), a new pleoanamorphic species from temperate rainforest in southern Chile

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Abstract *Cordyceps cuncunae* Palfner sp. nov. is reported from Valdivian rainforest in southern Chile, parasiting larvae of an unidentified ghost moth species (Lepidoptera, Hepialidae) which probably feed on roots of *Laureliopsis philippiana*. Morphology and anatomy of stromata as well as morphological and molecular characteristics of mycelium in pure culture which produces two anamorphs, one of them *Lecanicillium*-like, are described. The systematic position of the new taxon within the most recent generic concept is discussed. This is the first record of an endemic *Cordyceps* species from Chile.

Keywords *Cordyceps cuncunae* · Synanamorphs · *Lecanicillium* · Entomopathogenic fungi · Lepidoptera · Hepialidae · *Laureliopsis philippiana*

Introduction

Fungal parasites of the family Clavicipitaceae s. l. (Hypocreales, Ascomycota), which infect arthropods, are probably more than 130 my old (Sung et al. 2008). At present, more than 400 species are known from all continents except Antarctica (Sung et al. 2007). Known species richness of this particular group varies considerably between geographic regions, but to date seems to be correlated with local concentration of research efforts rather than with existing diversity. Most species have been reported from Asia, especially Japan and China (Kobayasi 1982; Sung et al. 2007), where the genus *Cordyceps* Fr. and related taxa have been studied extensively for decades and some species form an important part of traditional and

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modern medicine. On the other hand, there are still large and poorly studied areas, especially in the tropics and the southern hemisphere, where a high diversity of Clavicipitacean fungi can be expected (Aung et al. 2008). The temperate rainforests of southern Chile and Argentina are a striking example for still existing knowledge gaps: less than a handful of records have been published so far (Mujica et al. 1980; Mueller and Rajchenberg 1991) despite the extraordinary richness of potential arthropod hosts across highly diverse habitats, ecosystems and climate zones which characterize the austral part of South America. New records, hence, can be expected in the future and the discovered taxa may also be of economic interest, considering that some entomopathogenic fungi have been shown to be powerful agents in biological pest control (Meyling and Eilenberg 2007; Shah and Pell 2003) as well as producers of specific secondary metabolites to be used in new medical therapies (Holliday and Cleaver 2008; Paterson 2008).

Based on morphology and anatomy of stromata as well as morphological and molecular characteristics of two anamorphs observed in pure culture, we describe a new species of *Cordyceps* (Petch) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, discovered in temperate rainforest of the Valdivian Lake Region in southern Chile on large caterpillars of an unidentified ghost moth species (Lepidoptera, Hepialidae).

Material and methods

Macroscopical and microscopical characteristics of stromata with attached caterpillar carcasses were documented by digital colour photographs taken of fresh specimens in situ and in the laboratory with a Nikon Coolpix 950 (Nikon, Tokyo, Japan) camera. Microscopical features were studied on fresh and dried material mounted in water and 5% KOH, respectively, and documented by colour microphotographs and line drawings at 1000× magnification (bright field, oil immersion objective) on a Leitz Dialux (Leitz, Wetzlar, Germany) microscope equipped with a camera adapter and a drawing device (camera lucida). The host was identified to the family level based on morphological characteristics of the colonized caterpillar carcasses according to Stehr (1987). For conservation the specimens were desiccated for 48 hours at 50°C in a ventilated oven (Heraeus, Hanau, Germany). Dried reference material was deposited in the fungal collection of Concepción University (CONC-F).

For axenic culture, small explants from endosclerotia inside caterpillar carcasses were cut under a laminar flow hood (Factomet, Santiago, Chile) with a sterile scalpel and placed on Petri dishes with 2% Potato Dextrose Agar (PDA). Growing cultures were kept at 25°C in the dark in an incubator (Memmert, Schwabach, Germany). Mycelial structures were documented in the same way as the stromata. Reference cultures are kept at the Mycology Laboratory, Department of Botany of Concepción University.

For DNA extraction, amplification and sequencing (ITS region of ribosomal DNA), approximately 200 mg of cultured mycelium were placed in a mortar with liquid nitrogen and ground into fine powder. The powder was placed into Eppendorf tubes to perform the DNA extraction according the protocol described by Stange et al. (1998). Concentration of the purified DNA was measured in a ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The ribosomal ITS region was amplified by PCR reaction with primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′)/ ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as reported by Tao et al. (2008). The PCR assay was performed in a final volume of 12.5 μl. Each reaction contained PCR buffer 1X (Fermentas, Maryland, USA), 0.2 μg/μl of BSA, 200 μM dNTPs, 1.5 mM of Mg_2Cl, 0.5 μM of each primers, 0.1 U/μl of Taq Polimerase (Fermentas), and 13 ng/μl of DNA. Amplification was performed in a Verity Thermocycler (Applied Biosystems Inc., Carlsbad, USA). PCR conditions were 3 minutes initial denaturation at 95°C followed by 35 cycles of 50 seconds denaturation at 94°C, 50 seconds annealing at 56°C, 1 minute extension at 72°C, and a final 10 minutes extension at 72°C. PCR products were visualized via gel electrophoresis and documented using a digital image system (Discovery, UltraLum Inc., Claremont, USA). The PCR products were sequenced bidirectionally in an automatic ABI 3700 sequencer (Applied Biosystems) by Macrogen Inc. (Seoul, Korea). The bioinformatics analyses were carried out using Geneious Pro 5.1 software (Geneious, Auckland, New Zealand). A phylogenetic tree was constructed using PAUP from inside Geneious software according to the neighbor-joining (NJ) method based on the consensus sequence of *Cordyceps cuncunae* and other known sequences from Clavicipitaceae s. l. representing the three major clades (A, B, C) from the phylogenetic tree created by Sung et al. (2007). The data were bootstrapped 1000 times to estimate the internal stability of each node. *Glomerella cingulata* was used as outgroup .

Results

Taxonomy

Cordyceps cuncunae Palfner sp. nov.

Etymology: cuncuna is the native Chilean word for caterpillar.

Teleomorph (Figs. 1 and 2)

Stromata (Figs. 1a and 2a) ex capitibus chrysalidorum lepidopterarum (Hepialidarum) oriunda, singula vel rariter 2 aggregata, capitata, 80–110–120 mm longis; *receptaculum* ovalis vel subglobosum, ochraceum, leviter glutinosum, 15–18–21×12–15–18 mm; *stipes* cylindricus, albus, siccus, 59–92–105×7–8–10 mm; *peridium* (Fig. 2d) hymeniformis; *perithecia* (Fig. 1c) lageniformes, dense aggregata, immersa, perpendicularia ad superficiem, 772–793–829×257–279–314 µm; *asci* (Figs. 1e, f and 2b) 8-spori, hyalini, cylindrici, apice conspicue inspissato, porifero, 364–391–422×6–7–8 µm; *ascosporae* (Figs. 1e and 2b, c) filiformes, hyalinae, in cumulis albae, 340–375–414×1.5–2 µm, 63-septatae, diffrangentes; *sporae secundariae* (Figs. 1d and 2e) cylindricae vel subfusiformes, 4.3–6.3–8.6 µm longis;

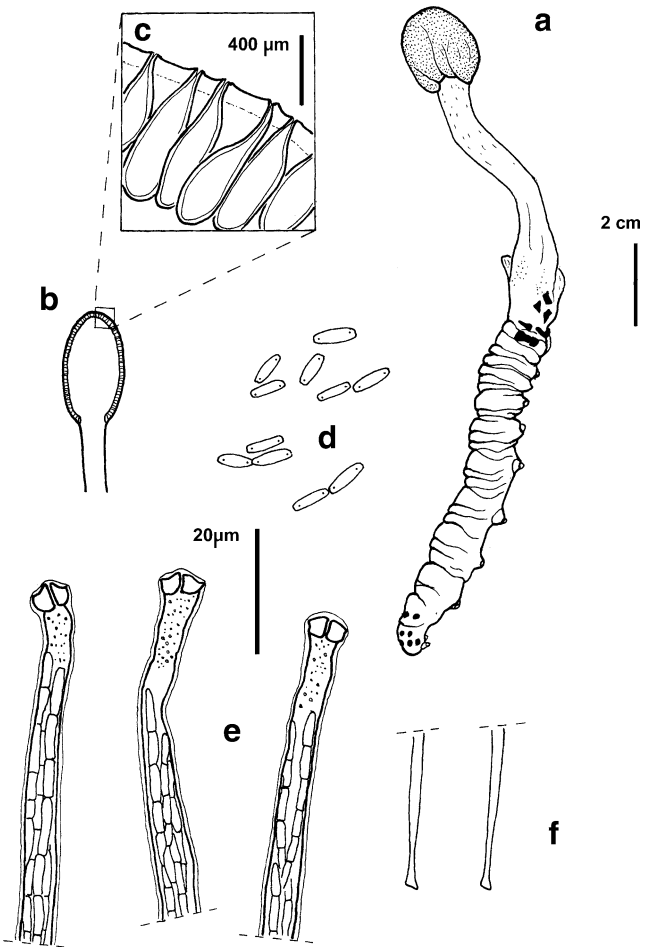

Fig. 1 Macro- and micromorphology of *Cordyceps cuncunae* sp. nov. teleomorph (I); **a**: stroma emerging from host carcass (holotype CONC-F0204); **b**, **c**: schematic drawing of receptacle in longitudinal section, insert (**c**) showing ordinal arrangement of perithecia; **d**: part spores; **e**: ascus tips with thickened apical caps; f: ascus bases

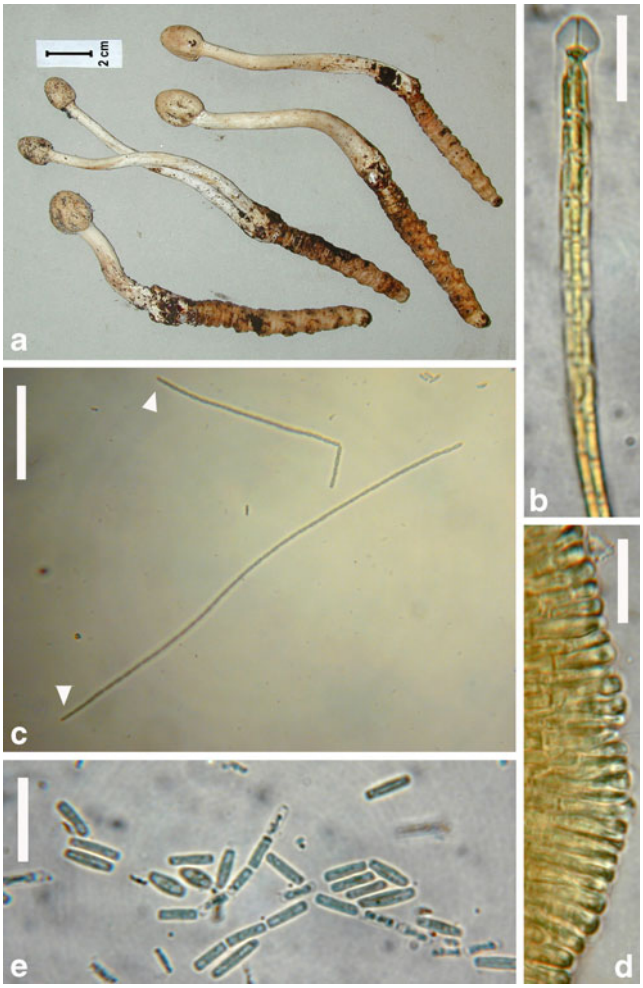

Fig. 2 Macro- and micromorphology of *Cordyceps cuncunae* sp. nov. teleomorph (II); **a**: stromata on host carcasses; **b**: ascus tip with apical cap (bar = 10 µm); **c**: broken ascospore consisting of 64 part spores, terminal segments (arrowheads) slightly longer and tapering (bar= 50 µm); **d**: peridium of receptacle in cross section with palisade-like arranged hyphae (bar=10 µm); **e**: part spores (bar=10 µm)

Anamorph (Figs. 3 and 4)

pleoanamorphigeris in cultivo; *anamorphe prima Lecanicillii* similis; *phialidae* (Figs. 3c and 4b) solitariae vel verticillatae, subulatae, aculeatae vel lanceolatae, hyalinae, 14.3–34.9–64.3×1.5–2.5–4.0 µm; *conidia* (Figs. 3d and 4b) basipetaliter in catenam formantur, hyalina, amygdaliformia vel ovata, 2.8–3.7–4.3×1.4–2.0–2.1 µm; *anamorphe secunda* (Figs. 3a and 4a, c) conidiophoribus columniformis, in circulis formantur, stipitibus cylindricis, candidis, 2–40×0.5–2 mm, apicis conidiigeris ochraciis, globosis, conicis, tuberiformibus vel irregulariter ramificatibus, 0.5–3×0.5–5 mm; *conidia* (Figs. 3b and 4d) copiosissima, in massis humidis aggregata, hyalina, globosa vel subglobosa, 1.5–2–2.5×1.5–2 µm in diam.; *habitat* in silvam Valdivianae ad terram et humum prope *Laureliopsis*

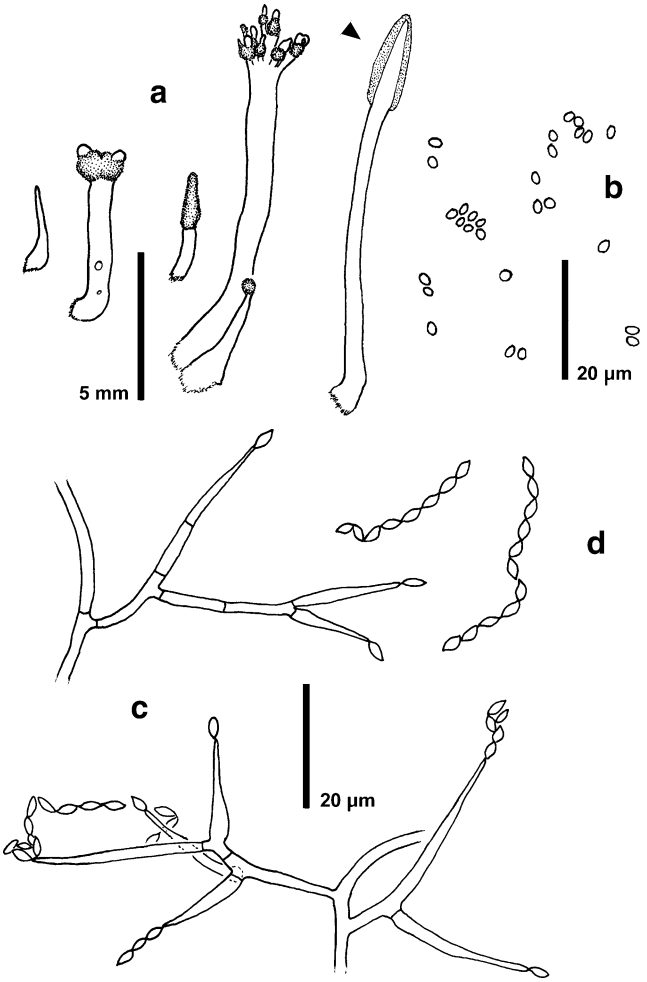

Fig. 3 Macro- and micromorphology of *Cordyceps cuncunae* sp. nov. synanamorphs (I); **a**: developmental stages of macroconidiophores, one in longitudinal section (arrowhead); **b**: conidia; **c**: *Lecanicillium* anamorph with single or verticillate philaides and conidia **d**: chains of conidia

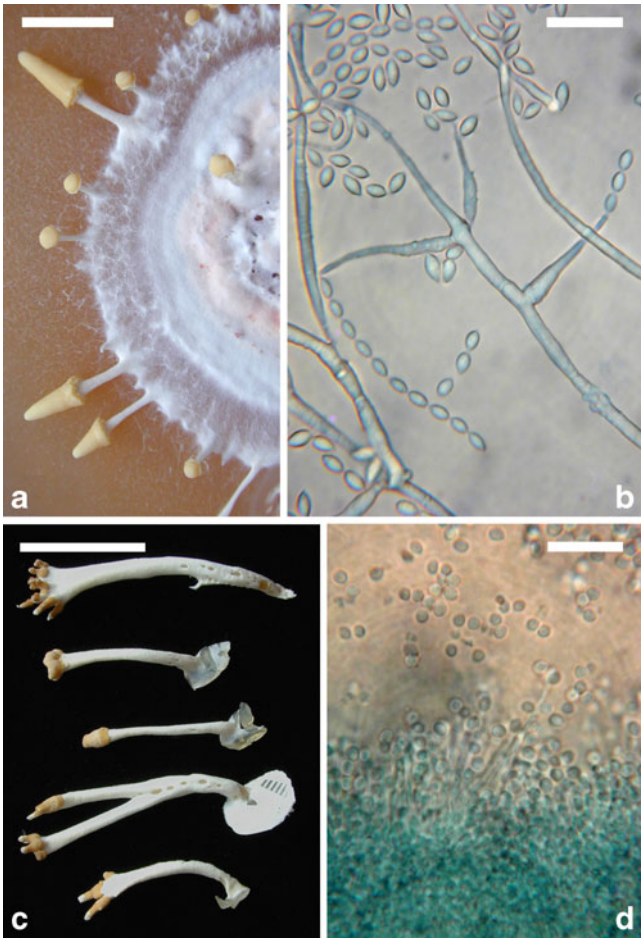

Fig. 4 Macro- and micromorphology of *Cordyceps cuncunae* sp. nov. synanamorphs (II); **a**: mycelial culture on 2% PDA with developing macroconidiophores (bar=5 mm) **b**: *Lecanicillium* anamorph with chains of conidia (bar=10 µm); **c**: macroconidiophores (bar=5 mm); **d**: surface of macroconidiophore in cross section with numerous conidia (bar=10 µm)

philippiana (Loser) Schodde, Chile, Panguipulli, San Pablo de Tregua, 39° 36′ 31′′ lat. austr., 72° 05′ 48′′ long. occid., 19-XII-2005, leg. G. Palfner, holotypus CONC-F0204 in CONC conservatur.

Teleomorph (Figs. 1 and 2)

Stromata (Figs. 1a and 2a) emerging from the heads of large hepialid larvae, vertically and head-up buried in the upper soil and litter, single or (rarely) double, capitate, 80–110–120 mm long; *receptacle* oval to subglobose, 15–18–21×12–15–18 mm, ochre, shiny and slightly glutinous when fresh, ostioles of perithecia appearing as densely arranged, fine, concolorous warts on the surface; *stipe* cylindrical, usually bent, white, dry, 59–92–105×7–8–10 mm; *peridium* (Fig. 2d) hymeniform (palisade-like); *perithecia* (Fig. 1c) flask-shaped, densely arranged, fully immersed, perpendicular to stroma surface, 772–793–829×257–279–314 µm; *asci* (Figs. 1e, f and 2b) 8-spored, cylindrical, tapering towards the base (Fig. 1f), with a gelatinous apical cap bearing a narrow, central pore, 364–391–422×6–7–8 µm; *ascospores* (Figs. 1e and 2b, c) filiform, hyaline, spore print white, 340–375–414×1.5–2 µm, 63-septate, disarticulating into 64 part spores; *part spores* (Figs. 1d and 2e) cylindrical to slightly spindle- or barrel-shaped 4.3–6.3–8.6 µm long, terminal part spores at both ends of ascospore slightly tapering;

Anamorph (Figs. 3 and 4)

pleoanamorphic in pure culture; *first anamorph Lecanicillium*-like, conidiophores mononematous, prostrate, *phialides* (Figs. 3c and 4b) solitary or verticillate (rarely more than two per whorl), subulate to lanceolate or aculeate,

hyaline, 14.3–34.9–64.3×1.5– 2.5–4.0 µm; *conidia* (Figs. 3d and 4b) forming in basipetal chains, hyaline, amygdaliform or oval, 2.8–3.7–4.3×1.4–2.0–2.1 µm; *second anamorph* (Figs. 3a and 4a, c) forming conspicuous, column-shaped conidiophores rising from a thin, tough subiculum, arranged in concentric rings, preferentially in the center and around the margin of older colonies, stalk 2–40×0.5–2 mm, white, conidia-bearing heads roundish, conical, tuberculous or irregularly ramified, 0.5–3×0.5–5 mm, formed by a dense, cream-coloured to ochraceous moist mass of conidia; *conidia* (Figs. 3b and 4d) globose to subglobose, hyaline, 1–2–2.5×1.5–2 µm in diam.; *habitat* in Valdivian rainforest on soil and litter near *Laureliopsis philippiana* (Loser) Schodde.

Examined material Chile, Panguipulli, San Pablo de Tregua, 39° 36′ 31′′ s. l., 72° 05′ 48′′ w. l., 19-XII-2005, leg. G. Palfner, holotype (1 stroma) in Concepción (CONC-F0204), further material (isotypes) studied (same location, same date): CONC-F0201 (1 stroma), CONC-F0202 (1 stroma), CONC-F0203 (2 stromata), CONC-F0205 (1 stroma).

The obtained ITS sequence (624 bp) was entered in the GenBank database (accession number: HQ661167). The closest matching sequence found belonged to *Cordyceps militaris* cf. var. *sphaerocephala* (96.1%, see Table 1).The neighbor-joining tree of *C. cuncunae* and representative taxa from the three major clades within the phylogenetic concept of Clavicipitaceae developed by Sung et al. (2007), places the new species in clade C (Fig. 5).

Discussion

According to the classic revision of *Cordyceps* by Kobayasi (1982), *C. cuncunae* fits in the subgenus *Eucordyceps*, section *Cystocarpon* due to its fragmenting spores, the apical receptacle of the stroma with immersed perithecia and its parasitism on arthropods. It keys out very close to *C. gracilis* (Grev.) Durieu & Mont. by its capitate shape with a roundish receptacle, perithecia size of approx. 800 µm, the palisade-like peridium and host type (hepialid larvae), but can clearly be distinguished from the latter species by the larger size and paler colours of the stromata and particularly by the simultaneous presence of two characteristic anamorphs in culture, one of them *Lecanicillium*-like, whereas in the case of *C. gracilis* only a *Paraisaria* anamorph is known (Samson and Brady 1983). The characteristic column-shaped macroconidiophores with apical, ochraceous masses of small conidia have, to our knowledge, not been reported for any other species of *Cordyceps*.

The most recent, extensive phylogenetic study of the Clavicipitaceae by Sung et al. (2007), based on sequence data from up to seven DNA regions as well as morphological and ecological characteristics, showed that *Cordyceps* s.l. is polyphyletic, splitting into three major clades (A, B, C), from which the emended or new genera *Cordyceps* s. str., *Elaphocordyceps*, *Metacordyceps* and *Ophiocordyceps* have been derived by the same authors.

Following the generic key which is provided within this new classification and which is principally based on colour and texture of stromata, host type and habitat, the new taxon described in this study can be readily assigned to *Cordyceps* Fr. emend. G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora which includes species of molecular clade C. Common morphological and ecological features in this clade which are matched by *C. cuncunae*, are fleshy to fibrous, pale-coloured, stipitate stromata growing from lepidopteran larvae in the upper soil layer. Additional and substantial support for this placement is provided by the presence of the *Lecanicillium* anamorph in pure culture and the ribosomal DNA sequence data which most closely

Table 1 Closest ITS sequence matches between *Cordyceps cuncunae* and other clavicipitaceous fungi obtained by BLAST search in GenBank database

Taxon	GenBank accession number	% identical sites
Cordyceps militaris cf. var. sphaerocephala MRCIF64	EU573344	96.1%
Paecilomyces sp. SJL0906	HM135164	94.1%
Paecilomyces sp. 97014	AB044644	93.5%
Cordyceps ramosopulvinata	AB027372	93.1%
Paecilomyces sp. NSP-2003	AY491998	93.0%
Ophiocordyceps crinalis	EF495104	92.3%
Cordyceps sp. LW-2007a	EU149921	91.7%
Cordyceps kanzashiana	AB027371	90.5%
Cordyceps chlamydosporia	AJ303054	85.3%
Cordyceps prolifica	AB027370	84.9%
Cordyceps cuboidea	AB296169	84.8%
Cordyceps sp. NBRC 101739	AB378668	84.7%

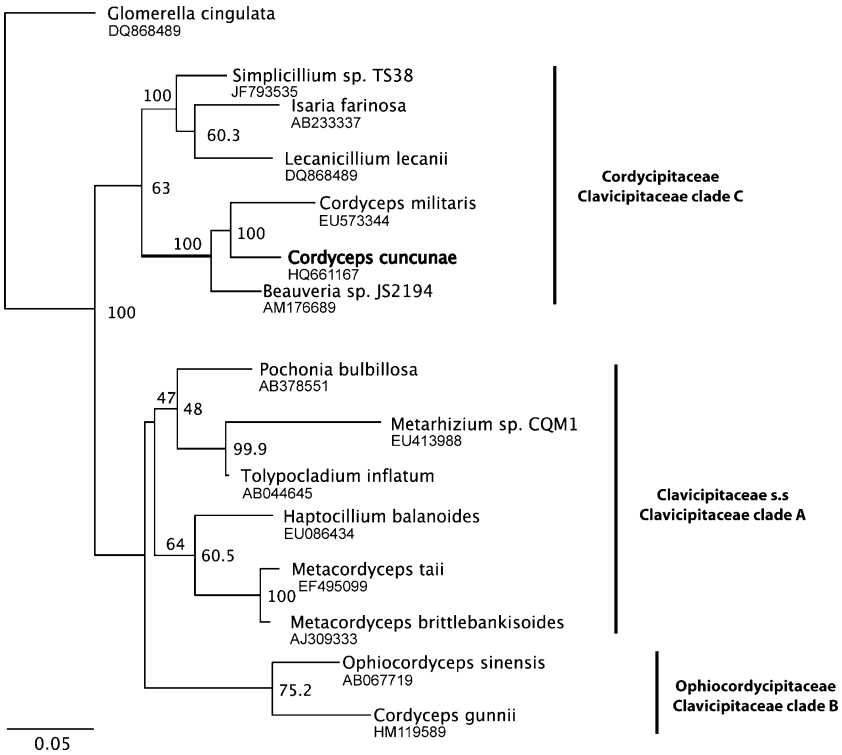

Fig. 5 Neighbor-joining tree (ITS1F/ ITS4 rDNA sequences) of *Cordyceps cuncunae* sp. nov. and selected taxa of main clades of Clavicipitaceae, according to the phylogeny established by Sung et al. (2007); outgroup: *Glomerella cingulata*

match a GenBank sequence of *C. militaris* cf. var. *sphaerocephala* (96,1% similarity, see Table 1): *C. militaris*, the type species of the genus, is also characterized by a *Lecanicillium* anamorph (Zare and Gams 2001). In the cladogram (Fig. 5), based on ITS sequences of selected taxa of the three main clades mentioned above, *C. cuncunae* appears in a well supported group together with *C. militaris* and relevant anamorphs including *Lecanicillium*, forming part of clade C and clearly separated from representative taxa of the clades A and B. Although the multiple-gene phylogenetic tree of Sung et al. (2007) does not include ITS sequences, our cladogram resembles well the three major clades with the exception of *Haptocillium balanoides* which appears in the group with clade A taxa instead of the clade B group.

The first published finding of *Cordyceps* s.l. from Chilean temperate rainforest dates back to 1924; the material was found by the Capuchine priest and amateur botanist A. Hollermayer at a rural site less than 40 km westward from the type locality of the taxon described in this study (Hollermayer 1937). He could not identify the species; however, his publication includes a black and white photograph, showing slender, wiry and dark stromata clearly distinct from *C. cuncunae*. Hollermayer sent his material to Werdermann at Berlin-Dahlem and it was later labelled as *C. robertsii* (Hook.) Gray by Sydow (1932). Considering that this species is mainly known from New Zealand (Cunningham 1921; Dingley 1953), Sydow´s identification seems somewhat doubtful. In fact, Dingley (1953) revised the material kept at the Commonwealth Mycological Institute (now CABI Bioscience) and found that the microscopical features did not support Sydow´s identification, but she did not suggest an alternative taxon. In 1991, *Cordyceps militaris* (Vuill.) Fr. growing on a lepidopteran larva was reported from *Nothofagus* forest of southern Argentina, close to the Chilean border (Mueller and Rajchenberg 1991). To date, there seem to be no further published findings of *Cordyceps* s.l. for the Andean-Patagonian region; however, there are reports of at least two species parasiting spiders (*C. ignota* Marchion., *C. singeri* Mains) from northern Argentina (Mains 1954).

One reason for the poor record of entomopathogenic fungi belonging to the Clavicipitaceae in southern South America and other regions of the Southern hemisphere may be the limited distribution of many species (Cunningham 1921) which in some cases can be restricted to a single location. On the other hand, some hosts may have complex and extensive life-cycles, opening only a narrow window in time for the parasite to attack and/ or to reproduce. Some hepialids have been reported to live up to several years in different larval stages in the ground (Nielsen et al. 2000), and possibly not all stages are equally susceptible to fungal infection. All collected specimens of *C. cuncunae* grew on caterpillars of similar size, morphology and position (vertically buried close beneath the soil surface), indicating that those were in the same developmental phase. Remark-

ably, all infected larvae were exclusively found in soil next to the stem bases of *Laureliopsis philippiana* (Looser) Schodde (Monimiaceae, Laurales), suggesting that they were feeding on the roots of this tree species. Root parasites are common among larval stages of the hepialid family (Nielsen et al. 2000). Possibly, this could be the first insight into a complex, tripartite association between plant (*L. philippiana*), parasite (hepialid larva) and fungal epiparasite (*C. cuncunae*) although to date, no hepialid larva has been described associated with this tree species (Baldini and Pancel 2002). Generally, immature stages of chilean ghost moths are still poorly known, although some species like *Dalaca pallens* Blanchard have been subject to closer investigation due to the serious damage they cause in agriculture. Adult hepialids in Chile have been reported mainly from *Nothofagus* forests in Central Southern Chile and further south from Patagonia (Nielsen and Robinson 1983). Some genera whose area of distribution coincides with the unidentified host of *C. cuncunae* are *Dalaca* Walker, *Callipielus* Butler, *Parapielus* Viette and *Andeabatis* Nielsen & Robinson. Clearly, further interdisciplinary studies are required, especially of the life cycle and trophism of the still unidentified lepidopteran and the ecology of the synanamorphs.

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