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Phylogeny and morphology of *Hirsutella tunicata* sp. nov. (Ophiocordycipitaceae), a novel mite parasite from Peru

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ABSTRACT

A new species of *Hirsutella* was isolated from unidentified mites on Petri plates inoculated with soil and root fragments collected from asparagus rhizosphere at Virú, Northern Peru. The fungus differs from other *Hirsutella* species by an envelope surrounding the conidium, conidia dimension and DNA sequences. In PDA cultures, the mycelium produced aerial hyphae with conidiogenous cells mainly at right angles, occasionally showing a secondary conidiophore. The solitary conidia are cymbiform, slightly apiculate, 5.0–6.0 × 3.0–4.0 μm. Phylogenetic analyses with partial rRNA and β-tubulin gene sequences confirmed the fungus as an *Hirsutella* (Ophiocordycipitaceae). Closest species shown by maximum likelihood and neighbor-joining trees were *H. nodulosa* and *H. aphidis*, from which the new species differs for conidium or conidiogenous cells dimensions, lack of synnemata and host type. A recombination event was also detected in the rRNA of the holotype strain, involving *Ophiocordyceps sinensis* as major parent and *O. cochlidiicola* as minor parent. A complement, inverted insertion was also found in its rRNA, involving part of the ITS2 and 5.8S regions, flanked by two short nucleotide arrays. Due to conidia dimension and phylogenetic position, the fungus is described as *Hirsutella tunicata* sp. nov. A review of mononematous *Hirsutella* species is provided.

1. Introduction

The biodiversity of invertebrate parasitic fungi is considered to be underestimated. Given the wide phylogenetic radiation of their hosts, it is expected that new species, specialized and/or linked by ecological or pathogenic host–parasite relationships, may be found in habitats only partially explored to date (Sung et al. 2007; Hoyos-Carvajal et al. 2009).

One main group of entomogenous species are the clavicipitaceous fungi, including *Cordyceps* and related genera. The classification of this group was revised by Sung et al. (2007) based on a phylogenetic analysis using five genes; they rejected the monophyly of *Cordyceps* and identified three clavicipitaceous clades, each characterized by a number of anamorph genera.

The genus *Hirsutella* Pat. includes anamorphs of specialized parasites belonging to the family Ophiocordycipitaceae, and

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related to *Ophiocordyceps unilateralis* clade B (Sung et al. 2007). Thus far, *Hirsutella* includes 90 valid species, mainly parasitic on insects, mites or nematodes (Table S1, supplementary materials), with the only exception *H. uncinata*, isolated from nuts of *Hakea* sp. (Seifert and Boulay 2004). Not all *Hirsutella* species are available in public collections, and in some cases (i.e., *H. piligena*, described from a fur-like substrate) the original description does not provide sufficient information for re-isolation, revision or re-description with modern taxonomic criteria. Available genetic data for a number of *Hirsutella* species include mainly partial sequences of rRNA and β-tubulin genes (Tigano et al. 2006), as well as translation elongation factor-1 α or DNA-dependent RNA polymerase (Sung et al. 2007).

Some of the most common species of *Hirsutella* have a practical importance, with potential in biological control of invertebrate pests, including insects, nematodes and mites (Jaffee et al. 1992; McCoy et al. 2009; Van der Geest 2010). Further applications are related to the production of several bioactive secondary metabolites (Mazet and Vey 1995; Liu et al. 1996; Lang et al. 2005).

During an epidemiological study on nematodes parasitic on asparagus in Peru, we isolated a mononematous *Hirsutella* sp. attacking unidentified mites in vitro. Attempts to identify the fungus showed that either its rRNA and β-tubulin gene sequences and morphological traits did not match any known *Hirsutella* species. The aim of the present study is to describe the fungus as a new species, *H. tunicata*, and to investigate its biology and phylogenetic position.

2. Materials and methods

2.1. Fungal culture and isolation

Soil and root samples were collected during the austral winter in Northern Peru, in the Virú district (Dept. La Libertad), from semi-desert coastal farms, planted with asparagus var. UC-157 or related hybrids. The samples were stored in Falcon tubes at 4 °C before transport and subsequent work. For fungal isolation and collection, approx. 5–10 g of asparagus root fragments and soil aggregates were spread on the surface of 1.5% water agar (WA) in 10 cm-diam Petri dishes, later incubated in the dark at 26 °C for 3–4 wk. The dishes were routinely examined for presence of invertebrates and predatory or parasitic fungi, under a stereomicroscope or a Leitz Orthoplan microscope (Wetzlar, Germany).

Hyphae with conidiogenous cells and conidia of the *Hirsutella* type were observed with light microscopy (LM) emerging from mite cadavers on Petri dishes. For isolation, the tip of an eyelash glued to a needle was sterilized in 95% ethanol, quickly air dried and then used to pick up conidia from hyphae emerging from mites on WA and then smeared on the surface of potato dextrose agar (PDA, Sigma, St. Louis, USA) with antibiotics (0.1% penicillin and 0.1% streptomycin). The smears were examined after 1–2 wk incubation at 25 ± 1 °C, a marginal mycelial fragment was removed from the small colonies, then transferred onto new PDA dishes to start pure cultures.

2.2. LM and SEM observations

For LM observations and imaging, a Leitz Orthoplan microscope provided with a Hamamatsu C5810 chilled CCD (Hamamatsu City, Japan) was used. Images from fresh mycelium water mounts or the PDA surface were captured from isolate Ht-002. Further, differential interference contrast (DIC) images were obtained from slide cultures examined at ×500 with a Zeiss Universal light microscope with a Neofluar Ph2 Plan objective. The captured images were edited and digitally contrasted with Paint Shop Pro v. 5.0.1 (Corel, Ottawa, Canada).

For SEM imaging of the same isolate, 1 cm wide agar blocks with hyphae of the fungus were cut from PDA cultures, placed on nitrocellulose filters in small Petri dishes and rapidly cooled for physical fixation by submersion in vapors of liquid nitrogen. The samples were then freeze dried by sublimation in a vacuum lyophilizer (Micromodulyo, Thermo Fisher Scientific, Pittsburgh, USA) to remove water with minimal exposure to surface tension forces, and then maintained overnight in a desiccator with $CaCl_2$ for subsequent examination. Agar blocks (5 mm wide) with freshly growing mycelium were also fixed for 24 h either in OsO_4 vapors at room temperature, or in 2% glutaraldehyde in phosphate buffer, at 4 °C. The blocks fixed in glutaraldehyde were dehydrated in a graded ethanol series (30, 50, 60, 70, 80, 90 and twice in 100%), infiltrated by hexamethyldisilazane (HMDS) in an ethanol-HMDS series (3:1, 1:1, 1:3) and twice in pure HMDS (Bray et al. 1993). Finally, for complete evaporation of HMDS, the staining blocks were left uncapped overnight in an open air cabinet. The blocks fixed with the two different methods were coated with Au (90 nm thick) for examination with a Stereoscan 360 SEM (Cambridge Instruments, Cambridge, UK) at 3 kV or S-420 SEM (Leica Microsystems, Heidelberg, Germany) at 1.5–2 kV.

2.3. DNA extraction and sequencing

For DNA extraction, the mycelium of isolate Ht-002 was grown in culture flasks in a liquid medium (glucose 1%, malt extract 0.3%, peptone 0.5% and yeast extract 0.3%) for 10 d at 26 °C. The fungal biomass was recovered on Whatman filter paper and approx. 500 μg of the mycelial fragments and conidia were crushed with a sterile micropestle for about 1–2 min, using the extraction buffer of the E.Z.N.A. Mag Bind Plant DNA kit (Omega Bio-Tek, Norcross, USA). The purified DNA was then used directly for PCR amplification of the internal transcribed spacer (ITS1-5.8S-ITS2) ribosomal region, with primers ITS1 and ITS4 (White et al. 1990). The final 50 μl PCR mixture contained 5.0 μl 10 × PCR buffer, 2 U of Taq polymerase, 200 μM each dNTPs, 1 mM of each primer and 10 μl of the template (100 ng). PCR was performed in a thermal cycler (BioRad, Hercules, USA) with the following cycles: 1 initial cycle at 95 °C for 7 min; 35 cycles with 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, with a single final extension cycle at 72 C for 4 min.

For PCR amplification of the β-tubulin gene, the primers βtubF and βtubR were used, using amplification conditions developed for *H. thompsonii* (Tigano et al. 2006). All amplicons were sequenced by an available commercial service (Microgen, Seoul, South Korea). The sequences obtained were

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checked to confirm species identity on the NCBI sequence databases with BLAST algorithm (Altschul et al. 1990).

2.4. *Molecular phylogeny*

Multiple alignments for either the rRNA (including a partial 18S, ITS1, 5.8S and a partial ITS2 region), and the β-tubulin gene region were performed using the nearest corresponding sequences identified by BLAST analyses available in GenBank, with a 80% nucleotide identity threshold.

To check the occurrence of nucleotide recombination events affecting the evolution of *Hirsutella* and related species, an analysis was performed using the program RDP3. For this purpose, a 710 position alignment with 104 entries was assembled first using the 536 bp rRNA sequences of the strain Ht-002 and closest accessions recovered by BLAST. The corresponding fragments of two further species, *H. guyana* (isolate ARSEF878 from *Saccharosydne saccharivora*), and *H. minnesotensis* (isolates CBS115627 and ARSEF2799 from juveniles of *Heterodera glycines*) were also included, although not listed by BLAST. For the 104 accessions used see Table S2 (supplementary materials). A Windows interface for the CLUSTAL W program (Thompson et al. 1997) implemented on software BioEdit ver. 7.0.9.0 (Hall 1999) was used with default options for the first multiple sequence alignment. Minor manual alignments were performed with BioEdit.

A subset of 29 species was used to infer the phylogenetic position of *H. tunicata* through their aligned rRNA genes (for alignment see TreeBASE entry: http://purl.org/phylo/treebase/phylows/study/TB2:S13332). For TreeBASE uploads, the files were checked using Mesquite (http://mesquiteproject.org/mesquite/mesquite.html). The evolutionary history was inferred by the maximum likelihood (ML) method with the Tamura–Nei model (Tamura and Nei 1993) or using the neighbor-joining (NJ) method (Saitou and Nei 1987). For ML analysis, initial trees were obtained using the maximum parsimony method when the number of common sites was <100 or less than one quarter of the total number of sites, otherwise using the BIONJ method (Gascuel 1997) with the Markov Cluster (MCL) distance matrix (Enright et al. 2002). All positions with less than 95% site coverage were eliminated, allowing fewer than 5% alignment gaps, missing data and ambiguous bases at any of the 386 positions in the final data set. For the NJ analysis, the evolutionary distances were computed using the Jukes-Cantor substitutions per site method (Jukes and Cantor 1969). The analysis involved the same 29 nucleotide sequence alignment, eliminating all positions with less than 95% site coverage, with a total of 387 positions in the final data set. The percentage of replicated trees in which the associated taxa clustered together was calculated with bootstraps resampled 500 times (Felsenstein 1985).

The β-tubulin amplicons were aligned with 14 closest entries listed by BLAST. The alignment (TreeBASE entry: http://purl.org/phylo/treebase/phylows/study/TB2:S13332), was used for NJ analysis, with the Jukes–Cantor substitutions per site method (Jukes and Cantor 1969). All analyses were carried out using MEGA5 software package (Tamura et al. 2011).

For analysis of DNA recombination, the 104 species rRNA and the β-tubulin gene fragments alignments were checked using the recombination tests provided by the program RDP3 (Martin et al. 2010), based on a pairwise scanning approach, with Bonferroni correction and no permutation. Recombination events were detected if identified by more than 3 methods. Detection algorithms used for analysis included RDP (Martin et al. 2005), Chimerae (Posada and Crandall 2001), Bootscan (Salminen et al. 1995), GENCONV (Padidam et al. 1999), MaxChi (Smith 1992), SiScan (Gibbs et al. 2000) and 3SEQ (Boni et al. 2007), at 0.05 highest *P* values.

2.5. *In vitro assay*

Larvae of *Tenebrio molitor* and *Musca domestica* (obtained from Microvita Azienda Agricola, Crespellano, Italy) were used to check the capability of *H. tunicata* to penetrate and parasitize insects. Larvae of *T. molitor* and pupae of *M. domestica* were individually hand picked, washed in sterile distilled water (SDW), and rolled gently on mycelium of *H. tunicata* on PDA for 1–2 min. Insects were then placed in pairs on a paper strip in 15 ml Falcon tubes with 50 μl SDW in five replicates, and incubated at 26 C in dark. Five replicated control pairs of each species were incubated as described. Ten specimens of each species were also placed on PDA with mycelium and then incubated as described. After 12 d, insects mortality was checked by counting pupae and adults of *M. domestica* and checking the *T. molitor* motility with a needle. Unhatched pupae of *M. domestica* and dead *T. molitor* larvae were surface sterilized for 10 s in 1% hypochloride and placed on WA with antibiotics, to check for inner hyphal growth.

3. Results

3.1. *LM and SEM observations*

Examination of mycelium emerging from dead unidentified mites (Fig. 1I) on WA showed conidiogenous cells with apical conidia typical of the genus *Hirsutella*. On PDA, the fungus produced creamy, pale-brown mycelium, and grew slowly (2 mm d^{-1} at 26 °C). Conidiogenous cells were phialidic, with necks showing the typical flask-shaped morphology (Figs. 1A–C, 2A, B), and occasionally originated also at hyphal tips. The conidiogenous cells were often located near a septum, and sometimes showed one or more secondary necks or one-two further conidiogenous cells stemming from the basal cell (Fig. lL), also visible on aerial mycelium. The necks were straight (Figs. 1A, E, 2A, B) and only rarely appeared distally twisted (Fig. 1B). At maturity, solitary conidia appeared at tips of necks. The conidia showed a covering envelope of variable size (Fig. 1C–H), which was kept after conidia secession (Fig. 1F, H). Free conidia on agar had a citriform, slightly asymmetric shape (Fig. 1D). No mature sexual stage was observed.

SEM examination of mycelium showed no difference among the different fixation procedures applied and confirmed the morphology observed with LM for hyphae and conidiogenous cells (Fig. 2A–D). The necks were mainly straight and occasionally showed a few knobs, 20–30 nm wide, along their distal portion (Fig. 2A). The conidial covering persisted after vacuum freeze drying (Fig. 2B–D) or fixation,

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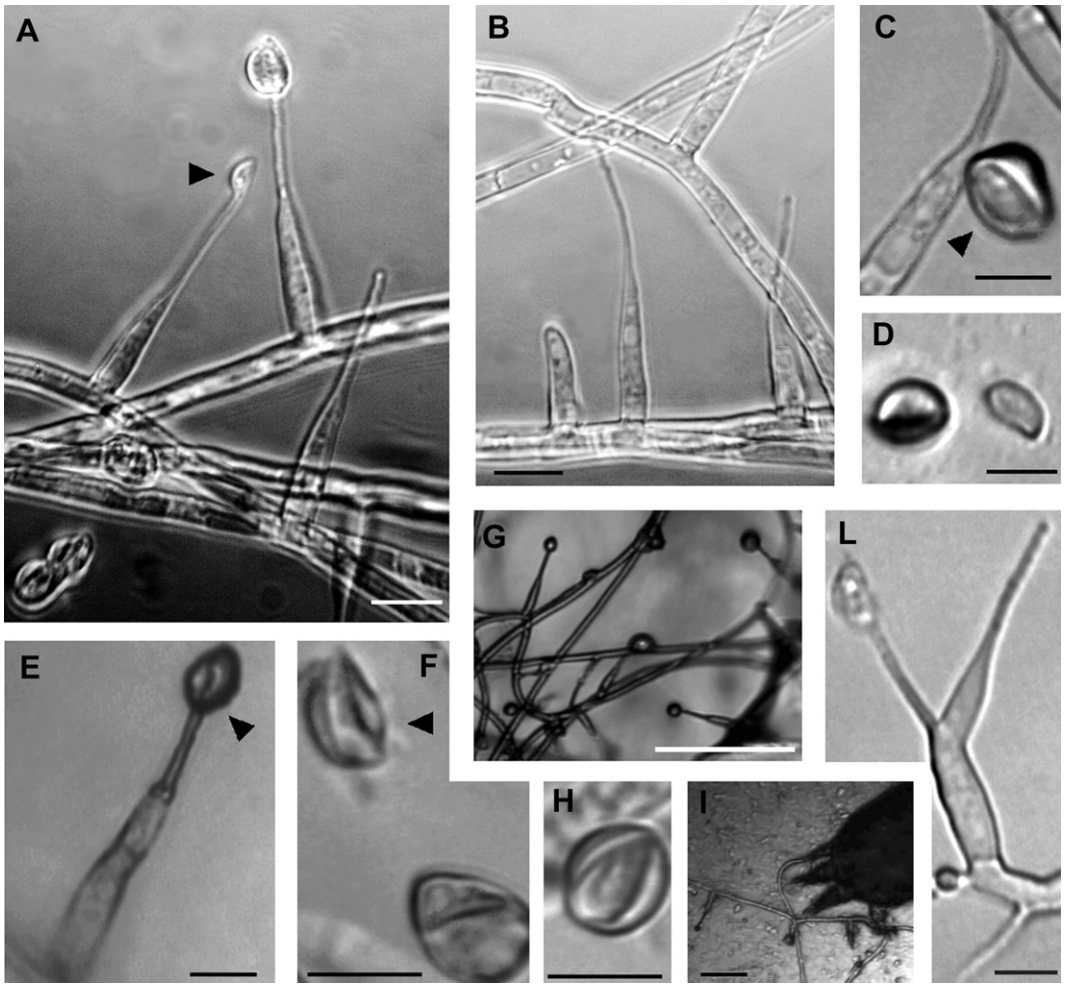

Fig. 1 – DIC (A, B) and LM (C–I, L) images of *Hirsutella tunicata* showing general morphology of conidiogenous cells and conidia. Conidiophores form at right angles on hyphae (A, B) and occasionally at a hyphal tip (L). Conidia develop at the apex of the phialidic conidiogenous cells (A, arrowhead). Aerial hypha provided with the enveloped apical conidium (E; arrowhead). At maturity, the conidia are enveloped (C, E, F: arrowheads; G, H). The envelope is maintained after conidia secession from the phialide (C, D, F, H), with a fissure visible either in lateral (F; arrowhead) and bottom side view (H). Free conidia show a citriform–cymbiform, slightly asymmetric and apiculated shape (D). General aspect of aerial mycelium (G). Hyphae growing from unidentified mite cadaver (I) and secondary phialides and neck (L). Bars: A, B: 10 μm; C–F, H, L: 5 μm; G: 50 μm; I: 30 μm.

and appeared as a thin hardened envelope around the conidium (Fig. 2D–H). Mature conidial envelope showed an apiculated tip on the side opposite to their insertion on necks (Fig. 2E, H) and an invaginated aperture or fissure along their central, longitudinal axis (Fig. 2E–G), with a wider opening at one end (Fig. 2G).

3.2. *Molecular phylogeny*

The phylogenetic analyses of the rRNA partial fragment of isolate Ht-002 (GenBank JN247824) yielded NJ and ML trees with similar structures, showing a grouping for *Elaphocordyceps* and related species and a further grouping including some *Cordyceps* species, *H. tunicata* and other *Hirsutella* species, *Ophiocordyceps cochlidiicola*, *O. crinalis* and *Cordyceps* sp. (Fig. 3A, B). In the latter group, the *Hirsutella*–host associations were shared only in closest species (*H. aphidis* and *H. nodulosa* originally isolated from insects, as well as *H. gregis* and *H. kirchneri*, isolated from eriophyid mites). More distant host associations were shown for other *Hirsutella* species parasitic on nematodes (Fig. 3). The trees confirmed the distinction of *H. tunicata* from the other species considered, with *H. nodulosa* and *H. aphidis* as closest taxa. Similar relationships for *H. nodulosa* were also shown by the NJ tree (Fig. 4) obtained with the 413 nt β-tubulin gene fragment produced (GenBank JQ062991).

An unusual inverted insertion in the rRNA fragment of *H. tunicata* was found in an rRNA product amplified with the ITS4-ITS5 primers. The fragment was inserted between nt positions 323 and 550 of the resulting closest sequence shown by BLAST (*O. cochlidiicola* AB027377). The inverted region included partial fragments of the ITS2 and 5.8S regions, flanked by two shorter (5 and 21 nt) insertions (Fig. 5).

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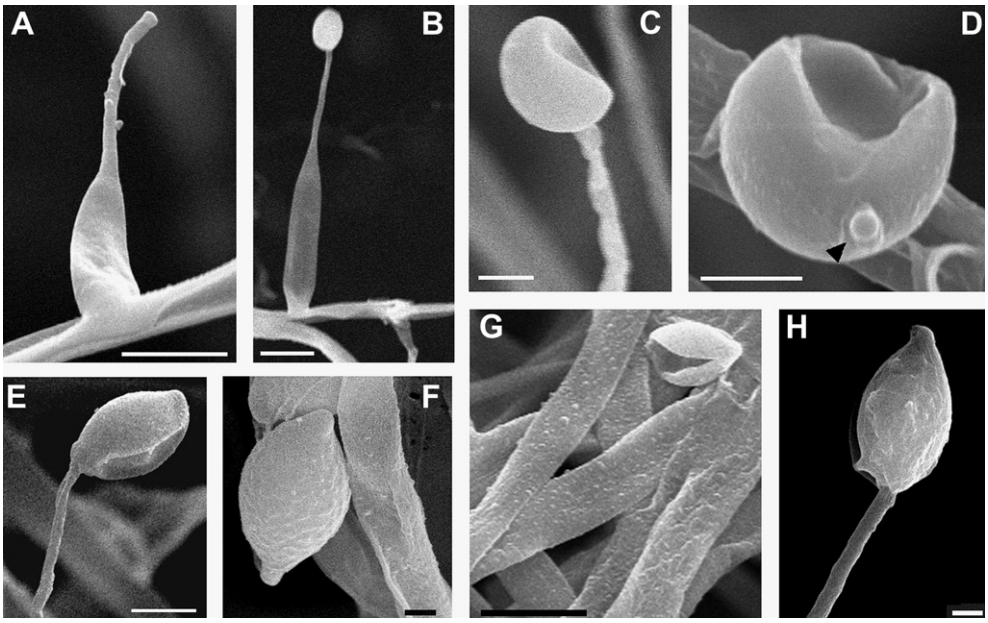

Fig. 2 – SEM images showing a conidiogenous cell of *Hirsutella tunicata* with a few sparse knobs on a straight neck (A), and the enveloped conidia development at its apex (B, C). Conidia detached from neck, as shown by the insertion site (D; arrowhead), show the envelope collapse (D). Close view of the conidial envelope shows its apiculated shape (F, H) and the invaginated longitudinal aperture or fissure (E, G). The mycelium was frozen-dried (A–D, G), treated with HMDS (E, F), or fixed in OsO_4 vapors (H). Bars: A, B, G: 5 μm; C, D: 2 μm; E: 3 μm; F, H: 1 μm.

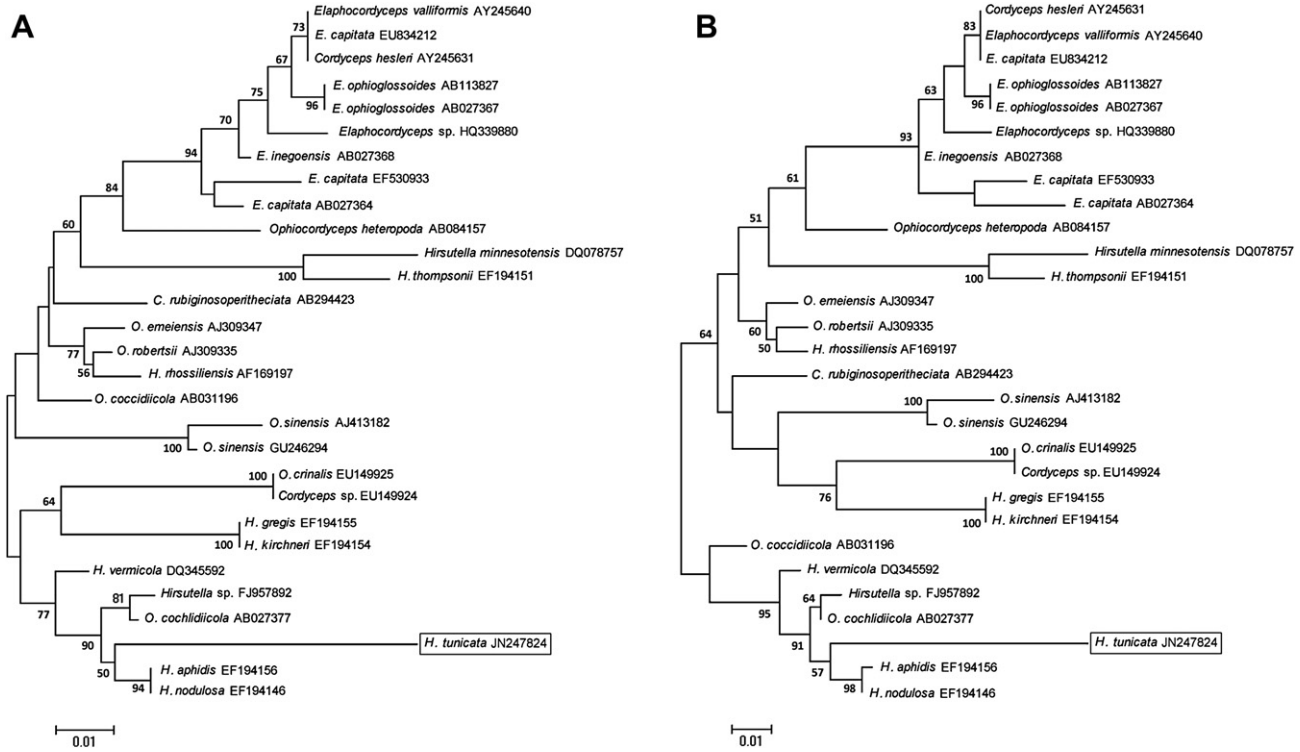

Fig. 3 – Phylogenetic analyses of *Hirsutella tunicata* based on the alignment of the rRNA amplified product with 28 closest GenBank accessions. (A) Neighbor-joining tree (sum of branch: 0.451467099) and (B) maximum likelihood tree (highest likelihood: −1902.6646). Both trees are based on the Jukes–Cantor method. The percentages of trees in which the associated taxa clustered together are shown next to branches (measured in substitutions per site, 500 replicates). For *Hirsutella* species and host associations, see Table S1.

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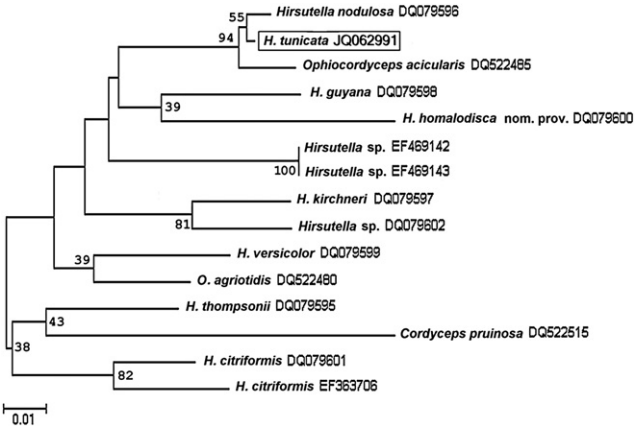

Fig. 4 – Neighbor-joining phylogenetic tree based on the β-tubulin gene amplicons of *Hirsutella tunicata* with 14 closest GenBank accessions shown by BLAST query. For *Hirsutella* species and host associations, see Table S1.

RDP3 analyses revealed two recombination events, detected by more than three methods in the 104 species alignment. The first event involved *H. tunicata* (JN247824), with *O. sinensis* (FJ654148) as a putative major parent and *O. cochlidiicola* (AB027377) as a minor parent. The recombination was found between positions 65 and 528 of the alignment by GENECONV (av. *P* value = 3.186×10^{-2}), Bootscan (2.336×10^{-1}), MaxChi (6.394×10^{-4}), Chimaera (4.326×10^{-4}), SyScan (5.818×10^{-5}) and 3Seq (3.385×10^{-4}) (see Fig. S1, supplementary material). A second recombination event was found for *Beauveria geodes* (U19037), with *Elaphocordyceps capitata* (EF530933) as a major parent and *Purpureocillium lilacinum* (GQ229079) as a minor parent (data not shown). When the β-tubulin gene fragment alignment was examined, RDP3 detected a further significant recombination between *H. citriformis* (DQ07960, EF363706) as a major parent and *H. nodulosa* (DQ079596) as a minor parent (data not shown).

3.3. *In vitro assay*

No parasitism or fungus effect was observed on emergence of adults from pupae of *M. domestica* placed in contact with the fungus. Some unhatched pupae showed hyphae emerging on WA, however they were different from *H. tunicata* when examined with LM. A mortality higher than the control (80 vs. 60%) was observed for treated *T. molitor* larvae, but it was related to a different fungal species, as observed on WA.

3.4. *Taxonomy*

Hirsutella tunicata Ciancio, Colagiero & Rosso, sp. nov. Figs. 1–3.

MycoBank no: MB 564955.

Differs from *H. nodulosa* in having solitary conidia, and from *H. aphidis* in having shorter conidia and a different host type. Conidia are covered at maturity by a thin envelope.

Typus: Peru, department La Libertad, Virú province, California district, farm Victor Raul (lat. –8.41667, long. –78.75, altitude 50 m a.s.l.), from hyphae emerging from cadavers of unidentified Acarina on water agar inoculated with soil and root fragments collected in the rhizosphere of asparagus (*Asparagus officinalis* L.), 27 August 2009, leg. A. Ciancio and S.N. Murga Gutierrez (holotypus: isolate Ht-002, in CBS 131647 and DSM 25513).

rRNA sequence ex holotypus: JN247824.

Etymology: covered, dressed (Lat. "*tunicata*"), in reference to the enveloped conidium.

Mycelium creamy white, pale brownish or yellowish on the lower side, with hyaline septate hyphae 2.0–4.0 μm wide. Hyphae septate, linearly diverging from cadavers of unidentified parasitized mites. Conidiogenous cells phialidic, mean 27.6 μm long ($n = 30$, range 20.0–40.0 μm), with an inflated awl-shaped lower portion 15.5 (10.0–23.0) × 3.5 (2.5–5.0) μm, gradually tapering to a slender neck 12.1

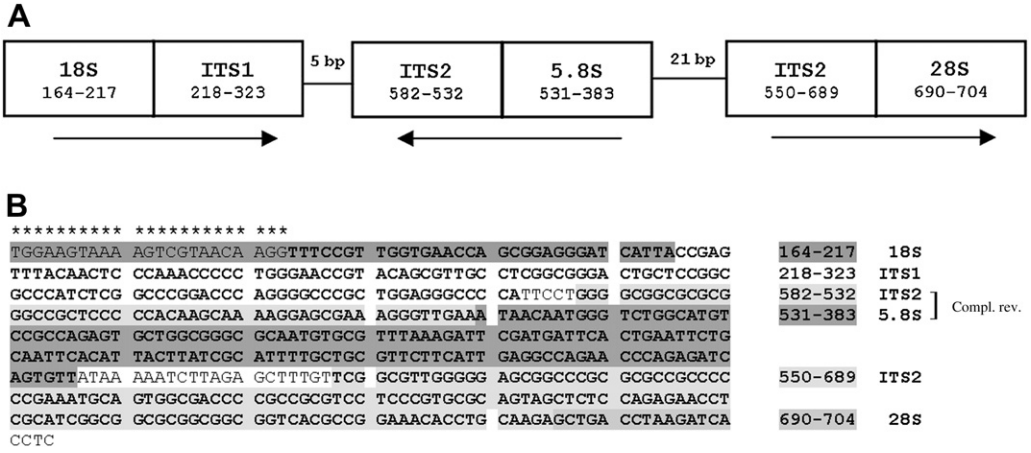

Fig. 5 – Structure (A) and sequence (B) of the inverted-repeat fragment detected in a 544 bp amplified product of rRNA of *Hirsutella tunicata*. The insertion of the complementary, inverted fragments of the ITS2 and 5.8S regions is flanked by two short nucleotide arrays. Arrows (a) show 5′–3′ direction. Numbers (A) show start codons in closest BLAST accession (*Ophiocordyceps cochlidiicola* AB027377). Asterisks (B) mark primer ITS5.

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(6.0–15.0) × 1.0–2.0 μm. Solitary conidiogenous cells originating on hyphae usually at right angles and equally spaced (Fig. 1A, B, E), or occasionally terminal of a hyphal tip (Fig. 1L). Conidiogenous cells occasionally branched, originating as one or more secondary conidiogenous cells or necks (Fig. 1L). Conidia solitary (Fig. 1A, E), cymbiform–citriform (the shape of an orange segment) sometimes with a more pointed apex, due to a slightly asymmetric curvature and less convex on one side, often with an apiculate tip (Fig. 1D). Conidia measuring 5.0–6.0 × 3.0–4.0 μm ($n = 30$, means: 5.4 ± 0.5 × 3.5 ± 0.4). On 3–5-wk-old cultures, with a thin, globular-apiculated envelope.

Teleomorph: unknown.

4. Discussion

Hirsutella tunicata is characterized by an envelope around the mature solitary conidia, after they have developed on the conidiogenous cells tip. It differs from the majority of mononematous *Hirsutella* species by conidiogenous cell and conidial dimensions, conidium numbers or shape, as well as for the kind of the host (see Table S1).

Both phylogenetic analyses applied showed that *H. aphidis* and *H. nodulosa* are the closest species, although distinct. Morphologically, *H. tunicata* can be distinguished from *H. aphidis* by the shape and dimension of the conidia, which are longer and narrower at both ends, usually in pairs and with 2–3 guttules in *H. aphidis*, as well by the shorter conidiogenous cells (Balazy 1985). *H. tunicata* can be distinguished from *H. nodulosa* by the twisted helical apex of the conidiogenous cell, which is characteristic of *H. nodulosa*, and by the conidium shape, being oval with a truncate base in *H. nodulosa* (Petch 1926). Furthermore, *H. nodulosa* conidia are grouped in clusters (Minter and Brady 1980), whereas in *H. tunicata* the conidium is solitary.

Morphological comparison with other *Hirsutella* species showed that the new species differs from *H. vermicola*, a species isolated from free living nematodes, by the gradual tapering of its conidiogenous cell from the swollen base toward the apex (more abrupt in *H. vermicola*). It also lacks the apical helical twist, typical of the latter (Xiang et al. 2006). *Hirsutella tunicata* also differs from the mite-parasitic *H. tydeicola* by the dimensions and shape of conidia (fusiform in the latter) and from *H. rhossiliensis* (a nematode parasite) by the more swollen conidiogenous cell base, host preference and sequence data. It also differs from the insect parasitic *H. abietina* by the number of conidia per conidiogenous cell (four in *H. abietina*), as well as by their shape (oval or subcymbiform with obtuse ends in the latter) and lack of synnemata (Petch 1931).

Considering species isolated from insects, *H. besseyi* differs from *H. tunicata* by its narrowly elongated conidia, as originally described, which are surrounded by a gelatinous matrix, and by its insect host specificity (Fisher 1950). In the subsequent re-description of *H. besseyi* based on the original material, Minter and Brady (1980) stated that the lower part of the conidiogenous cell is almost cylindrical in this species, tapering quite abruptly near the top to a short neck up to 10 μm long, and that this character was also visible when more than one conidiogenous cell was present. This character was also reported by Evans and Samson (1982), who compared an isolate of *H. besseyi* obtained from an insect host from the Galapagos Islands with the holotype specimen, and confirmed both the lack of synnemata and the structure of the conidiogenous cells, as reported by Minter and Brady (1980). The conidiogenous cell shape is hence a character clearly distinguishing the two species.

In comparison with *H. acridiorum*, an insect parasite, *H. tunicata* can be distinguished by the production of more than two secondary necks on conidiogenous cells, a key character for *H. acridiorum* for which no more than two necks are produced (Minter and Brady 1980). Also, in *H. acridiorum* the conidia are present in groups (as opposite to only one per conidiogenous cell in *H. tunicata*) embedded in a mucus sheath. Finally, a further distinction is given by the *H. acridiorum* insect host.

Considering related species isolated from mites (Minter et al. 1983), *H. tunicata* differs from *H. necatrix* by conidiogenous cell and conidial shapes (shorter, globose with ovoid–ellipsoid or verrucose conidia in *H. necatrix*), and from *H. gregis* and *H. kirchneri* by its longer, more elongated conidiogenous cells, and a different rRNA sequence.

The conidia of *H. tunicata* are solitary at the tips of the conidiogenous cells (Figs. 1A, E, 2A, B) and when free of their covering, show an apiculate, citriform shape (Fig. 1D) similar to the conidia of *H. satumaensis*, a species isolated from the silkworm, *Bombyx mori*. A further similarity with *H. satumaensis* is the occasional formation of conidiogenous cells at the end of the hyphal tip (Aoki et al. 1957), a character occasionally observed in *H. tunicata*. However, the latter can be distinguished from the synnematous *H. satumaensis* by its mononematous conidiophores, as well as its mite host.

The reliable proposal of new *Hirsutella* species is often biased by the unavailability of ex-type cultures, or by the unavailability or poor condition of historical holotype material deposited in permanent insect or mite collections. Similarly, a better understanding of the phylogeny of *Hirsutella* anamorphs within Ophiocordycipitaceae is also constrained by the lack of living material in collections, or by the possible extinction of some teleomorphs (Sung et al. 2007). Hence, the reconstruction of the *Hirsutella* phylogenetic radiation(s) requires the full exploration of their biodiversity from different environments and geographic areas, as well as the sequencing or revision of cryptic species (i.e., *H. piligena*), currently not yet available. Original data and descriptions often represent the only available data for species distinction, and provide the only comparative information available for identification or taxonomic purposes.

A schema of all *Hirsutella* species described thus far, based on conidial dimensions from original descriptions or revisions and other morphological and biological data, was produced to support the taxonomy and the species status of *H. tunicata* (see Table S1). Within the framework of the available data for the genus, either the two phylogenetic trees and both genes sequenced confirmed the status *H. tunicata* as a genuine new species, as did morphological analysis. Considering the three clades identified in clavicipitaceous fungi (Sung et al. 2007), and the proximity of *H. tunicata* to *O. cochlidiicola*, *O. coccidiicola*

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(Fig. 3), and *O. acicularis* (Fig. 4), the fungus is assigned as an anamorph member of the family Ophiocordycipitaceae, and in particular of the *O. unilateralis* subclade of the phylogenetic clade identified as "Clavicipitaceae clade B" (Sung et al. 2007). Whether *Ophiocordyceps* or *Hirsutella* is chosen as the generic name in the single-name system remains a decision to be discussed by taxonomic specialists. We have maintained use of the anamorph genus name here pending the outcome of these discussions.

The phylogenetic proximity of *H. tunicata* with *O. cochlidiicola* also marks one or more recombination events. Recombination events have been detected in some groups of fungi, like the asexual arbuscular mycorrhizal species (Gandolfi et al. 2003; Croll and Sanders 2009), or may occur among isolates of species whose sexual stage is rare (Souza-Paccola et al. 2003) as well as in basidiomycetes (Hughes and Petersen 2001). The homologous recombination of group I introns in *Cordyceps* species was considered an important evolutive mechanism involved in gains and losses of nuclear rRNAs (Nikoh and Fukatsu 2001).

In vitro assays with insects showed that *H. tunicata* must be considered a specialized parasite of mites, from which it was originally isolated. Analyses in this study suggest multiple host-association radiations for the *Hirsutella* species examined. Together with inter-kingdom host jumping (Nikoh and Fukatsu 2000), recombination events may have provided an important source of variability during *Hirsutella* speciation paths.

Disclosure

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.myc.2013.01.002.

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