



Universitat Autònoma de Barcelona

FACULTAT DE CIÈNCIES

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UNITAT DE BOTÀNICA

TRICOMICETS IBÈRICS



Memòria presentada per:

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per optar al grau de Doctor en Ciències Biològiques

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Bellaterra, Desembre 2004

ANNEX III (PÀGS. 307-328)

VALLE & SANTAMARIA. 2004. The genus *Smittium* (Trichomycetes, Harpellales) in the Iberian Peninsula. *Mycologia* 96:682-701.

The genus *Smittium* (Trichomycetes, Harpellales) in the Iberian Peninsula

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Abstract: A study of larval Diptera (Chironomidae, Culicidae and Simuliidae) from Spain has been carried out to fulfill a catalogue of species of the genus *Smittium* (Harpellales: Legeriomycetaceae) present within these hosts. Among the reported taxa, eight are new species: *Smittium brevisporum*, *S. bulbosporophorus*, *S. gracilis*, *S. hecatei*, *S. heterosporum*, *S. inexpectans*, *S. prostratum* and *S. pseudodimorphum*. We also report six previously described species, which are new for the Iberian Peninsula: *S. alpinum*, *S. dipterorum*, *S. megazygosporum*, *S. pusillum*, *S. typhellum* and *S. fecundum*. Three other species (*S. simulii*, *S. culicis* and *S. culisetae*) previously were reported from Spain. In two of them (*S. fecundum* and *S. culicis*), we describe for the first time the presence of zygospores. Some of the included species have been artificially cultured as well as ultrastructurally studied using scanning electron microscopy (SEM), with the purpose of observing the surface of both trichospores and the trichospore collar and the morphology of the trichospore appendage.

Key words: Dipteran larvae, Legeriomycetaceae, scanning electron microscopy, Spain, taxonomy, trichospores, zygospores

INTRODUCTION

The first species described in the genus *Smittium* was *S. arvernense* by Poisson (1936). Some other species actually related to the genus previously were reported with other generic names. Poisson (1932) described earlier the genus *Dixidium*, with the single species *D. dixae*. In agreement with what is mentioned by Lichtwardt (1986), this taxon might belong to the genus *Smittium*, although it was found in an undetermined larva of *Dixa* (Diptera, Dixidae), an unusual host for *Smittium*. Manier and Lichtwardt (1968) rejected the

genus *Dixidium* for its poor description and the absence of illustrations.

Other species currently placed in *Smittium* formerly were described as *Orphella* Léger & Gauthier (Léger and Gauthier 1931, Tuzet and Manier 1947), *Rubetella* Tuzet, Rioux and Manier (Tuzet et al 1961, Manier et al 1961, Manier and Mathiez 1965, Manier 1963), *Genistella* Léger & Gauthier (Léger and Gauthier 1932, 1935) and *Typhella* Léger & Gauthier (Léger and Gauthier 1935, Manier and Mathiez 1965). The presence of zygospores in *Smittium* (Poisson 1936) initially was used as the diagnostic character to distinguish species of this genus from those of the presumably related genera. Afterward, the taxonomic revision made by Manier and Lichtwardt (1968) and Manier (1970), where many of the species conceived in other genera were transferred to *Smittium*, the taxonomy of the genus became more clear and reliable.

In the study of the genus *Smittium*, where the number of morphological attributes used for classification often is critically reduced, there is a natural tendency of weighting sporangial characters. This is justified by the mostly conservative behavior of these reproductive structures within one species, while other thallial features, such as the basal cell morphology, or branching patterns, would be more variable. The emphasis on such scarce characters was not a handicap in the past because relatively few species were described and they were more or less easily identifiable on the basis of spore morphometry. Nonetheless, to date about 60 species of *Smittium* are accepted, some of them being published with descriptions that are too brief and that produce low predictability because spore morphometrics, by themselves, do not allow a reliable identification. At any rate, these characters can be used for a preliminary classification, as we do in the preliminary key to the species included here, and for providing additional information of basal cell and holdfast morphology.

All of these inherent difficulties in *Smittium* classification have led to an increased use of phylogenetic data, based on comparative studies of rDNA sequences, accompanying the classical phenetic taxonomy. Using molecular analyses, the polyphyletic origin of the genus *Smittium* recently has been postulated to include at least five distinct lineages

Accepted for publication October 8, 2003.

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(Lichtwardt et al 2001, Misra and Horn 2001, Gottlieb and Lichtwardt 2001).

In this study we follow the classical concept of the genus *Smittium* established by Poisson (1936). We have tried to include as many characters as possible in the descriptions for each of the reported species, all of them accompanied by photographs.

Twenty species of *Smittium* have been found in the Iberian Peninsula, eight of which correspond to new species here described: *Smittium brevisporum*, *S. bulbosporophorus*, *S. gracilis*, *S. hecatei*, *S. heterosporum*, *S. inexpectans*, *S. prostratum* and *S. pseudodimorphum*, all them dissected from the hindgut lining of Chironomidae larvae. Among the others, some represent first reports for our territory of study.

SEM techniques for the study of Trichomycetes have not been used frequently (Moss and Lichtwardt 1976), and some of the features observed have not been reported previously (e.g., the coat of unspecified material surrounding both trichospores and trichospore collars of *S. culicis* Manier [FIGS. 62, 63], the verrucose surface and band-like appendage of *S. heterosporum* [FIGS. 38, 39], or the vesicle-like bodies adhered to the appendage surface in *S. hecatei* [FIGS. 26, 27]).

MATERIALS AND METHODS

The description of all taxa is based on material collected in several localities from the Iberian Peninsula. Dipteran hosts, including Chironomidae, Culicidae and Simuliidae larvae, have been captured using the methods described by Lichtwardt et al (2001). The microscopic slides have been mounted as described by Valle and Santamaria (2002a, b) and deposited in BCB-Mycotheca (herbarium at the institutional address of authors).

Some cultured species were studied with SEM. For this purpose, the axenically cultured material of *S. culicis*, *S. fecundum* Lichtw. & M.C. Williams, *S. hecatei*, *S. heterosporum* and *S. megazygosporum* Manier & Coste, were fixed with glutaraldehyde 2.5% in Cacodylate buffer 2M (pH 7). The thalli were washed with the same cacodylate solution and dehydrated in ethanol series (15 min in each 10–20–40–60–80%) to absolute ethanol and immediately transferred to acetone before being critical-point dried and gold-coated. Grids were examined with an HITACHI S-570.

Axenic cultures of *S. culicis*, *S. fecundum*, *S. hecatei*, *S. heterosporum*, *S. megazygosporum* and *S. simulii* Lichtw. were grown on stationary Petri dishes with Brain-Heart Infusion agar (BHIa) 1/10, with added vitamins (biotin, thiamin), following the methods proposed by Lichtwardt (1986).

For a more consistent study, type specimens were borrowed from the Farlow Herbarium (FH) and from the Museum d'Histoire Naturelle de Paris (PC). These types have been examined and sporangial features have been measured to compare to Spanish specimens.

NEW SPECIES

Smittium brevisporum L.G. Valle & Santam., sp. nov. FIGS. 1–5

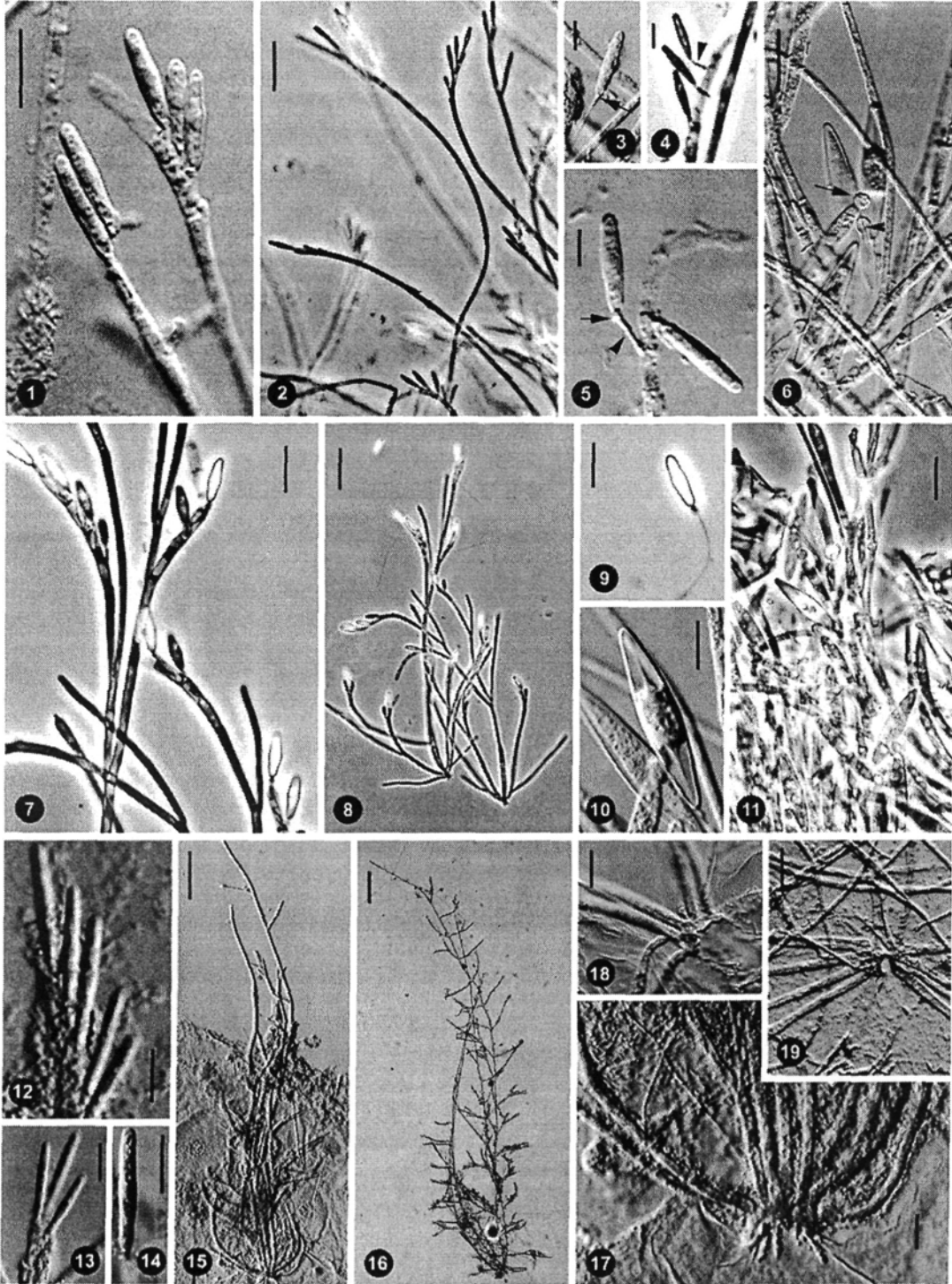
Thallus basi profuse ramosus, arcte crescens aggregatosque formans ubi hyphales apices arcuati sunt. Basalis cellula secernens simplex disciforme tenaculum. Trichosporae elongato-ellipsoidales ad ellipsoidales, (9–)12–14.5(–19) × 2–2.5 μm, praebentes brevem atque latam, plicatam in longitudinem appendicem, parvum collare, 0.5–1 μm longum. Unaquaeque fertilis rama cum 4–8(–12) genitalibus cellulis. Zygosporae ignotae. In Chironomidarum larvarum proctodaeo incolii.

Thalli profusely branched from the base, with an indeterminate pattern, tightly growing to form aggregates in which curved hyphal apices can be observed. Basal cell with a simple disk-like holdfast. Trichospores long-ellipsoidal to ellipsoidal, (9–)12–14.5(–19) × 2–2.5 μm, each showing, after released from generative cells, a short and thick, longitudinally folded, appendage (FIGS. 4, 5), and a nearly inconspicuous collar (FIG. 5), 0.5–1 × 1.5 μm, that becomes observable when the appendage unfolds (FIG. 3). Each fertile branch with 4–8(–12) generative cells (FIGS. 1, 2). Zygosporae not found.

Etymology. L., *brevi* = small, short; and *sporum* = spore.

Specimens examined. SPAIN. BARCELONA: Fogars de Montclús; Santa Fe del Montseny, Santa Fe stream, 3-IX-2000, L.G. Valle, BCB-Tr0345; same locality, 25-IX-2001, L.G. Valle, BCB-Tr1085–1087; same locality, 7-XI-2001, L.G. Valle, BCB-Tr1152 (HOLOTYPE), BCB-Tr1150 (ISOTYPE). LLEIDA: Espot; Estany de Ratera (Parc Nacional d'Aiguestortes i Estany de Sant Maurici), in a little unnamed stream, 19-VI-2001, L.G. Valle, BCB-Tr0853. All prepared from Chironomidae Orthocladinae (*Cricotopus* spp. and other) larvae.

This species displays morphological affinities with *S. microsporum* M.C. Williams & Lichtw., an Australian species having smaller trichospores (8–10 × 2–2.3 μm) with inconspicuous appendages (Lichtwardt and Williams 1992a), different by the short and appreciable appendage of the species here described (FIGS. 3–5). The number of generative cells is larger in *S. microsporum*, with more than 15 cells per fertile branch. *Smittium paludis* M.C. Williams & Lichtw. is comparable to *S. brevisporum*, with its small trichospores (12–14 × 2.5–3 μm), although the typical branching pattern of *S. paludis* is verticillate and the trichospore appendage is inconspicuous (Lichtwardt and Williams 1990). *Smittium dipterorum* Lichtw. also is similar but may be separated from our species by the appendage morphology and trichospore size, longer in *S. dipterorum* (Lichtwardt 1997). The longer collar of *S. parvum* Lichtw. (2–6 μm according to our measurements from the type specimens, photomicrographs KU-204-35 *Holotype* [FH], and KU-204-36 *Iso-*



FIGS. 1-19. *Smittium brevisporum*, *S. bulbosporophorus*, *S. gracilis* and *S. delicatum*. 1-5. *S. brevisporum* (BCB-Tr1150). 1-2. Fertile branches showing terminal trichospores. 3. Free trichospore with a small collar (arrow) and an unfolded appendage. 4-5. Trichospores in different stages of release; when released, showing the thick folded appendage (arrowhead) and the minute collar (arrow). 6-11. *S. bulbosporophorus* (FIGS. 6, 9-11, BCB-Tr1285; FIGS. 7-8, BCB-Tr1284). 6. Peduncle, bulbous zygospore (arrow), appendage (arrowhead) and zygospores. 7. Fertile branches with trichospores. 8. Overall view of a

type [FH]) is the best character to differentiate this species from *S. brevisporum*.

This species has been observed growing with *S. bulbosporophorus* and *S. inexpectans*, described below.

Smittium bulbosporophorus L.G. Valle & Santam.,
sp. nov. FIGS. 6–11

Thallus cum sparsim ramificanti principali axe et aliquot lateralibus secundariis ramis. Basalis cellula aliquando lata facta in maturo thallo, secernens disciforme tenaculum. Trichosporae obovato-ellipsoidales ad late ellipsoidales, (9–)12–14.5(–18) × 3.5–5.5 μm, praebentes singulas conspicuas appendices atque elongatum, cylindricum vel leviter decrescens versus extremum collare, 1.5–3 μm. Unaquaeque fertilis rama cum 2–6(–8) genitalibus cellulis, 5–7.5 μm in longitudine. Zygosporae biconicae, 35–40 × 7–8 μm; zygosporophorum globosum, fere sphaericum, in extremo quadrante zygosporae longitudinis locatum. Liberae zygosporae cum globoso collari, 3.5–4 × 3.5 μm, atque facile visibili appendice. In Chironomidarum larvarum proctodaeo incoli.

Thallus with a sparsely branched main axis, occasionally pinnate, and with secondary lateral branches arising from the base proximity (FIG. 8). Basal cell often broadened in mature thalli, not differentiated in young individuals, secreting a disk-like holdfast. Trichospores obovate-ellipsoidal to broadly ellipsoidal, (9–)12–14.5(–18) × 3.5–5.5 μm, each showing, after released from generative cells, a conspicuous, easily distinguishable, appendage, measuring about three times the trichospore length and a collar that is long, cylindrical or slightly narrowing toward the end, 1.5–3 × 2 μm (FIG. 9). Each fertile branch with 2–6(–8) generative cells, 5–7.5 μm length (FIG. 7). Zygosporae biconical, 35–40 × 7–8 μm; zygosporophore globose, nearly spherical, located at the last quarter of the zygosporae length (FIGS. 6, 10–11). Once released, the zygosporae shows a globose collar, 3.5–4 × 3.5 μm, and an easily observable appendage (FIG. 6).

Etymology. L., *bulbo* = globose, bulbous; *sporophorus* = sporophore (referring to zygosporophore).

Specimens examined. SPAIN. BARCELONA: Montseny, Tordera river, prepared from Chironomidae Diamesinae larvae, 13-II-2002, L.G. Valle, BCB-Tr1284 (HOLOTYPE), BCB-Tr1285 (with zygosporae!), Tr1289, Tr1291 (ISOTYPES); Fogars de Montclús; Santa Fe del Montseny, Santa

Fe stream, prepared from Chironomidae Diamesini larvae, 25-IX-2001, L.G. Valle and S. Santamaria, BCB-Tr1087, Tr1089, Tr1095, Tr1106–1107; same locality, 7-XI-2001, L.G. Valle, BCB-Tr1150, Tr1153, Tr1156. BIZKAIA: Trucíos, Agüera river, prepared from Chironomidae Orthoclaadiinae larvae, 29-V-2002, L.G. Valle, BCB-Tr1434–1435, Tr1438–1439, Tr1441, Tr1443. GIRONA: Agullana, La Guilla stream, prepared from Chironomidae Orthoclaadiinae and Diamesiinae larvae, 14-VI-2001, L.G. Valle, BCB-Tr0836–0839.

The most noticeable feature of this species probably is the presence of a globose zygosporophore (FIG. 6), that inspired its specific epithet. In other species, zygosporophores can be slightly broadened under the zygosporae, as *Smittium cylindrosporum* Lichtw. & Arenas, but not as extremely as in this species. On the other hand, zygosporae measurements are larger in *S. cylindrosporum* (51–52 × 10–11 μm, according to Lichtwardt and Arenas 1996) than in *S. bulbosporophorus*, which represents, along with *S. arvernense*, the smallest zygosporae described in the genus. Poisson's species differs from ours by trichospore measurements (20–25 × 5 μm) and by zygosporae characteristics, which are even slightly shorter and wider in the medial part (30–35 × 8–10 μm), with a small collar (Poisson 1936), not as eccentric as that of *S. bulbosporophorus*. Another difference between both species is the trichospore layout on fertile branches, being more tidily arranged in *S. arvernense* and untidily in *S. bulbosporophorus*, which shows few trichospores at the very last portions of the sparse and disarranged long branches (FIG. 8). When sexual reproduction is present, the conjugated thalli grow in such a dense manner that zygosporae observation can be very difficult (FIG. 11).

Morphologically, *S. morbosum* Sweeney is probably the closest species. It is the only species described as pathogenic and even lethal against its hosts (Sweeney 1981a, b). *Smittium bulbosporophorus*, apparently, is not pathogenic to hosts and differs from *S. morbosum*, not only by its ecology and host relationship but also by its sporangial features, mainly by trichospore collar morphology, longer and not outwardly flared in *S. morbosum* but cylindrical or narrowing toward the end in the new species. In addition, the thallus is more densely branched in *S. morbosum* and lacks a

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thallus. 9. Free trichospore showing the collar and appendage. 10. Zygosporae. 11. Sexual conjugant hyphae producing zygosporae. 12–18. *S. gracilis* (FIG. 12, BCB-Tr0448; FIGS. 13–14, 18, BCB-Tr1331; FIGS. 15, 17, BCB-Tr0441; FIG. 16, BCB-Tr0438). 12–13. Terminal fertile branches bearing trichospores. 14. Free trichospore showing the short collar. 15–16. Thalli habitus. 17–18. Basal cells with holdfast. 19. *S. delicatum* (FH AUS-24-7, type). Detail of basal cell. All FIGS. photographed from lactophenol cotton-blue mounted slides. FIGS. 1, 3, 5–6, 12–14, 17–19, by DIC. FIGS. 2, 4, 7–11, by phase contrast. FIG. 16 by bright field. Scale bar FIG. 1 = 10 μm; Scale bar FIGS. 2, 6, 8, 11 = 20 μm; Scale bar FIGS. 3–5 = 5 μm; Scale bar FIGS. 7, 9–10, 12–14, 17–19 = 10 μm; Scale bar FIGS. 15–16 = 50 μm.

broadened basal cell, which otherwise is typical of most mature thalli of *S. bulbosporophorus*.

Smittium bulbosporophorus was found sharing the host hindgut with *S. brevisporum*, described above.

***Smittium gracilis* L.G. Valle & Santam., sp. nov.**

FIGS. 12–18

Thallus cum (2–)3–4(–6) ramis ortis e basali cellula, hippocrepica atque laterales expansiones ferens. Secundariae atque tertiariae ramae verticillatim in supera area dispositae. Secreta tenaculi materia in concava area basalis cellulae locata. Trichosporae subcylindricae, (18–)20–26(–29) × 2–3.5 μm, cum tenuissima appendice, circa bis magisve longiores quam trichosporae longitudo, inconspicuum collare circa 1 μm longum. Unaquaeque fertilis rama cum 2–8 genitalibus cellulis. Zygosporae ignotae. In Chironomidarum larvarum proctodaeo incolli.

Thalli with (2–)3–4(–6) branches arising directly from the basal cell, which is horseshoe-shaped and has lateral expansions (FIGS. 17, 18). A small amount of secreted material of the holdfast can be seen in the concavity of the basal cell in its middle part. Secondary and tertiary branches verticillately arranged at the upper zone (FIGS. 15, 16). Basal hyphae measuring 3–4 μm diam, to 2–3 μm in distal ones. Trichosporae subcylindrical, (18–)20–26(–29) × 2–3.5 μm, each showing, after released from generative cells, a very thin appendage about twice or more as long as the trichospore length, and an inconspicuous collar of approximately 1 × 1 μm (FIG. 14). Each fertile branch with 2–8 generative cells (FIG. 12). Zygosporae not found.

Etymology. L., *gracile* = delicate (referring to the general aspect of the thin thallus).

Specimens examined. SPAIN, BARCELONA: L'Estany, l'Estany stream, prepared from Chironomidae Chironomini (*Chironomus* spp.) larvae, L.G. Valle, 21-XI-2000, BCB-Tr0414 (HOLOTYPE), Tr0409, Tr0412, Tr0415 (ISOTYPES); same locality, 19-XII-2000, L.G. Valle, BCB-Tr0438, Tr0440–0444, Tr0446–0448; Moià, Moià stream, prepared from Chironomidae (*Chironomus* sp.) larvae, 13-V-2002, L.G. Valle, BCB-Tr1352; Palas de Torrella; Cardener river, prepared from Chironomidae Diamesinae larvae, 24-IV-2002, L.G. Valle, BCB-Tr1331.

An outstanding characteristic of this species is the peculiar horseshoe-shaped basal cell (FIGS. 17, 18), similar to that of another species that we consider closely related, *S. delicatum* Lichtw., described from Australia in *Chironomus* larvae (Lichtwardt and Williams 1990). We have studied the type of this species (microscope slide AUS-24-7, *Holotype* [FH]) and both may be distinguished by thallial and sporangial features. In *S. delicatum*, there is a main hyphal axis clearly continuous with the basal cell and some lateral, basally septate branches (2–4), arising more or less symmetrically from both sides of this axis (FIG. 19). In *S. gracilis*, there is not a definite main axis

(FIGS. 15, 16). *Smittium delicatum* secondary branches are longer, thinner and more sparsely arranged than those of *S. gracilis*. The trichosporae, although comparable, are quite different, being slightly longer and narrower in *S. delicatum*.

Smittium gracilis could be related, as well, to species of the genus *Furculomyces* M.C. Williams & Lichtw. by its thallial characteristics, but we have not found the zygosporae, the most important element that defines the genus *Furculomyces*, with its furcula-like conjugation tubes (Lichtwardt and Williams 1992b). We have examined the type of *Furculomyces boomerangus* (M.C. Williams & Lichtw.) Lichtw. & M.C. Williams (microscope slide AUS-42-M-2 *Holotype* [FH]) for comparison with our species.

An unidentified species of *Smittium* recently has been found in Norway (Lichtwardt, pers comm), sharing many characteristics with *S. gracilis*, in its horseshoe-shaped basal cell, and trichospore features. We think that those specimens may correspond to the species here proposed.

***Smittium hecatei* L.G. Valle & Santam., sp. nov.**

FIGS. 20–27

Thallus a basali cellula ramosus, verticillatus in mediis atque superis areis. Basalis cellula ramificans atque lato pedis similem structuram formans (usque 12 μm lata) in maturo thallo secreta tenaculi materia tectam. Trichosporae subcylindricae ad elongato-ellipsoidales, cum duabus magnitudinibus: typus α, 30–37(–49) × 3.5–5.5 μm, cum collari 3.5–6 × 3.5 μm; typus β, 14.5–24 × 2.5–3.5 μm, cum collari 1.5–2.5 × 2–2.5 μm, in utroque typo cylindricis ad leviter decrescentibus versus extremum. Unaquaeque fertilis rama cum 8–14 magisve genitalibus cellulis, variabili magnitudine (longiores cum trichosporas α formant) atque decrescentibus versus distalia extrema (circa 2.5–3.5 μm diam). Zygosporae ignotae. In Chironomidarum larvarum proctodaeo incolli.

Thalli branched from the basal cell, verticillate in the medial and upper areas. Basal cell not distinguished in young thalli, ramified and differentiated in a kind of broadened-leg (≤12 μm wide) coated with secreted holdfast material in mature thalli (FIG. 22). Bulbous lateral protuberances often can arise from the basal cell, occasionally slightly perforating the hindgut lining. Trichosporae subcylindrical to long-ellipsoidal, with two size ranges (FIGS. 20, 24): type α, 30–37(–49) × 3.5–5.5 μm, with a collar of 3.5–6 × 3.5 μm; type β, 14.5–24 × 2.5–3.5 μm, with a collar of 1.5–2.5 × 2–2.5 μm, cylindrical or slightly narrowing to the end in both types (FIG. 21). Each fertile branch with 8–14 or more generative cells (FIG. 20), variable in size (longer when producing trichosporae α), and narrowing to the distal ends (ca. 2.5–3.5 μm diam). Zygosporae not found.

Etymology. Gr., *hecate* = Greek goddess (who was

venerated by different people, among them, by fishers who asked her for a good fishing; we could also "venerate" her to get the best trichomycete hosts).

Specimens examined. SPAIN. BARCELONA: Moià, Moià stream, prepared from Chironomidae Diamesini (Diamesinae) larvae, 13-III-2002, L.G. Valle, BCB-Tr1348 (HOLOTYPE), BCB-Tr1349-1350, Tr1361-1362 (ISOTYPES); same locality, 14-III-2002, L.G. Valle and S. Santamaria, BCB-Tr1364-1371, Tr1373-1378, Tr1380-1384; same locality, prepared from axenic cultures (Moià pq7), BCB-Tr1494; Gualba, Gualba de Baix, Gualba stream, prepared from Chironomidae Orthocladinae (*Cricotopus* spp.) larvae, 28-III-2001, L.G. Valle, BCB-Tr0656-0659.

This species is characterized by the presence of a wide trichospore size range, which we have considered separable into two forms. We define these two forms as type α , the larger, and type β , the smaller, which measures nearly half the length of the former type. Both types are found randomly on the same thallus but arising from different fertile branches (FIG. 20). The absence of small, ovate trichospores can be used to segregate this species from other dimorphic species as *S. dimorphum* Lichtw. & M.C. Williams (Lichtwardt and Williams 1983), which also show a longer collar in the larger, subcylindrical trichospores than those present in trichospores α of *S. hecatei*.

Another diagnostic characteristic of *S. hecatei* is the presence of numerous generative cells per fertile branch, usually longer in those branches giving rise to trichospores of type α . The basal cell is also a very peculiar feature to separate this species from others, showing an elephant leg-like morphology (FIG. 22), with a coat of secreted holdfast material covering its perimeter.

The trichospores α of *S. hecatei* are similar to trichospores of *S. megazygosporum* (Manier and Coste 1971) but differentiated by the slightly flared outward collar in the latter species. Also, the morphology of the basal cell is an important character to be considered for the separation of both species, when trichospores β are not observed in *S. hecatei*.

Smittium elongatum Lichtw. also can be compared with the species here described, although the differences can be easily discerned when closely observed because *S. elongatum* has a markedly verticillate pattern of ramification, with many branches arising from each verticil (observations from the examined type, microscope slide COL-4-15, *Holotype* [FH]), while in *S. hecatei*, less ramified, just a few branches are observed per verticil, and these have more or less pinnate secondary ramifications. Otherwise, *S. elongatum* does not show the long series of generative cells observed in *S. hecatei*. The presence of the small trichospores of type β and the peculiar base are other

characters to distinguish both species. *Smittium elongatum* also displays a wide trichospore size range, with larger maximums, $(20-34(-44)) \times 3-6 \mu\text{m}$, and a collar of $2-4 \mu\text{m}$ (Lichtwardt 1972).

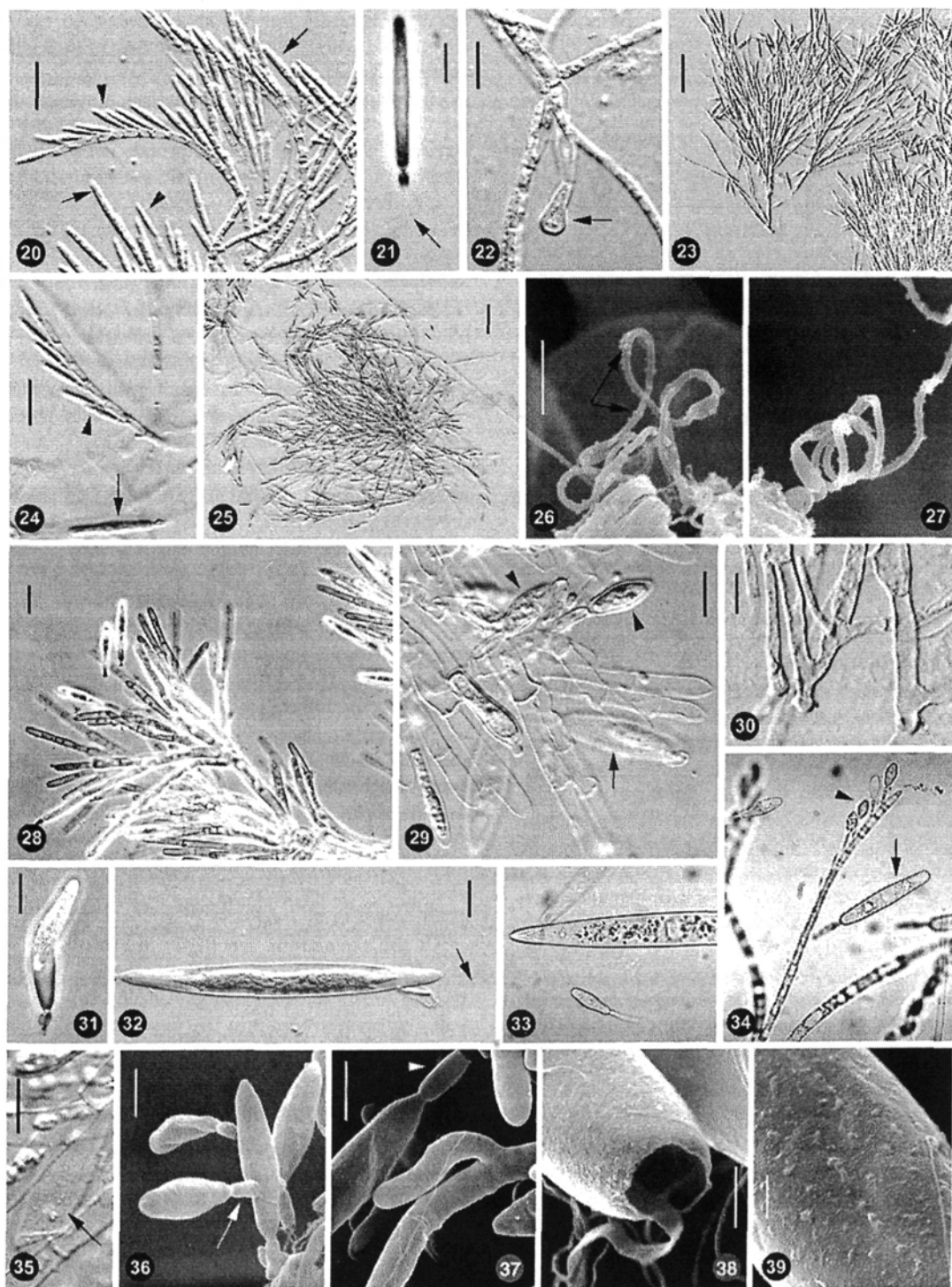
The observations with SEM revealed the cylindrical shape of the trichospore appendage in all its length. Some vesicular bodies can be seen on the appendage surface (FIGS. 26, 27), which probably are related with the presence of adherent materials allowing the trichospore to be attached in the vicinity of the host environment.

***Smittium heterosporum* L.G. Valle & Santam., sp. nov.** FIGS. 28-39

Thallus ramosus, verticillatus in mediis atque superis areis. Basalis cellula cum simplici, disciformi vel campanulato tenaculo. Trichosporae dimorphae: typus α , elongato-ellipsoidales, $(36-50-62(-74)) \times (7.5-9-13 \mu\text{m})$, cum subtiliter granulata pagina et collari leviter decrescenti versus extremum, $(10-12.5-17(-20)) \times 3-4(-5.5) \mu\text{m}$; typus β , parvae, ovato-ellipsoidales ad pyriformes, $14-27 \times 8.5-11 \mu\text{m}$, cum subtiliter vel nullimodo granulata pagina et collari $8-9 \times 1.5 \mu\text{m}$. Zygosporae fusiformes, $(160-170-180(-190)) \times 15-18 \mu\text{m}$; zygosporophorum excentricum, prope unum extremorum polarium locatum. Liberae zygosporae cum collari $17-20(-30) \times 10-13 \mu\text{m}$. In Chironomidarum larvarum proctodaeo incolit.

Thalli branched, verticillate in the medial and upper areas. Some broadenings and constrictions are observed in the basal segment with hyphal cells highly variable diameter ($6-14 \mu\text{m}$ or more). Basal cell with a simple, discoid or slightly campanulate holdfast (FIG. 30). Trichospores dimorphic: type α , long-ellipsoidal, $(36-50-62(-74)) \times (7.5-9-13 \mu\text{m})$, with a delicately granulated surface (more or less apparent depending on the individual, FIGS. 29, 35 and 39), collar slightly narrowing toward the end, $(10-12.5-17(-20)) \times 3-4(-5.5) \mu\text{m}$, filiform appendage (but ribbon-like under SEM! FIG. 38), measuring up to eight times the trichospore length; type β , smaller, ovate-ellipsoidal or pyriform, $14-27 \times 8.5-11 \mu\text{m}$, with the surface slightly granulated or not, collar $8-9 \times 1.5 \mu\text{m}$, appendage identical to that of type α . At the lowermost and medial areas of fertile branches, the generative cells may not arise directly from the main axis but from lateral peduncles, variable in size ($18-50 \mu\text{m}$). Zygosporae fusiform, $(160-170-180(-190)) \times 15-18 \mu\text{m}$; zygosporophore eccentric, located near one of the polar ends (FIG. 32). Once released, the zygosporae shows a collar of $17-20(-30) \times 10-13 \mu\text{m}$, and a large appendage (FIG. 32). Specialized conjugation hyphae giving rise to angulous conjugation bridges, from which a peduncle develops, supporting both zygosporophore and zygosporae.

Etymology. Gr., *hetero* = different; L., *sporum* = spore (referring to trichospore).



FIGS. 20–39. *Smittium hecatei* and *S. heterosporum*. 20–27. *S. hecatei* (FIGS. 20–21, 23–25, BCB-Tr1365; FIG. 22, BCB-Tr1372; FIGS. 26–27, from axenic culture). 20. Fertile branches with trichospores α (arrow) and β (arrowhead). 21. Free trichospore showing the collar and appendage (slightly out of focus, arrow). 22. Basal cell covered with holdfast material (arrow). 23. Fertile branches. 24. Fertile branches with trichospores β (arrowhead) and one free trichospore α (arrow). 25. Overall view of a thallus. 26–27. SEM images of the collar and cylindrical appendage, with adhered globose vesicles (arrows). 28–39. *S.*

Specimens examined. SPAIN. BARCELONA: Sant Llorenç Savall; Vall d'Horta stream, prepared from Orthoclaadiinae (Chironomidae) larvae, 6-III-2001, L.G. Valle, BCB-Tr0598 (HOLOTYPE), BCB-Tr0601 (ISOTYPE); same locality, prepared from axenic cultures (SmV.H.-pq3), 11-XII-2001, BCB-Tr1175-1177, 5-II-2002, BCB-Tr1242-1243, Tr1249; same locality, 7-II-2002, BCB-Tr1266; same locality, prepared from axenic cultures (SmV.H.-pq2), 28-II-2002, BCB-Tr1298; same locality, prepared from axenic cultures (SmV.H.-pq2), 5-III-2002, BCB-Tr1301-1302; Marganell, Marganell stream, prepared from Chironomidae Orthoclaadiinae and Diamesiinae (*Symphothastia* spp., *Potthastia* spp.) larvae, 13-I-2002, L.G. Valle, BCB-Tr1194, Tr1199; same locality, 6-III-2002, L.G. Valle, BCB-Tr1309-1315; same locality, prepared from axenic cultures (SmV.H.-pq1), 13-II-2002, BCB-Tr1288; same locality, prepared from axenic cultures (SmV.H.-pq4), 28-II-2002, BCB-Tr1293, Tr1295-1297. GIRONA: Boadella d'Empordà, Muga river, prepared from Chironomidae Orthoclaadiinae (*Cricotopus bicinctus* Meig.) larvae, 14-VI-2001, L.G. Valle, BCB-Tr0840. TARRAGONA: Horta de St. Joan, Ports de Beseit, Mas de la Franqueta, prepared from Chironomidae Diamesiinae larvae, 24-III-2001, L.G. Valle, BCB-Tr0762-0765, Tr0767-0768, Tr0772, Tr0775-0777.

As defined by the specific epithet, this species is characterized by the dimorphic trichospores, here named α and β . Some characters of *S. heterosporum* are shared with other species, but the composite features were enough to segregate this from others. *Smittium macrosporum* Kobayasi may be considered as the most closely related species but having somewhat shorter trichospores (extreme size $42 \times 7.5 \mu\text{m}$, according to Kobayasi et al 1969). *Smittium macrosporum* trichospores resemble those of *S. heterosporum* by its finely coarse surface and by the presence of branches (or peduncles) growing intercalated between generative cells. *Smittium macrosporum* shows few generative cells per branch, usually two, while we have distinguished up to eight cells per branch in *S. heterosporum*. The typical dimorphism in *S. heterosporum*, and the presence of trichospores measuring up to $70 \mu\text{m}$ long are other important characters that can be used to determine this species. Another spe-

cies with trichospores showing a punctate wall, but different in other trichospore features, is *S. incrasatum* Kobayasi (Kobayasi et al 1971).

The zygospores of *S. heterosporum* are the longest ever described in a species of *Smittium*, and are characterized, as well, by their extremely eccentric collar (FIG. 32). The conjugating filaments are formed from specific hypha that resemble the furcula-like structures of *Furculomyces* (Lichtwardt and Williams 1992b), a genus described for the previously named *Smittium boomerangus* M.C. Williams & Lichtw. (Lichtwardt and Williams 1990). For comparison we have examined the type of *Furculomyces boomerangus* (microscope slide AUS-42-M2, *Holotype* [FH]).

We have observed an interesting phenomenon, already reported in other species, which is the presence of released sporangiospores initiating their development prematurely in the midgut and sometimes with the young thalli having the ability to perforate the peritrophic matrix. *Smittium perforatum* M.C. Williams & Lichtw. also reveals this capacity (Williams and Lichtwardt 1987, Lichtwardt et al 1997). The thallial morphology of the initial developmental stages are similar in both species, but the features of mature individuals are entirely different, with smaller trichospores ($[33-]38[-45] \times [7-]7.9[-8.2] \mu\text{m}$) and a shorter collar ($[6-]7[-8] \times [3-]4.2[-4.6] \mu\text{m}$) in *Smittium perforatum* (type examined!, microscope slide RMBL-28-15 *Holotype* [FH]). Sweeney (1981a) reported that *S. morbosum*, along with its pathogenic action, perforate the hindgut lining. *Smittium longisporum* M.C. Williams, Lichtw. & S.W. Peterson (Williams et al 1982) is another species in which the precocious sporangiospore extrusion has been observed (Lichtwardt et al 1997).

All the collections are highly uniform in trichospore features. The trichospores α represent the main type in nearly all the studied samples. It might be of some interest to mention the peculiar holdfast found in some thalli from Tarragona (Tr0765), which show

←

heterosporum (Figs. 28, 31, BCB-Tr1242, from axenic culture; Figs. 29, 35, BCB-Tr1298, from axenic culture; FIG. 30, BCB-Tr0765; Figs. 32-33, BCB-Tr0529; FIG. 34, BCB-Tr0598; Figs. 36-39, from axenic culture). 28. Fertile branches. 29. Trichospores β (arrowhead) and one free trichospore α showing the verrucose surface (arrow). 30. Basal cells and holdfasts. 31. Trichospore extruding the sporangiospore. 32. Zygospore carrying its eccentric collar and appendage (arrow). 33. Detail of a zygospore with its thickened extreme, and a trichospore of type β . 34. Fertile branch with trichospores β (arrowhead) and one free trichospore type α (arrow). 35. Detail of the verrucose surface of an empty trichospore (arrow). 36-37. Trichospores showing collars (arrows). 38. Detail of a trichospore collar from which the ribbon-like appendage comes out. 39. Detail of the verrucose ornamentation on trichospore outer wall. All FIGS. photographed from lactophenol cotton-blue mounted slides, except FIGS. 21, 28, 30-31, 33-34, photographed from water mounted slides. FIGS. 20, 22-25, 29-30, 32, 35, by DIC. FIGS. 21, 28, 31, by phase contrast. FIGS. 33-34, by bright field. FIGS. 26-27, 36-38, by SEM. Scale bar FIGS. 20, 24, 28-34 = $20 \mu\text{m}$, FIGS. 33-34 with the same scale of FIG. 32; Scale bar FIGS. 21-22, 35-37 = $10 \mu\text{m}$; Scale bar FIG. 23 = $40 \mu\text{m}$; Scale bar FIG. 25 = $50 \mu\text{m}$; Scale bar FIGS. 26-27 = $2 \mu\text{m}$; Scale bar FIGS. 38-39 = $1 \mu\text{m}$.

a globose base and a skirt-like membrane partially covering it (FIG. 30).

Sporangiospore extrusion has been frequently observed in axenically cultured material, without additional help (e.g., induced pH changes [Horn 1989]). The sporangial content (sporangiospore) is released through the apical pole (opposite to the collar, FIG. 31). Once empty, the observation of the granulated surface of the outer sporangial (trichospore) wall is easier (FIGS. 29 and 35). In addition, the sporangiospore extrusion and posterior germination has been viewed in natural conditions inside the chironomid gut lumen. A less frequent episode has been noticed: the extrusion of the zygospore content. The released endospore has been recognized as a fusiform material, ejected through one of the acute poles. We have not detected the whole extrusion because part of the endoplasm remained inside the zygospore.

Observations with SEM revealed a very interesting characteristic, namely the presence of a ribbon-like appendage (FIG. 38), unique among the described species of *Smittium*. In addition, the observation with electronic microscopy shows the coarse outer surface of trichospores (FIG. 39).

***Smittium inexpectans* L.G. Valle & Santam., sp. nov.**

FIGS. 40–44

Thallus e basali cellula pinnatus ad verticillatim ramosum. Basalis cellula leviter lata facta cum simplici, disciformi, secreto tenaculo, inter laterales basales ramas locato. Trichosporae subcylindricae, aliquando cum media protuberatione, 23–27 × 2–2.5 μm, collare cylindricum, 5.5–6 μm longum. Unaquaeque fertilis rama cum 4–6 genitalibus cellulis, 15–28 × 3.5–4 μm. Zygosporae ignotae. In Chironomidarum larvarum proctodaeo incolli.

Thalli pinnate to verticillately branched, with a main and other hyphal axes arising from the basal cell and tapering to the tips. The basal cell is slightly broadened with a simple, disk-like secreted holdfast, located between the lateral basal branches. Trichospores subcylindrical, often with a median bulge, 23–27 × 2–2.5 μm, each showing, after released from generative cells, a cylindrical collar, 5.5–6 × 2 μm (FIG. 41). Each fertile branch with 4–6 generative cells, variable in length. Zygosporae not found.

Etiology. L., *inexpectans* = not expected.

Specimens examined. SPAIN. BARCELONA: Fogars de Montclús; Santa Fe del Montseny, Riera de Santa Fe, prepared from Chironomidae Orthocladiinae larvae, 25-X-2001, L.G. Valle and S. Santamaria, BCB-Tr1156 (HOLOTYPE); same locality, 7-XI-2001, L.G. Valle and S. Santamaria, BCB-Tr1095, BCB-Tr1089.

Smittium inexpectans is characterized by its relatively long collar (FIG. 41). Thallial characters are somewhat peculiar, because the pattern of basal ramification is not common, with its laterally emerging

branches, tapering toward the apex, as wide as the basal cell, without any septa at the base; and the holdfast, placed in the middle of these lateral branches (FIG. 40). *Smittium kansense* Lichtw. & Grigg has similar trichospores but differs in its shorter collar (2 μm, according to Lichtwardt and Grigg 1998). Other species with similar-size trichospores are *S. phytotellum* Lichtw. (Lichtwardt 1994), *S. delicatum* (Lichtwardt and Williams 1990), *S. typhellum* Manier & Coste (Manier and Coste 1971), and *S. angustum* M.C. Williams & Lichtw. (Lichtwardt and Williams 1992a), all separable from our species by the presence of shorter trichospore collars, as well as by other thallial features.

***Smittium prostratum* L.G. Valle & Santam., sp. nov.**

FIGS. 45–48

Thallus prostratus super proctodaeo, cum lateralibus ramis verticillatas ramificationes in superis areis ferentibus. Secreta tenaculi materia secus thalli paginam hanc adhaerens cum proctodaeo. Basalis cellula variabilis, plerumque cum lateralibus ramis. Trichosporae subcylindricae ad elongato-ellipsoidales, 26–30 × 3.5–4.5 μm, cum campanulato collari, decrescenti versus extremum, 4.5–5.5 × 2.5–3.5 μm. Quoque fertile ramae 2–4(–6) genitales cellulas. Zygosporae ignotae. In Chironomidarum larvarum proctodaeo incolli.

Thalli prostrate on the hindgut lining, with lateral branches that form verticillate ramifications at the upper areas. Holdfast material secreted along the thallial surface keeping it in contact with the gut lining, functioning as glue (FIG. 48). Basal cell variable, usually with lateral branches. Trichospores subcylindrical to elongate-ellipsoidal, 26–30 × 3.5–4.5 μm, with a campanulate collar, narrowing toward the end, 4.5–5.5 × 2.5–3.5 μm (FIGS. 46–47). Each fertile branch with 2–4(–6) generative cells. Zygosporae not found.

Etiology. L., *prostratum* = prostrate (referring to the position of the thallus).

Specimen examined. SPAIN. HUESCA: Puente de la Reina de Jaca, Asabón river, prepared from Chironomid Orthocladiinae larvae, 264-IX-2002, L.G. Valle, BCB-Tr1550 (HOLOTYPE).

The main diagnostic characteristic of this species undoubtedly is the position of thalli inside the hindgut, being prostrate (i.e., horizontally in relation to the gut lining). The holdfast not only is concentrated in the basal cell but covers the entire thallial axis that remains in contact with the gut lining (FIG. 48). An increase of the basal surface may improve its capacity to develop a larger and more ramified thallus, allowing more trichospore production.

Trichospore morphology in *S. prostratum* is similar to that of other species, but collar morphology is a good character for its isolation, being wider and

more campanulate than in the related species. Trichospores of *S. cylindrosporum* are somewhat larger ([21–]26–33[–41] × 4–6 μm, according to Lichtwardt and Arenas 1996) and more cylindrical. *Smittium typhellum* also shows similar trichospore sizes but has shorter and cylindrical collars (Manier and Coste 1971, Manier and Mathiez 1965).

Another species where a lateral direction of the thallial growth has been reported is *S. fecundum*, although in this example the lateral extension is not so evident and the holdfast material is not continuous but secreted at the tip of small pits laterally produced from the axial cells. Other genera of Harpellales also have similar patterns of thallial development (e.g., *Lancisporomyces vernalis* Santam., where the main axis lies on the gut lining, being fixed to it by numerous discontinuous peg-like holdfasts and profuse lateral branches [Santamaria 1997]). In addition, *Baltomyces styrax* Cafaro (Cafaro 1999) has a similar layout. In all these cases the increase in the contact surface could be a result of a phenomenon of evolutionary convergence.

***Smittium pseudodimorphum* L.G. Valle & Santam., sp. nov.** FIGS. 49–55

Thallus e basali cellula ramosa, verticillatus in mediis atque superis areis. Basalis cellula simplex vel bilobata, cum secreta tenaculi materia. Trichosporae dimorphae: typus α, subcylindricae, (45–)50–55(–66) × (4–)5.5(–7) μm, cum cylindrico vel convergentibus marginibus collari 9–11.5 × 3.5 μm; typus β, late ellipsoidales, (12–)14–16.5 × 5.5–6 μm, cum collari cylindrico, 12.5–18 × 1.5–2 μm. Genitales cellulae longiores in ramis trichosporas typi α ferentibus, breviores atque plures in ramis trichosporas typi β ferentibus. Zygosporeae biconicae, 82–97 × (14–)16–18(–20) μm, zygosporeophorum in extremo quadrante zygosporeae longitudinis locata. Liberae zygosporeae cum collari, 16.5–20 × .5–7 μm atque conspicua appendice. In Chironomidarum larvarum proctodaeo incolit.

Thalli branched from the basal cell, verticillate at the medial and upper areas. Basal cell simple or bilobulated (FIG. 53), with secreted holdfast material. Trichospores dimorphic: type α, subcylindrical, (45–)50–55(–66) × (4–)5.5(–7) μm, with a thickened apex, collar cylindrical or with convergent margins (FIGS. 49–50), 9–11.5 × 3.5 μm; type β, broadly ellipsoidal, (12–)14–16.5 × 5.5–6 μm, collar cylindrical, 12.5–18 × 1.5–2 μm (FIGS. 52, 54–55). Both trichospore types growing in the same thallus but on different branches, the trichospores of type β being less common. Generative cells longer in branches producing the trichospores of type α, shorter and more numerous in fertile branches producing the trichospores of type β. Zygospores biconical, 82–97 × (14–)16–18(–20) μm (FIGS. 50–51). Once released,

the zygospore shows a collar of 16.5–20 × .5–7 μm, and a visible appendage.

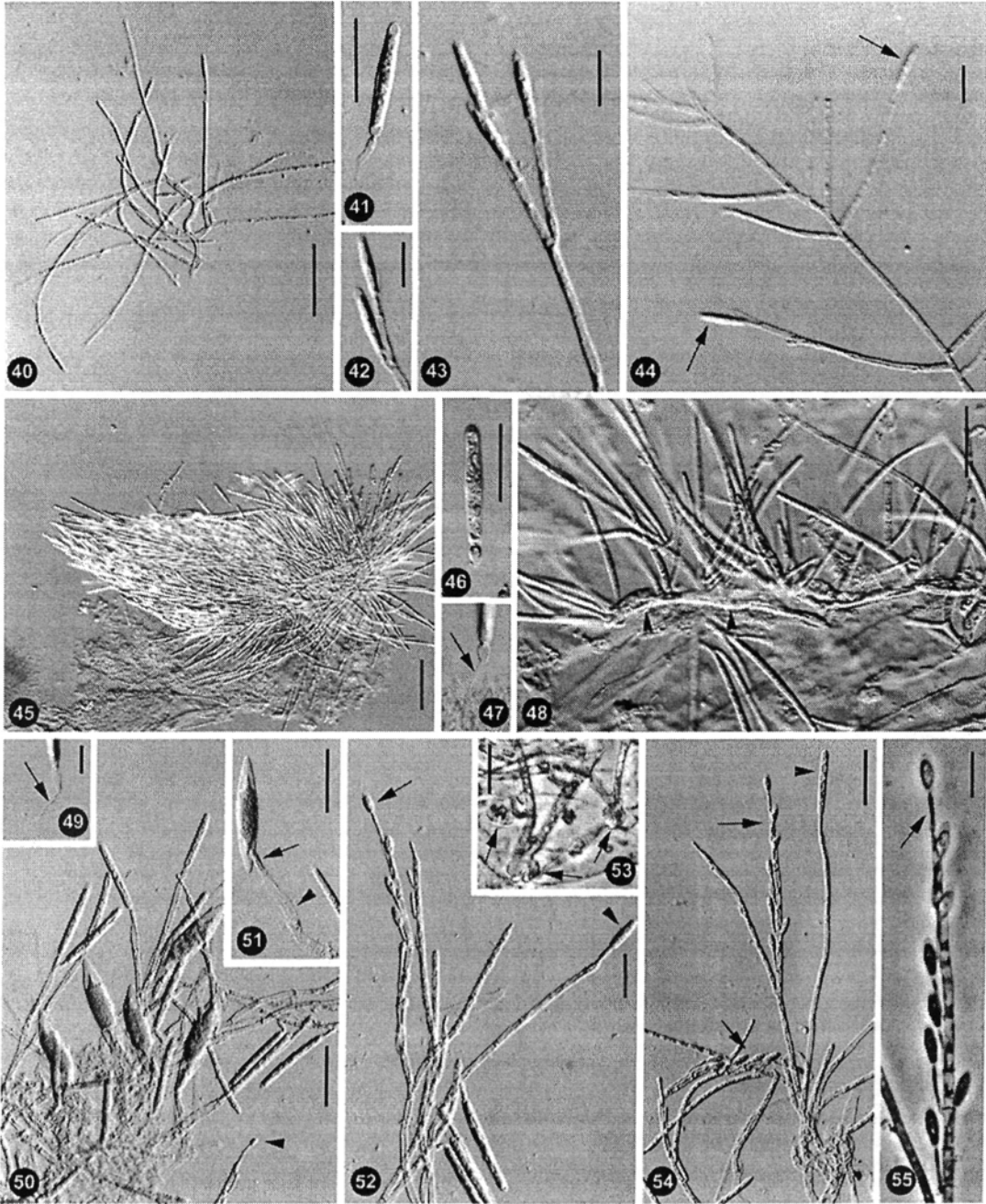
Etymology. L., *pseudo* = false, similar to; *dimorphum* = dimorphic (referring to the similarity with *Smittium dimorphum*).

Specimens examined. SPAIN. BARCELONA: Palas de Torrella, Cardener river, prepared from Chironomidae Diamesiinae larvae, 15-III-2001, L.G. Valle, BCB-Tr0605 (HOLOTYPE). TARRAGONA: Prades; Riudabella, La Milana stream, prepared from Chironomidae Diamesiinae larvae, 19-III-2001, L.G. Valle and S. Santamaria, BCB-Tr0622, Tr0624–0625.

As inferred by the specific epithet, this species is similar to *S. dimorphum*, one of the several dimorphic species described within the genus. Trichospores of the types α and β are similar in size range in both species, but the main difference between them is the presence, in *S. pseudodimorphum*, of a longer collar in both trichospore types (FIGS. 49, 55), most obvious in trichospores of type β (2.5–4 μm in *S. dimorphum*, Lichtwardt and Williams 1983). In *S. pseudodimorphum*, trichospores α are slightly longer (38–50 × 5.5–6.5 μm in *S. dimorphum*, Lichtwardt and Williams 1983). Zygospores of *S. pseudodimorphum* are broader and more biconical ([82–97 × [14–]16–18[–20] μm) than those of *S. dimorphum* (71–96 × 13–14 μm). The zygospore collar is broader and campanulated in the American species (12–17 μm diam, according to Lichtwardt and Williams 1983). The basal cell is different between the two species, being bilobulate in most of the individuals of *S. pseudodimorphum* (FIG. 53). *Smittium pseudodimorphum* presents a well-distinguished and differentiated holdfast, while it is inconspicuous in *S. dimorphum* (Lichtwardt and Williams 1983). For comparison we have examined the type of *Smittium dimorphum* (microscope slide MBL-62-7 *Holotype* [FH]).

Trichospores of type β may be compared to those found on *S. alpinum* Lichtw. (Lichtwardt 1984), although in *S. pseudodimorphum* they are somewhat larger ([23–]33[–44] × [10–]12[–14] μm) and the collar is likewise larger ([10–]14[–19] × [2–]3[–4] μm). In both species zygospores are nearly identical.

Smittium esteparum Ferrington, Lichtw. & López Lastra (Lichtwardt et al 1999) is another dimorphic species, although it is distinguished from *S. pseudodimorphum* by its trichospore and holdfast features, as well as by its smaller zygospores. *Smittium orthocladii* Manier is similar to the Spanish species, although α trichospores are more ellipsoidal and shorter and the β trichospores are smaller than in *S. pseudodimorphum*. Zygospores in *S. orthocladii* were observed to be more fusiform (Manier 1970) when we examined the type (microscope slide COUL 9.63 *Holotype* [PC]).



FIGS. 40-55. *Smittium inexpectans*, *S. prostratum* and *S. pseudodimorphum*. 40-44. *S. inexpectans* (FIG. 40, BCB-Tr1156; FIGS. 41-42, BCB-Tr1095; FIGS. 43-44, BCB-Tr1084). 40. Overall view of the thallus. 41. Free trichospore showing the collar. 42-43. Fertile branches with few trichospores. 44. Aspect of a secondary axis with few trichospores (arrows). 45-48. *S. prostratum* (BCB-Tr1550). 45. Overall view of a thallus. 46. Free trichospore with its collar. 47. Detail of trichospore collar and appendage (arrow). 48. Lateral view of the basal prostrate area, with holdfast material secreted all along the contact surface (arrowheads). 49-55. *S. pseudodimorphum* (FIGS. 49-52, 54-55, BCB-Tr0605; FIG. 53, BCB-Tr0623). 49. Detail of trichospore collar and appendage (arrow). 50. Conjugant hyphae with zygospores and trichospores of type α , young trichospore type β is focused at the lower part (arrowhead). 51. Aspect of a zygospore with its zygospore (arrow) and part of the supporting cell (arrowhead). 52, 54. Fertile branches, some producing trichospores type β (arrow) and others, from the same thallus, producing trichospores type α (arrowhead). 53. Lobulate basal cells (arrows) with holdfasts. 55. Detail

PREVIOUSLY KNOWN SPECIES

Smittium alpinum Lichtw. FIG. 56

Specimens examined. SPAIN. TARRAGONA: Horta de Sant Joan; Mas de la Franqueta, prepared from Chironomidae Diamesini larvae, 24-V-2001, L.G. Valle, BCB-Tr0773, Tr0778.

Other collections examined. USA, Glacier National Park, prepared from *Diamesa* sp., 11-VIII-1975, Lichtwardt, microscope slide MBL-13-10 (HOLOTYPE, FH).

Species reported from the USA and Europe (Sweden, France and Switzerland; Lichtwardt 1984). Some variations have been noticed between the American and the European collections, with longer trichospores in the former (Lichtwardt 1984). A remarkable characteristic is the presence of a long cylindrical collar (FIG. 56), especially in some of the individuals studied ($14\text{--}17 \times 2 \mu\text{m}$). Trichospores measure $30.5\text{--}36 \times 9\text{--}12 \mu\text{m}$, being somewhat narrower but mostly coincident with other European thalli of *S. alpinum* ($[23\text{--}33\text{--}44] \times [10\text{--}12\text{--}14] \mu\text{m}$, according to Lichtwardt 1984). We have not found zygospores, which have been reported only from the American samples. This is the first report of *S. alpinum* from the Iberian Peninsula.

Smittium chironomi Tuzet & Manier FIG. 57

Specimens examined. SPAIN. SEGOVIA: Vegas de Matute, Moros river, prepared from Chironomidae Diamesinae larvae, 24-IX-2001, L.G. Valle, BCB-Tr1009, Tr1012-1013, Tr1015.

This species was described from France (Tuzet and Manier 1953) and last reported but not definitely identified from England, where several experimental studies were carried out with cultured material (Moss 1972). This species previously was reported from Spain, where smaller trichospores than typical were described (Santamaria and Girbal 1997). The Spanish samples have slightly shorter and broader trichospores ($13\text{--}20 \times 3\text{--}4 \mu\text{m}$) and the collar is slightly longer ($2\text{--}3 \mu\text{m}$) than those of the original description.

Smittium culicis Manier FIGS. 58-63

Specimens examined. SPAIN. BARCELONA: Viladrau; Collpregon stream, prepared from Chironomidae Orthoclaadiinae (*Eukiefferiella* sp.) larvae, 23-I-0, L.G. Valle, BCB-Tr1219, Tr1227-1228, Tr1230; Moià, Moià stream, prepared from Chironomidae Orthoclaadiinae (*Chironomus* sp.) larvae, 13-III-2002, L.G. Valle, BCB-Tr1318; same locality, L.G. Valle and S. Santamaria, 14-III-2002, Tr1356, Tr1360, Tr1379. GIRONA: Tor, Llosa river, prepared from Culicidae

larvae, 7-VIII-2000, L. Ribas, L.G. Valle and S. Santamaria, BCB-Tr0198.

Other collections examined. FRANCE. Montpellier, prepared from *Culex pipiens*, 21-V-1959, Manier, microscope slide TYPE M.17C (HOLOTYPE, PC).

Smittium culicis is a cosmopolitan species, since it has been reported nearly from all continents where Trichomycetes have been searched (France, Tunisia [Manier et al 1964], Canada, New Zealand and USA [Lichtwardt 1986], Chile [Lichtwardt and Arenas 1996], Argentina [Lichtwardt et al 1999]). It comprises thalli and collections with a broad trichospore size range and morphometric variations that have been solved taxonomically creating what often has been designed as a species complex (Lichtwardt et al 2001). Our specimens include individuals at the lower limits of trichospore size range ($[17\text{--}]20\text{--}25.5 \times 4\text{--}6 \mu\text{m}$, with a collar of $4\text{--}7.5 \mu\text{m}$ long).

The typical hosts for *S. culicis* are the Culicidae larvae, although it also has been reported and isolated in axenic cultures from Chironomidae (*Chironomus* spp.) in France and from Simuliidae in the USA (Lichtwardt 1986, Lichtwardt et al 2000). Our specimens were found in both Chironomidae and Culicidae, with zygospores observed in the Orthoclaadiiinae *Eukiefferiella* sp., an unusual host. No remarkable differences have been observed between the examined populations in spite of host variation.

We report for the first time the presence of zygospores (FIG. 61), which typically are biconical, measuring $47\text{--}55 \times 11\text{--}12 \mu\text{m}$, with a collar of $4.5\text{--}8 \times 3.5\text{--}5.5 \mu\text{m}$, having cylindrical or slightly out-flared margins. In those thalli where zygospores develop, trichospore size is smaller ($14.5\text{--}19.5 \times 5\text{--}6 \mu\text{m}$) and the number per fertile branch decreases in comparison with those seen in nonsexual phases. Zygospore and zygospores develop at the top of a peduncle formed from one of the conjugants, next to the conjugation tube (FIG. 58).

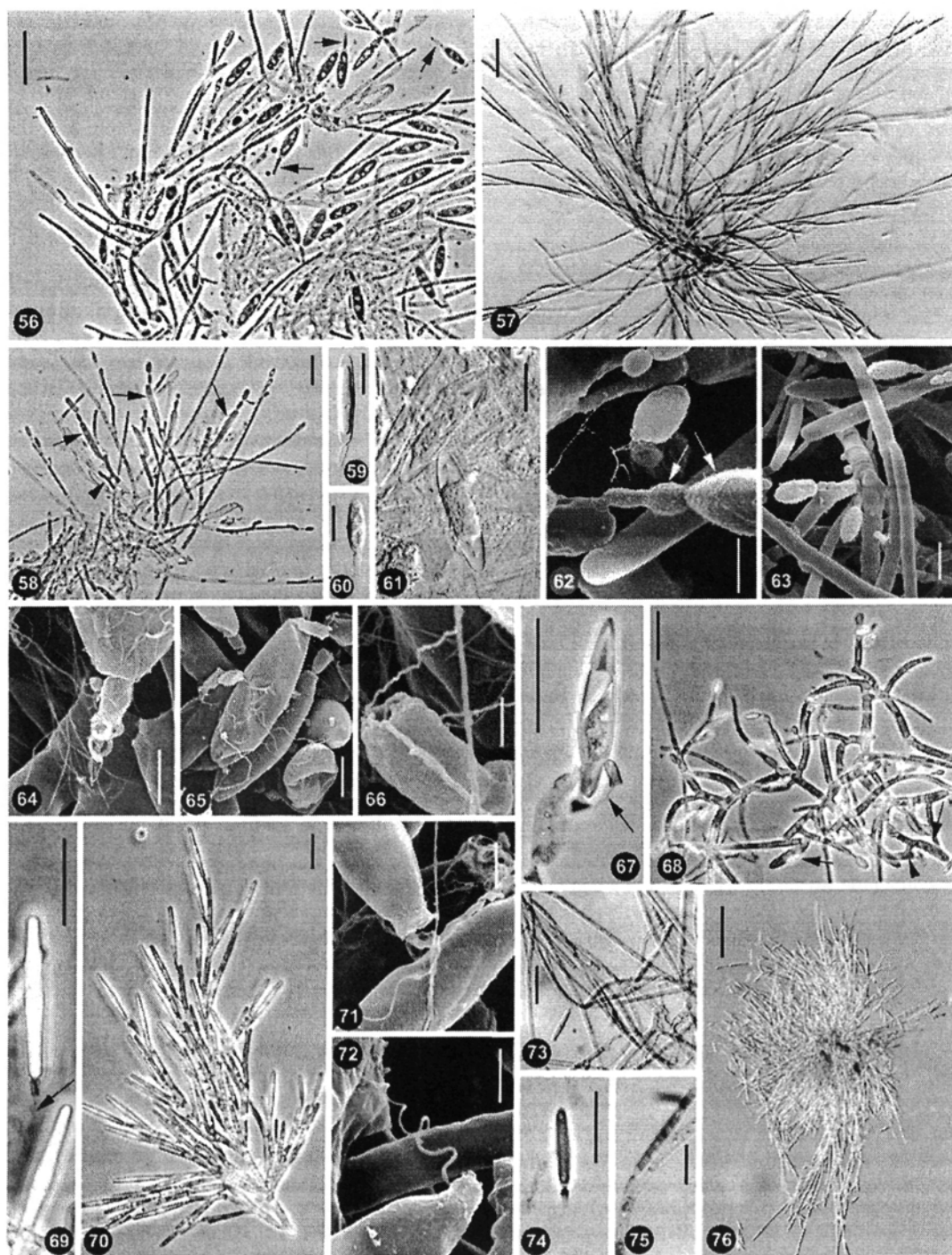
The observation of a coat-like substance on both trichospores and trichospore collars, when studied with scanning electronic microscopy (FIGS. 62, 63), is remarkable.

Smittium culisetae Lichtw.

Specimens examined. SPAIN. BARCELONA: Caldes de Montbui, prepared from Culicidae larvae, 13-X-94, J. Girbal, BCB-Tr0049-0056; Cerdanyola del Vallès, Bellaterra, UAB, prepared from Culicidae larvae, 15-VII-2002, L.G. Valle, Tr-BCB-1502.

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of a fertile branch bearing trichospores type β , showing its long collars (arrow). All FIGS. photographed from lactophenol cotton-blue mounted slides. FIGS. 40-54, by DIC. FIG. 55, by phase contrast. Scale bar FIGS. 40, 50-51, 54 = $50 \mu\text{m}$; Scale bar FIGS. 41-44, 46-47, 52-53, 55 = $20 \mu\text{m}$; Scale bar FIGS. 45, 48 = $40 \mu\text{m}$; Scale bar FIG. 49 = $10 \mu\text{m}$.



FIGS. 56-76. Previously known species of *Smittium*. 56. *S. alpinum* (BCB-Tr0773). Fertile branches with trichospores, some of them detached and showing its collars (arrows). 57. *S. chironomi* (BCB-Tr1015). Overall view of a thallus. 58-63. *S. culicis* (FIGS. 58-61, BCB-Tr1219; FIGS. 62-63, from axenic culture). 58. Thalli with conjugation bridges (arrowhead) and young zygospores on supporting cells (arrows). 59-60. Free trichospores showing their variability within one thallus. 61. Zygospores. 62. Trichospore and collar with their coat. 63. Some coated trichospores on generative cells. 64-68. *S. fecundum* (FIGS. 64-66, from axenic culture; FIGS. 67-68, BCB-Tr1268). 64. Detail of a trichospore showing the collar and a cylindrical and

The species is widely distributed (Lichtwardt 1986). We have studied the material previously reported by Santamaria and Girbal (1997). These specimens perfectly match the trichospore size range proposed for the species ($[11-]16[-30] \times [3-]4[-7] \mu\text{m}$, according to Lichtwardt 1964). *Smittium culisetae* is distinguished from *S. culicis* by its characteristic maximum breadth located in the basal section of the trichospore.

Smittium dipterorum Lichtw.

FIG. 76

Specimens examined. SPAIN. BARCELONA: Gualba de dalt (Parc del RACC), Gualba stream, prepared from Chironomidae Orthocladiinae larvae, 7-XI-2001, L.G. Valle, BCB-Tr1122.

Other collections examined. COSTA RICA. Monteverde, prepared from *Simulium* sp., 11-XI-1991, Lichtw., CR-260-2 (HOLOTYPE, FH), CR-17-3 (PARATYPE, FH).

This is the second world record of this species, previously known only from Costa Rica (Lichtwardt 1997) in the tract of Simuliidae and Chironomiidae (*Orthocladius* spp.). Our specimens have almost cylindrical trichospores measuring $15-20 \times 2.5-3.5 \mu\text{m}$, with a collar of $1.5-3 \mu\text{m}$, slightly flared outward ($[10-]12-18[-26] \times 2-3[-4] \mu\text{m}$, in Lichtwardt 1997). The thallus is profusely branched at the base, showing many radiating branches with verticillate ramifications (FIG. 76). Each fertile branch includes 4-6(-8) generative cells. We have not clearly observed the basal cell because the only mature thalli collected were crowded.

Smittium fecundum Lichtw. & M.C. Williams

FIGS. 64-68

Specimens examined. SPAIN. BARCELONA: L'Espunyola; Can Macià, in a little stream proceeding from a pond, prepared from Chironomidae Orthocladiinae larvae, 4-II-2002, L.G. Valle, BCB-Tr1245-1248, Tr1251, Tr1254-1265, Tr1267-1277; same locality, prepared from axenic cultures (SmMacià-pq4 and pq7), same locality, 28 Feb 2002, BCB-Tr1294, Tr1299, Tr1320, Tr1322.

Other collections examined. USA. COLORADO: Gunnison Country, prepared from *Psectrocladius* sp., 27-VII-1995, Lichtwardt and Williams, RMBL-61-8 (HOLOTYPE, FH).

This species was known only from the type locality in the USA (Lichtwardt and Williams 1999). The Spanish specimens show trichospores with a narrower collar ($5.5-7.5 \mu\text{m}$) in comparison to the type, although they absolutely are comparable and the differences are not important. Iberian specimens have trichospores measuring $(14-)25-28(-31) \times 7-7.5(-9.5) \mu\text{m}$, similar to those described for the American specimens ($17-20[-27] \times 5-8 \mu\text{m}$, in Lichtwardt and Williams 1999), although we have observed some trichospores longer and wider. The original description was prepared, in part, on the basis of cultured material, where the formation of long series of generative cells was observed. Likewise, we have seen this arrangement only in cultured specimens. In the natural hosts, the number of generative cells per fertile branch clearly decreases (4-6[-8]). The characteristic peg-like holdfasts have been observed in some but not in all of the examined thalli.

We report for the first time the presence of zygospores, which are biconical, slightly asymmetric, rounded at the medial section, measuring $70-85 \times 18-19 \mu\text{m}$, with the zygosporophore eccentrically placed at approximately one-third the distance from one apex (FIG. 67). Zygosporophores and zygospores grow at the top of a sterile peduncle measuring $42-54 \times 8-10 \mu\text{m}$ (FIG. 68). Zygospore collar measures $9-12 \mu\text{m}$ in length. These sexual spores are comparable to those of *S. alpinum*, which also are biconical with a pronounced rounded or broadened middle section (Lichtwardt 1984), as well as those of *S. ouseli* M.C. Williams & Lichtw. (Williams and Lichtwardt 1984).

Observations using SEM techniques revealed the presence of a cylindrical appendage, with a microgranulation on the surfaces of both trichospores and trichospore collars. The margins of the collar are seen clearly, being inwardly folded at the distal end (FIG. 66).

Smittium megazygosporum Manier & Coste

FIGS. 69-72

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 helically folded appendage. 65. Trichospore and its collar with smooth surface. 66. Detail of a collar with convergent margins. 67. Zygospore with collar and appendage (arrow), extruding its content. 68. Sexual conjugant hyphae with bridges (arrowhead) and supporting cells (arrows). 69-72. *S. megazygosporum* (FIG. 69, BCB-Tr0765; FIG. 70, BCB-Tr1194; FIGS. 71-72, from axenic culture). 69. Free trichospores with collar and appendage (arrow). 70. Overall view of a thallus. 71-72. Trichospores with broken collars and emerging cylindrical appendages. 73-74. *S. pusillum* (BCB-Tr1591). 73. Branches and trichospores. 74. Free trichospore with collar. 75. *S. typhellum* (BCB-Tr1443). Trichospore attached to the generative cell. 76. *S. dipterorum* (BCB-Tr1122). Overall view of a mature thallus. All figures photographed from lactophenol cotton-blue mounted slides, except Figs. 58, 67-70, photographed from water mounted slides. Figs. 57, 59-61, by DIC. Figs. 58, 67-70, 76, by phase contrast. Figs. 74-75, by bright field. Figs. 62-66, 71-72, by SEM. Scale bar FIG. 56 = $40 \mu\text{m}$; Scale bar FIGS. 57-58, 67, 76 = $50 \mu\text{m}$; Scale bar FIGS. 59-60, 75 = $10 \mu\text{m}$; Scale bar FIG. 61 = $25 \mu\text{m}$; Scale bar FIG. 68 = $30 \mu\text{m}$; Scale bar FIGS. 69-70, 73-74 = $20 \mu\text{m}$; Scale bar FIGS. 62, 64, 71-72 = $2 \mu\text{m}$; Scale bar FIGS. 63, 65 = $5 \mu\text{m}$; Scale bar FIG. 66 = $1 \mu\text{m}$.

Specimens examined. SPAIN. BARCELONA: Avià, Clarà stream, prepared from Chironomidae Diamesini (Diamesinae) larvae, 2-IV-2001, *L.G. Valle*, BCB-Tr0669-0671, Tr0673-0675; Rupit, Rupit stream, prepared from Chironomidae Orthocladiinae (*Eukiefferiella* gr. *minor*) larvae, 26-IV-2001, *L.G. Valle*, BCB-Tr0559-0562; Marganell, Marganell stream, prepared from Chironomidae Orthocladiinae larvae, 13-I-2002, *L.G. Valle*, BCB-Tr1188-1200; same locality, prepared from axenic cultures (SmMarg-pq4), 28-II-2002, BCB-Tr1293, Tr1295-1297, Tr1324. TARRAGONA: Riudabella, Milana stream, prepared from Chironomidae Orthocladiinae (*Cricotopus* sp.) larvae, 19-III-2001, *L.G. Valle* and *S. Santamaria*, BCB-Tr0623.

Other collections examined. FRANCE. Hérault, prepared from *Syncricotopus paquiventris*, XI-1965, *Manier and Coste*, TYPE LIR-11.65 (HOLOTYPE, PC).

Smittium megazygosporum was described in France from the Orthocladiinae *Syncricotopus rufiventris* (*Manier and Coste* 1971) and also was reported from Argentina in *Cricotopus* sp. (*Lichtwardt et al* 1999). The description of this species is wide enough to include several forms with overlapping trichospore size ranges. Taking this into account, we could consider this as a species complex, comparable to *S. culicis*. Until now, the descriptions for the specimens found in France and Argentina comprise individuals with extreme trichospore sizes of 36-49 × 3.5-6 µm, and collar of 3.5-7 µm (from *Manier and Coste* 1971, and *Lichtwardt et al* 1999). We have found trichospores measuring (35-)40-47(-53) × 3.5-5.5 µm, with collar of (3-)4.5-5.5 × 2.5-4 µm. Some of the collections of *S. megazygosporum* examined surpass the trichospore length of those previously described. The trichospore size differences seem not to be very important, we consider them as intraspecific variations. We did not find zygospores, an essential feature to fully confirm the identity of the species, however, all the thallial and sporangial characters indicate that these Iberian collections correspond to the description of *S. megazygosporum*.

We have studied some cultured material (from Marganell collection) with SEM techniques, it is worth mentioning that the cylindrical appendage is helically arranged just after release (FIG. 72). We also mention the smooth trichospore outer wall (FIG. 72). Most of the trichospore collars strangely have been broken and appear incomplete (FIG. 71). These events have been observed in other species (*S. culicis* and *S. hecatei*), giving rise to abnormal morphologies (i.e., shorter collars, usually also somewhat outwardly flared).

Cultured material shows trichospores that frequently extrude their content with no external help. These apically released sporangiospores are viable and produce new thallial colonies.

Smittium pusillum Manier & Coste FIGS. 73-74

Specimens examined. SPAIN. CANTABRIA: Cabezón de Liébana, Bullo river, prepared from Chironomidae Orthocladiinae larvae, 30 Sep 2002, *L.G. Valle*, BCB-Tr1591.

Other collections examined. FRANCE. Hérault, prepared from *Procladius* sp., 2-IV-1968, *Manier and Coste*, TYPE VEND 4.68 (HOLOTYPE, PC).

This is the third record of *S. pusillum*, which originally was described from France. The second report occurred in the Rocky Mountains (USA), within Chironomidae Orthocladiinae (*Cricotopus* spp. and *Orthocladius* spp.) larvae (*Williams and Lichtwardt* 1987, *Lichtwardt and Williams* 1988). A peculiar character of this species that we have not observed is the presence of a pseudorhizoidal basal cell, reported in the original description, although without any illustration (*Manier and Coste* 1971) and not mentioned in the second record of the species (*Williams and Lichtwardt* 1987). We have observed only some lateral and nonseptate branches arising from the basal cell in some individuals. The spore features of the Spanish specimens are coincident with those described in France (14-22 × 2.5-3 µm, in *Manier and Coste* 1971), although we have observed some larger and broader trichospores (15-24 × 2.5-3.5 µm) as reported from the American specimens (14-25 × 3.5-4 µm, according to *Williams and Lichtwardt* 1987). The collar measures 3-4 × 1.5-2 µm in our specimens but reaches 4.5 µm in those described by *Williams and Lichtwardt* (1987). We have not found zygospores.

Smittium simuli Lichtw.

Specimens examined. SPAIN. BARCELONA: El Brull, Montseny, La Castanya, la Castanya stream, prepared from Simuliidae larvae, 3-X-2000, *L.G. Valle* and *S. Santamaria*, BCB-Tr0326, Tr0328; Fogars de Montclús, Sta. Fe del Montseny, Riera de Sta Fe, prepared from Simuliidae larvae, 14-IX-94, *J. Girbal* and *S. Santamaria*, BCB-Tr0066; Cerdanyola del Vallès, UAB, prepared from Culicidae larvae, 15-VII-2002, *L.G. Valle*, Tr-BCB-1502-1513; same locality, prepared from axenic cultures, 10-XI-2002, BCB-Tr1682. GIRONA: Setcases; Còma d'Orri, Ter river, prepared from Chironomidae larvae, 12-IX-2000, *L.G. Valle* and *S. Santamaria*, BCB-Tr0273.

This is a frequent and cosmopolitan species with a wide host range, from the more typical Simuliidae through the less typical Chironomidae (*Lichtwardt et al* 1987) to the more infrequent Culicidae (*Lichtwardt et al* 2001). We also have found this species in all of these hosts, the more remarkable being those individuals found in Culicidae larvae. This species was reported previously from Spain (*Girbal and Santamaria* 1998), only from Simuliidae tracts. The specimens found in Culicidae larvae show a slightly different thallus, with more prolific and densely ramified fertile branches. In fact, thallial features some-

what resemble those of *S. culisetae* but the trichospores are clearly in concordance with those described for *S. simulii* ([19–]23–27 × 5–5.5 μm carrying a collar of [1.5–]2–3[3.5] × 2–3 μm in our specimens), lacking the diagnostic broadening at the submedian section of *S. culisetae*. Moreover, some of the individuals display the distinctive horseshoe-shaped basal cells.

Smittium typhellum Manier & Coste FIG. 75

Specimens examined. SPAIN. BIZKALA: Trucíos, Agüera river, prepared from Chironomidae Diamesinae larvae, 29-V-2002, A. Elosegui and L.G. Valle, BCB-Tr1433, Tr1436; Gorbea, Badaya river, prepared from Chironomidae Diamesinae larvae, 15-V-2002, I. Salcedo and L.G. Valle, BCB-Tr1409.

Other collections examined. FRANCE. Herault, prepared from *Chironomus* complex *plumosus*, 6-II-1964, Manier and Coste, COUR 1.68 (ISOTYPE, PC).

This species has been reported from France (Manier and Mathiez 1965, Manier and Coste 1971), USA (Williams and Lichtwardt 1987) and Australia (Lichtwardt and Williams 1990). The specimens collected in Spain are somewhat more delicate, with long terminal sparse branches. Trichospore features are equal to those reported by Manier and Coste, although the French specimens were more cylindrical and reached 30 μm long (24–29 × 3–3.5[–4] μm in our specimens).

Trichospore collars attain intermediate lengths (2–3.5 × 1.7–2 μm) between these reported for the French specimens (3–3.5 × 1.5–2 μm, Manier and Coste 1971) and the Australian ones (5 μm, in Lichtwardt and Williams 1990). We have found *S. typhellum* specimens growing in the same host tribe (Orthocla-diinae) as the Australian and American specimens.

KEY TO THE IBERIAN SPECIES OF *SMITTIIUM*

- 1. Trichospores dimorphic (two types, different by size and/or by shape) 2
- 1'. Only one trichospore type 4
 - 2. Trichospores similar by morphology (subcylindrical to elongate-ellipsoidal) but different by size *S. hecatei*
 - 2'. Trichospores different by morphology and size 3
- 3. Zygospores <100 μm in length [82–97 × [14–]16–18[–20] μm). The larger trichospores subcylindrical and 4–7 μm width. The smaller widely ellipsoidal to ovate-ellipsoidal and (12–)14–16.5 × 5.5–6 μm, with a collar of 12.5–18 μm *S. pseudodimorphum*
- 3'. Zygospores >150 μm in length ([160–]170–180[–190] × 15–18 μm). The larger trichospores subcylindrical and (7.5–)9–13 μm width. The smaller ovate-pyriform and 14–27 × 8.5–11 μm, with a collar of 8–9 μm long *S. heterosporum*
 - 4. Collar of trichospores <4 μm in length 5
 - 4'. Collar of trichospores >4 μm in length 12
- 5. Trichospores ≥5 μm in width 6
- 5'. Trichospores <5 μm in width 7
 - 6. Trichospores elongate-ovoid, measuring 18–27 × 5–5.5 μm, and a collar of 1–2 μm *S. culisetae*
 - 6'. Trichospores elongate-ellipsoidal, measuring 13–15.5 × 5–5.5 μm, and a collar of 3–3.5 μm *S. simulii*
- 7. Trichospores with a short or inconspicuous collar (≤1 μm in length) 8
- 7'. Trichospores with a conspicuous collar (>1 μm in length) 9
 - 8. Trichospores subcylindrical, measuring (18–)20–26(–29) × 2–3.5 μm, with a thin appendage. Holdfast cell horseshoe-shaped *S. gracilis*
 - 8'. Trichospores ellipsoidal, measuring (9–)12–14.5(–19) × 2–2.5 μm, with a thick and short appendage, longitudinally folded after the trichospore release. Holdfast cell simple *S. brevisporum*
- 9. Trichospores ovate-ellipsoidal to broadly ellipsoidal, measuring (9–)12–14.5(–18) × 3.5–5.5 μm, and a collar of 1.5–3 μm. Zygospores measuring 35–40 × 7–8 μm, with a globose collar of 3.5–4 × 3.5 μm *S. bulbosporophorus*
- 9'. Different characters 10
 - 10. Trichospores elongate-ellipsoidal, measuring 15–24 × 2.5–3.5 μm, and a collar of 3–3.5 × 1.5–2 μm. Basal cell slightly enlarged or with lateral projection *S. pusillum*
 - 10'. Different characters 11
- 11. Trichospores subcylindric to elongate-ellipsoidal, measuring 24–29 × 3–3.5(–4) μm, with a collar of 3–3.5 μm *S. typhellum*
- 11'. Trichospores subcylindric to elongate-ellipsoidal, measuring 15–20 × 2.5–3.5 μm, with a collar of 1.5–2 μm *S. dipterorum*
 - 12. Trichospores length:width ratio ≤5:1 13
 - 12'. Trichospores length:width ratio >5:1 15
- 13. Trichospores collar long, measuring 14–17 μm *S. alpinum*
- 13'. Trichospores collar shorter than 14 μm 14
 - 14. Zygospores measuring 47–55 × 11–12 μm. Trichospores measuring (17–)20–25.5 × 4–6 μm, and a collar of 4–7.5 μm *S. culicis*

- 14'. Zygosporos measuring $70-85 \times 18-19 \mu\text{m}$. Trichosporos measuring $(14-)25-28(-31) \times 7-7.5(-9.5) \mu\text{m}$, and a collar of $5.5-7.5 \mu\text{m}$ *S. fecundum*
15. Thalli horizontally attached on the internal gut cuticle, with holdfast material secreted along the contact area
..... *S. prostratum*
- 15'. Thalli erect, with a simple holdfast 16
16. Trichosporos measuring $23-27 \times 2-2.5 \mu\text{m}$, and a collar of $5.5-6 \mu\text{m}$ *S. inexpectans*
- 16'. Trichosporos measuring $(35-)40-47(-53) \times 3.5-5.5 \mu\text{m}$, and a collar of $(3-)4.5-5.5 \mu\text{m}$ *S. megazygosporum*

TABLE I. Comparison of dimorphic species of *Smittium* with sporic characters

Species	Trichospore shape	Trichospore size (μm)	Trichospore collar size (μm)	Zygosporos size (μm)
<i>S. dimorphum</i>	Long-ellipsoidal (α)	$38-50 \times 5.5-6.5$	$(2.5-)5$	$71-96 \times 13-14$
	Oval (β)	$10-12 \times 5.5-6.5$	$12-17$	
<i>S. pseudodimorphum</i>	Subcylindrical (α)	$(45-)50-55(-66) \times (4-)5.5(-7)$	$9-11.5 \times 3.5$	$82-97 \times (14-)16-18(-20)$
	Widely ellipsoidal (β)	$(12-)14-16.5 \times 5.5-6$	$12.5-18 \times 1.5-2$	
<i>S. esteparum</i>	Subcylindrical (α)	$31-18 \times 4.5-6.5$	$4-6$	50×10
	Ellipsoidal (β)	$12-14 \times 4-5$	2	
<i>S. heterosporum</i>	Long-ellipsoidal (α)	$(36-)50-62(-74) \times (7.5-)9-13$	$(10-)12.5-17(-20) \times 3-4(-5.5)$	$(160-)170-180 (-190) \times 15-18$
	Oval-ellipsoidal (β)	$14-27 \times 8.5-11$	$8-9 \times 1.5$	
<i>S. orthocladii</i>	Long-ellipsoidal (α)	$(25-)30(-36) \times (6-)7(-8)$	$5-10$	$(81-)87(-98) \times (9-)10(-11)$
	Oval (β)	$8-10 \times 5-6$	$5-10$	

DISCUSSION

This study has explored localities of central and southern Spain, while the northern and northeastern regions have been more intensively prospected. The objective of successive collections was to collect not only hosts infested with *Smittium* species but also other species of Trichomycetes. Nonetheless, we have focused our attention first on this particular genus due to its complexity, species richness and wide distribution. Successive surveys will be necessary to achieve a better knowledge of *Smittium* species that inhabit the Iberian Nematocera.

This study has revealed the presence of several dimorphic species, meaning species with two differentiated trichosporos in size and/or morphology. This sporic dimorphism might represent an ecological adaptation, where each of the forms would have some advantages under particular environmental conditions. In all the species where this sporic variation has been reported, the morphology of both trichospore types are comparable, always with a more or less cylindrical (or long-ellipsoidal) form that we have called α and the other, shorter and more oval-ellipsoidal that we have called β form. We should consider *S. hecatei* as an exception, not included in this pattern, because the treatment of its trichosporos in two differentiated types is more practical than natu-

ral, being dissimilar in size but with comparable shapes.

In TABLE I, *S. dimorphum*, *S. esteparum* and *S. orthocladii* are included with a summary of their spore features. It is notable that in all of these dimorphic species, zygosporos have been found. Among all of the 64 described species of *Smittium*, zygosporos have been reported only from 21 species (32.8%), 27% if we don't consider the dimorphic species. We have observed, in *S. heterosporum* and *S. pseudodimorphum*, that one of the trichospore types is dominant in some individuals and even one of them may not be present at all. This circumstance stimulates one to postulate the possible existence of other cryptic dimorphic species.

The use of SEM has been helpful in observing some ultrastructural features. The band-shaped appendage of *S. heterosporum* could be compared to those of some *Stachylina* species (*S. nana* Lichtw., Lichtwardt 1984), although it has not been reported previously from any *Smittium* species. The larger surfaces of this flat appendage might improve its adherent capacity to avoid being flushed away. The vesicular bodies attached to the appendage surface of *S. hecatei* also could be related to the adherent capacity of trichosporos.

The coat-like substance on both trichosporos and

trichospore collars of *S. culicis*, observed when using SEM, is another interesting feature. It appears to be some kind of amorphous material, of uncertain nature and function, probably related with phenomena of dispersal (adhesive substances) and/or host recognition (protein molecules functioning as lectins might be embedded in the coat). It is perceived as a secretion of the trichospore outer wall layer. The location of this coat is explained by the common origin of both collar and trichospores, the latter developing holoblastically from the collar, which is not continuous with the generative cell wall (Moss and Lichtwardt 1976). It is not the first time that *S. culicis* has been subjected to SEM observation; Moss and Lichtwardt (1976) made some ultrastructural studies on this species and *S. culisetae*, mainly using transmission electron microscope (TEM) as well as SEM. In the published SEM images, the trichospore surface is not easily observed (because of low magnification) since the focus of the photograph was the extruded sporangiospore (FIGS. 19–20, Page 2357 in Moss and Lichtwardt 1976).

The observation of these structures has provided us with both taxonomic and ecological information. Because the use of SEM is not always possible, the micromorphologic structures have not been used as important diagnostic characters in any of the described species and we have based the descriptions on light-microscope observable characteristics, such as trichospores, zygospores and thallial features. Among these, holdfast characteristics have been considered as an important element for the classification of *Smittium* species, although it is important to mention the possible morphological variations within one species. This variation has been reported and noted in the descriptions, when observed. Of course, sporic characters are essential in the identification of the species, with trichospores being the most relevant. Zygospores are of great help for a more consistent characterization.

Among the studied Spanish *Smittium* species, few have been cultured and are actually growing in our laboratory; these are *Smittium fecundum*, *S. hecatei*, *S. heterosporum*, *S. megazygosporum* and *S. simulii*. The thallial and spore production of axenically cultured *S. heterosporum* is the largest observed among the cultured *Smittium* spp., producing colonies that reach about 7 mm diam and 4 mm in depth. The Brain-Heart Infusion agar (BHIa) medium is specially recommended to attain a high trichospore production; the major fungal biomass proportion is due to spores, while hyphal mass is more reduced. The morphologic characters of cultured specimens can be slightly different from natural individuals.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to R.W. Lichtwardt, for helpful comments and suggestions on the manuscript, as well as for his assistance in providing samples of Trichomycetes fungi for our study; to M.J. Cafaro, for sending many cultures of *Smittium* to compare with our material; also to E. Beard for letting us observe his cultured *S. megazygosporum*; to the curatorial staff of FH (Farlow Herbarium, Harvard University, Cambridge, Massachusetts) for the loan of specimens; to F. Pando (MA Herbarium, Madrid, Spain) for arranging the loan of types from PC (Herbarium at Museum d'Histoire Naturelle, Paris, France); to the staff of the Botanic Department at the University of the Basque Country (UPVEHU, Leioa) and the Real Jardín Botánico at Madrid for their kind support and shelter for one of us (L.G. Valle) in their laboratories; to N. Prat (University of Barcelona, Department of Ecology) for providing bibliographic information as well as valuable suggestions relating to invertebrate aquatic fauna; to J. Fortes for translating and providing the Latin diagnoses; to the personnel of Servei de Microscòpia Electrònica at our university for their assistance in studies with scanning electronic microscopy. This research has been financed by MCYT and FEDER funds by means of project No. REN2002-04068-C02-02 (Flora Micològica Ibèrica V).

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ANNEX IV

(PÀGS. 329-337)

VALLE & SANTAMARIA. 2002b. *Tectimyces*, a new genus of Harpellales on mayfly nymphs (Leptophlebiidae) in Spain. Mycol. Res. 106:841-847.

Tectimyces, a new genus of *Harpellales* on mayfly nymphs (*Leptophlebiidae*) in Spain

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Received 2 March 2002; accepted 13 June 2002.

The genus *Tectimyces* gen. nov. (*Harpellales*: *Legeriomycetaceae*) is described with two species, *T. leptophlebiidarum* and *T. robustus* spp. nov., collected on the hindgut of the mayfly nymph *Habroleptoides confusa* (*Ephemeroptera*: *Leptophlebiidae*) in northern Spain. This is the second report of a trichomycete inhabiting a member of this family of ephemerids. Diagnostic for the new genus are type II zygospores and unappendaged trichospores, borne on long generative cells and carrying a very short collar after release. The position and morphological traits of the newly described taxa are discussed and compared with other genera and species, such as *Bojamyces repens* and *Orphella* spp.

INTRODUCTION

Two new species in a new genus of *Trichomycetes* (*Zygomycota*) are reported and described from Catalonia (Spain). These fungi have the characteristic features of the *Legeriomycetaceae* (*Harpellales*) which includes species with branched thalli producing trichospores (asexual spores) and zygospores (sexual spores), and typically develop on the hindgut lining of several kinds of arthropods, mainly aquatic insect larvae.

Tectimyces leptophlebiidarum was discovered first, and proved to be a rather common fungus, having a broad distribution in the northeastern Iberian Peninsula. Initial observations on this fungus showed such a unique thallial structure that it encouraged us to do an intensive survey of *Habroleptoides* nymphs throughout the Catalan region. Meanwhile, in prospecting amongst *Leptophlebiidae* hosts, a second species, *T. robustus*, was collected, so named because of its stout thallus. When compared, the two species seem to be too different to be included in the same genus, but some shared features support this choice: (1) the trichospores are unappendaged and bear an inconspicuous collar upon release; (2) the generative cells are long, appearing inflated below the trichospore before its release; (3) the trichospores follow the same pattern of development and differentiation; and (4) the hosts and ecology are identical.

Ephemeropteran nymphs of the family *Leptophlebiidae* are common in streams with pebble gravel, sand, and bank vegetation, mostly fallen leaves from the deciduous riparian forest. This organic debris is the basic nourishment of the nymphs and is consumed together with the accompanying saprophytic fungi which decompose it. *Habroleptoides confusa* is the most widespread species of this family in the area studied, and occurs in both calcareous and siliceous streams. It extends from the Pyrenees to the Mediterranean littoral and prelittoral mountain streams, and is also rather common around all the Mediterranean, central and eastern European countries (Putz 1978).

MATERIALS AND METHODS

Descriptions of the new taxa are based on material found in various localities of northeastern Spain. Collections of ephemeropteran nymphs were made by hand picking from the stream substrate, and also by dragging aquatic nets under rocks and sand in the edge of both wide rivers and little mountain streams, all of them preferentially in clean or low organically-polluted waters, from 50 to 2000 m altitude. The insect larvae were dissected to extract and clean the guts under a stereomicroscope and then the fungi were mounted in water on a slide and photomicrographed using phase contrast and interference contrast optics. Slides were fixed with lactophenol cotton blue and preserved in BCB herbarium, of the Universitat Autònoma de Barcelona.

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TAXONOMY

Tectimyces L. G. Valle & Santam., gen. nov.

Etym.: From Latin *Tectus* = hidden; and Greek *myces* = fungus. Based on the concealed condition of the thalli among abundant bacteria and gut debris.

Trichosporae sine appendicibus, inconspicuum collarem post liberatas ferentes. Genitales cellulae elongatae et distaliter latae factae. Zygosporae biconicae, zygosporophoro oblique et in submedio affixae. Zygosporae post liberatas cum laterali collare et una mucilaginosa appendicis simili structura. Ad cuticulam proctodaei nympharum Leptophlebiidarum affixi.

Typus generis: *Tectimyces leptophlebiidarum* L. G. Valle & Santam. 2002.

Trichosporae without appendages and bearing an inconspicuous collar upon release. *Generative cells* elongate and broadened distally. Biconical *zygosporae* of type II (Moss, Lichtwardt & Manier 1975), sub-medially and obliquely attached to the *zygosporophore*. Upon release the *zygosporae* bear a lateral collar and a single mucilaginous appendage-like structure. *Thalli* irregularly pinnate or umbellate. Attached to the hindgut cuticle of *Leptophlebiidae* nymphs.

Tectimyces leptophlebiidarum L. G. Valle & Santam., sp. nov. (Figs 1–25)

Etym.: nov. From Latin *leptophlebiidarum*, referring to the host family where the fungus is found.

Thallus pinnatus aut irregulariter ramosus, cum secundariis vel tertiariis ramis ex ambobus lateribus principalis axis ortis. Una aut plures laterales, conspicuae, rhizoidei similes ramae principali axe ortae. Cellulae basales latae et aliquando lobulatae, cum disciformi pede. Omnes fertiles ramae cum 1–2(–4) ovato-ellipsoidalibus et sine appendicibus trichosporis, 45–52 × 10.5–12.5 µm, cum late rotundato apice et complanata base. Genitales cellulae usque ad 110 µm in longitudinem. Zygosporae 41–47 × 6–8 µm metientes, cum laterali extrinsecus flammiformi collare 3–4 × 3–4 µm et 11–14(–80) µm in longitudinem appendicis simili flammiformi structura. Ad cuticulam proctodaei nympharum *Habroleptoidis confusae*, *Leptophlebiidae* (*Ephemeroptera*) affixi.

Typus: **Hispania orientalis**: *Barcelona*: Cantonigròs, Els Aiats, Torrent de La Rotllada, UTM 31T DG5156, alt. 920 m, ad *Habroleptoides confusa*, 26 Feb. 2001, L. G. Valle (BCB Tr0540 – holotypus; BCB Tr0538, BCB Tr0539, BCB Tr0541, BCB Tr0542 – isotypi).

Thalli pinnate (in young thalli) to irregularly branched (in old thalli), attached to the host gut cuticle by means of a broadened and sometimes lobulate basal cell, with a disk-like secreted holdfast. A variable number of lateral, rhizoid-like branches, arising from the main axis, probably functioning as 'subsidiary' holdfasts (Figs 3–7). Remaining branches projecting laterally from the main axis showing typically arcuate tips. Each fertile branch giving rise to 1–4 ovate-ellipsoidal unappendaged *trichosporae* of 45–52 × 10.5–12.5 µm, with a rounded apex and a flattened base (Figs 14, 17–19). *Trichosporae* developing at the tips of up to 110 µm long generative cells, or from a lateral outgrowth

(3.5–)8–15(–25) µm of intermediate *generative cells*. Free *trichosporae* bearing a very short collar, 1.5–2 µm, remainder of the upper part of the swollen generative cell (Fig. 14). Biconical *zygosporae* of type II, measuring 41–47 × 6–8 µm, arising up from the scalariform conjugation area, developing on long and thin specialized hyphae. Once released, *zygosporae* show a lateral outwardly flared collar 3–4 × 3–4 µm. A single mucilaginous flame-shaped appendage-like structure, with a total length of 11–14(–80) µm, was found in most of the detached *zygosporae* (Figs 21–25). *Habitat*: Found on the hindgut cuticle of *Habroleptoides confusa*.

Additional specimens: **Spain**: *Barcelona*: Rupit, Sant Julià de Cabrera, Font de Cabrera, UTM 31T DG5057, alt. 850 m, from *Habroleptoides confusa*, 19 Feb. 2001, L. G. Valle (BCB Tr0519 to BCB Tr0528); *Idem*, 26 Feb. 2001, L. G. Valle (BCB Tr0543 to BCB Tr0550); Rupit, Riera de Rupit, UTM 31T DG5552, alt. 890 m, from *H. confusa*, 26 Mar. 2001, L. G. Valle (BCB Tr0554 to BCB Tr0557); El Brull, La Castanya, Riera de La Castanya, UTM 31T DG4625, alt. 650 m, from *H. confusa*, 30 Oct. 2000, L. G. Valle (BCB Tr0387); Fogars de Monclús, Santa Fe del Montseny, Riera de Santa Fe, Font de Passavets, UTM 31T DG5425, alt. 1250 m, from *H. confusa*, 24 Oct. 2001, L. G. Valle (BCB Tr1105); Fogars de Monclús, Santa Fe del Montseny, Torrent de Can Ramis, UTM 31T DG5524, alt. 1100 m, from *H. confusa*, 23 Jan. 2002, L. G. Valle (BCB Tr1214 to BCB Tr1216); Figols, Peguera, Font del Coix, UTM 31T CG9968, alt. 1600 m, from *H. confusa*, 31 May 2001, L. G. Valle (BCB Tr0818, BCB Tr0819). *Girona*: Susqueda, Coll de Condreu, Riera de l'Om, UTM 31T DG5855, alt. 1020 m, from *H. confusa*, 1 Mar. 2001, L. G. Valle (BCB Tr0576); Osor, Riera d'Osor, Font Bunyola, UTM 31T DG5939, alt. 780 m, from *H. confusa*, 1 Mar. 2001, L. G. Valle (BCB Tr0577). *Lleida*: Bellver de Cerdanya, Riu de la Vall del Pi, UTM 31T DG9787, alt. 1300 m, from *H. confusa*, 9 Aug. 2000, L. G. Valle (BCB Tr0213); Farrera, Riu de la Vall de Burg i de Ferrera, UTM 31T CH1229, alt. 1229 m, from *H. confusa*, 22 June 2001, L. G. Valle (BCB Tr0895 to BCB Tr0897); Vielha, Conangles, Riu Noguera Ribagorçana, UTM 31T CH1621, alt. 1580 m, from *H. confusa*, 30 July 2001, L. G. Valle (BCB Tr0940); Les Bordes, Artiga de Lin, Barranc de la Betum, Riu Jòeu, UTM 31T CH1230, alt. 1160 m, from *H. confusa*, 31 July 2001, L. G. Valle (BCB-Tr0949). *Tarragona*: Capafonts, Riu Brugent, UTM 31T CF3573, alt. 700 m, from *H. confusa*, 11 Jan. 2001, L. G. Valle (BCB Tr0456); *Idem*, 30 Jan. 2001, L. G. Valle (BCB Tr0461); Farena, Riu Brugent, UTM 31T CF3975, alt. 600 m, from *H. confusa*, 9 Jan. 2001, L. G. Valle (BCB Tr0453, BCB Tr0455, BCB Tr0556). *Teruel*: Beseit, El Parrissal, Riu Matarranya, UTM 31T BF6320, alt. 650 m, from *H. confusa*, 24 Mar. 2001, L. G. Valle (BCB Tr0639).

Tectimyces robustus L. G. Valle & Santam., sp. nov. (Figs 26–36)

Etym.: From Latin, *robustus*, referring to the stout thallus habitus.

Thallus umbellatus, axes distaliter lati facti, usque ad quintum ordinem ramas ferentes. Ramae apicales angustatae et cum arcuatis apicibus. Cellulae basales latae, cum parvo disciformi pede, tantum distinguibili in juvenibus thallis. Omnes fertiles ramae cum 1–3 obovato-ellipsoidalibus et

sine appendicibus trichosporis, (33–)38–40(–44) × 9–11 µm. Genitales cellulæ usque ad 118 µm in longitudinem, abrupte contractæ infra apicem. Zygosporæ ignotæ. Ad cuticulam proctodæi nympharum *Habroleptoidis confusæ*, *Leptophlebiidae* (*Ephemeroptera*) affixi.

Typus: Hispania orientalis: Barcelona: El Brull, La Castanya, Riera de la Castanya, UTM 31T DG4625, alt. 650 m, ad *Habroleptoides confusa*, 7 Nov. 2001, L. G. Valle (BCB Tr1129 – holotypus; BCB Tr1126 to BCB Tr1128, BCB Tr1147 to BCB Tr1149, BCB Tr1051 – isotypi).

Thalli umbellate, with distally broadened axes, with 3–7 branches arising per node (Fig. 26), reaching up to a fifth order of ramification, attached to the host gut cuticle by a rounded basal cell with a disc-like holdfast (in young thalli), but later, when thalli mature, the holdfast is no longer observed and bidirectional growth occurs, giving rise to a napiform basal hypha, from which generative cells and trichospores can develop (Figs 26–28). Basal and medial cells broader than those of the apex, with a swollen zone at the apex, giving rise to multiple tapering branches with curled tips. Each fertile branch giving rise to 1–4 obovate-ellipsoidal unappendaged trichospores of (33–)38–40(–44) × 9–11 µm (Figs 30–35). Trichospores developing at the tips of generative cells that are abruptly constricted at the apex like a bottleneck (Figs 30–31). Free trichospores carrying a very short collar, 1.5–2 µm, consisting of the remainder of the upper part of the swollen generative cell (Figs 34–35). Terminal generative cells can reach up to 118 µm long. Intermediate generative cells very variable in size, with trichospores developing from lateral outgrowths of 15–90 µm. Zygosporæ unknown.

Habitat: Found on the hindgut cuticle of *Habroleptoides confusa*.

Additional specimens: Spain: Barcelona: Riera de Santa Fe del Montseny, Font de Passavets, UTM 31T DG5425, alt. 1250 m, from *Habroleptoides confusa*, 25 Oct. 2001, L. G. Valle (BCB Tr1083, BCB Tr1084, BCB Tr1090 to BCB Tr1094).

DISCUSSION

Tectimyces is the second harpellid genus described from the ephemeropteran family *Leptophlebiidae*, after *Bojamyces repens* collected on *Leptophlebia intermedia* (Longcore 1989). Moreover, another unnamed harpellid, similar to *Bojamyces* was found on a *Leptophlebiidae* (*Australonisia* sp.) from Tasmania (Lichtwardt & Williams 1992b). Trichospores of *Bojamyces* are interspersed with vegetative (non sporulating) cells in a long thallus, are unappendaged, have a collar of 3–5 × 3–5 µm after release, and no zygosporæ

were described (Longcore 1989). In contrast, *Tectimyces* trichospores grow from generative cells, which arise from fertile branches, have extremely short collars (Figs 14, 34–35), and zygosporæ of type II. Longcore (1989) also reported that cells of *B. repens* separate disarticulating from the thallus. This event is not observed in *Tectimyces*.

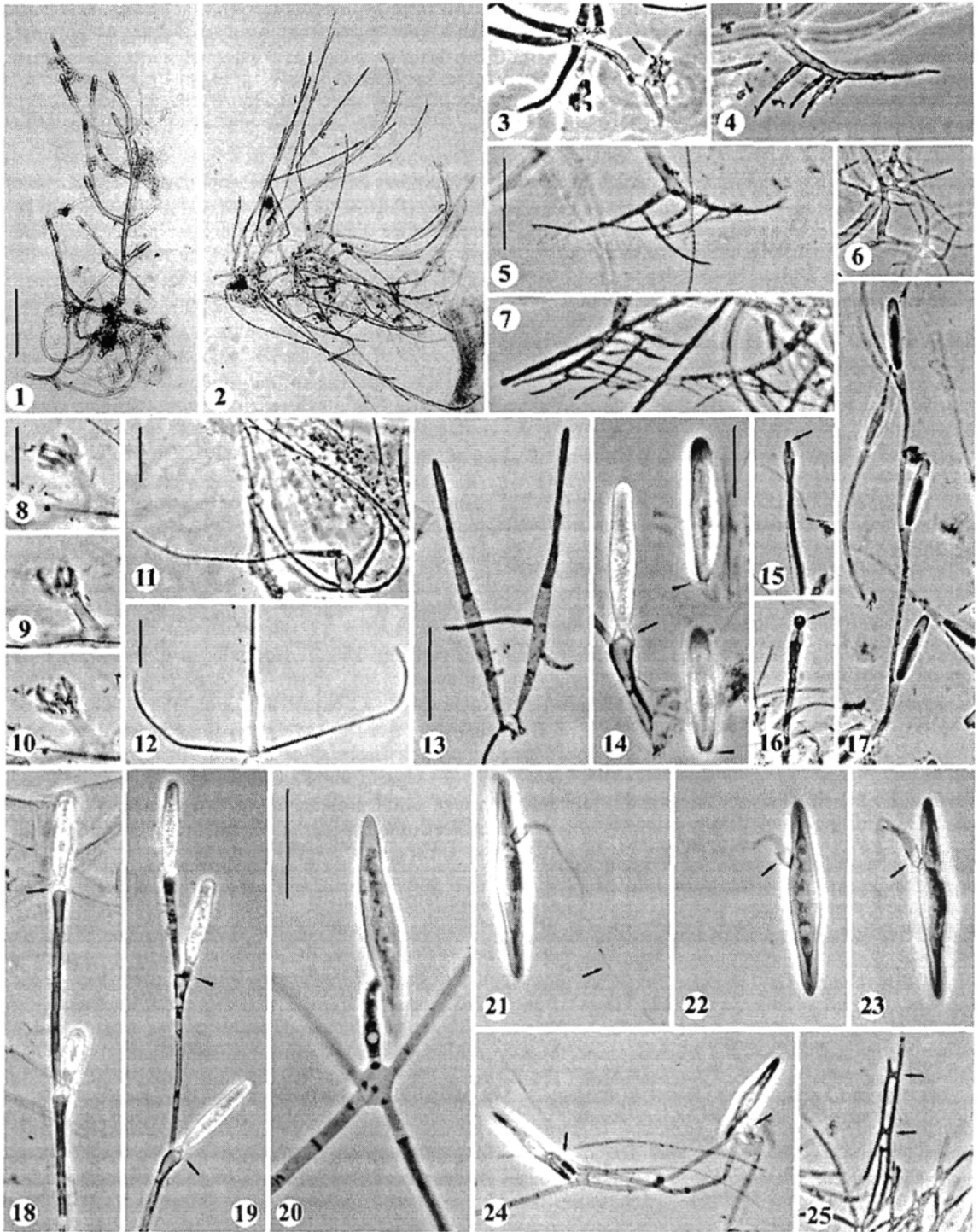
Zygosporæ have only been observed in *T. leptophlebiidarum* (Figs 20–24). These sexual spores seem to be formed after conjugation from specialized, long and very thin hyphae, following a scalariform pattern that results in the production of numerous zygosporæ, arising from the centre of the conjugation tube, at the tip of elongate zygosporophores (Figs 20, 24). The production of specialized hyphae for conjugation has been also reported in *Furculomyces* (Lichtwardt & Williams 1992a), where zygosporæ develop from conjugating branches that fuse at their tips in a furculum-like structure.

In *Tectimyces*, fertile hyphae produce series of swellings, which correspond to the apical wider apices of the future generative cells. Afterwards, septa form to delimit each generative cell. Terminal trichospores develop directly from the top of generative cells; intercalary trichospores grow at the tip of lateral outgrowths (Figs 17–19), which are very variable in size, reaching lengths (specially in *T. robustus*) only comparable to those of *Caudomyces japonicus* (Lichtwardt, Kobayasi & Indoh 1987). The fragile appearance of the narrow and long generative cells is remarkable, especially those of *T. leptophlebiidarum*.

Trichospores of *Tectimyces* lack appendages, although a differentiated appendage-associated structure has been noted inside the generative cell, just underneath the trichospore (Figs 14, 30–31). These appendage structures are comparable to those of other species of *Harpellales*, where a refractive material is displayed alongside the wall of the generative cell apex. Just before the trichospore release the refractive appearance of this material disappears (Fig. 19, arrowhead; Figs 32–33). As in *Zygopolaris*, these structures will not develop into an appendage (Moss *et al.* 1975, Moss & Lichtwardt 1976, Lichtwardt & Williams 1984). The reason for the late degradation observed in mature trichospores of *Tectimyces* has not yet been clarified. However, some material of the appendage structure frequently appears attached to the very recently released trichospores (Figs 32–33). This phenomenon was also reported on *Z. ephemeridarum* (Moss *et al.* 1975). The absence of appendages could be related to the ecology of the host, which prefers hush zones of flowing streams where plant debris tends to accumulate. In this

Key to species of *Tectimyces*

- 1 Thalli umbellate, generative cells abruptly constricted at the apex, like a bottleneck. Trichospores (33–)38–40(–44) × 9–11 µm. *robustus*
- Thalli pinnate to irregularly branched, generative cells uniformly inflated. Trichospores 45–52 × 10.5–12.5 µm. *leptophlebiidarum*



Figs 1–25. *Tectimyces leptophlebitidarum* (Figs 1, 4, 18, BCB-Tr1130; Fig. 2, BCB-Tr0895; Fig. 3, BCB-Tr0526; Fig. 5, BCB-Tr0461; Figs 6, 19, BCB-Tr1159; Figs 7, 25, BCB-Tr0819; Figs 8–10, BCB-Tr0546; Fig. 11, BCB-Tr0897; Fig. 12, BCB-Tr0902; Fig. 13, BCB-Tr0387; Fig. 14, 20–24, BCB-Tr0525; Figs 15–16, BCB-Tr1141; Fig. 17, BCB-Tr0455; Fig. 19, BCB-Tr1159). **Figs 1–2.** Overall view of two branching thalli. **Fig. 3.** Holdfast and rhizoid-like branch (arrow). **Figs 4–7.** Different models of lateral rhizoid-like branches. **Figs 8–10.** Lobulate basal cell at three different focusing levels to demonstrate its three-dimensional structure. **Figs 11–13.** Young thalli in different stages of development, showing progressive cell elongation and branch production. **Fig. 14.** At left, trichospore attached to its generative cell showing refractive contents (arrow); at right, two released trichospores showing the inconspicuous collars (arrowheads). **Figs 15–16.** Trichospore development at the tip of fertile branches (arrows). **Figs 17–19.** Fertile branches bearing trichospores.

environment, trichospores could remain secure from the drift effect of fast waters. Hence, no appendages need be present to guarantee stability, necessary to allow spore ingestion by another host. The absence of appendages in other species, such as *Bojamyces repens*, a host with similar ecology, support this possibility, as mentioned by Longcore (1989).

Young thalli show a pattern of development similar to those of other genera of *Legeriomycetaceae*. Initially, a hypha grows upwards from the newly attached sporangiospore (Figs 29, 36). Later, new hyphae develop laterally from the base of the sporangiospore, close to the hindgut cuticle. At this stage, a typical structure with several young branches radiating from the initial sporangiospore is seen (Figs 11–13). This pattern was observed in both species of *Tectimyces*, with slight differences observable in later development. The presence in *T. robustus* of a mucilaginous skirt covering the young germinating thalli is remarkable (Fig. 36).

The final umbellately branched structure in thalli of *T. robustus* (Figs 26–28) could be compared to that of *Orphella* species, which are also apically ramified, with multiple branches arising from a main axis. Species of *Orphella* have a main axis consisting of numerous cells that can be laterally branched, notably *O. avalonensis* (Lichtwardt, White & Colbo 2001), *O. catalaunica* (Santamaria & Girbal 1998), *O. coronata* (Léger & Gauthier 1931), *O. haysii* (Williams & Lichtwardt 1987), and *O. hiemalis* (Lichtwardt, Peterson & Huss 1991). However, in *T. robustus*, merely one or two cells, shorter and wider than those of *Orphella* species, will give rise to all the umbellately branched structure.

Affinities within both species in *Tectimyces* are evident when trichospores are observed, albeit generative cells and thallial structure allow a clear specific distinction between them. In *T. leptophlebiidarum*, the generative cell apex (collar region) appears uniformly inflated (Fig. 18), whereas it is constricted or bottleneck-shaped in *T. robustus* (Figs 30–33). Once released, only the distal part of the generative cell is carried with the trichospore as a short collar, nearly identical in both species (Figs 34–35). The appendage-associated structure inside generative cells is longer and thinner in *T. robustus* (Fig. 31), while is shorter and broader, as well as closely crowded to the apex in *T. leptophlebiidarum* (Fig. 19).

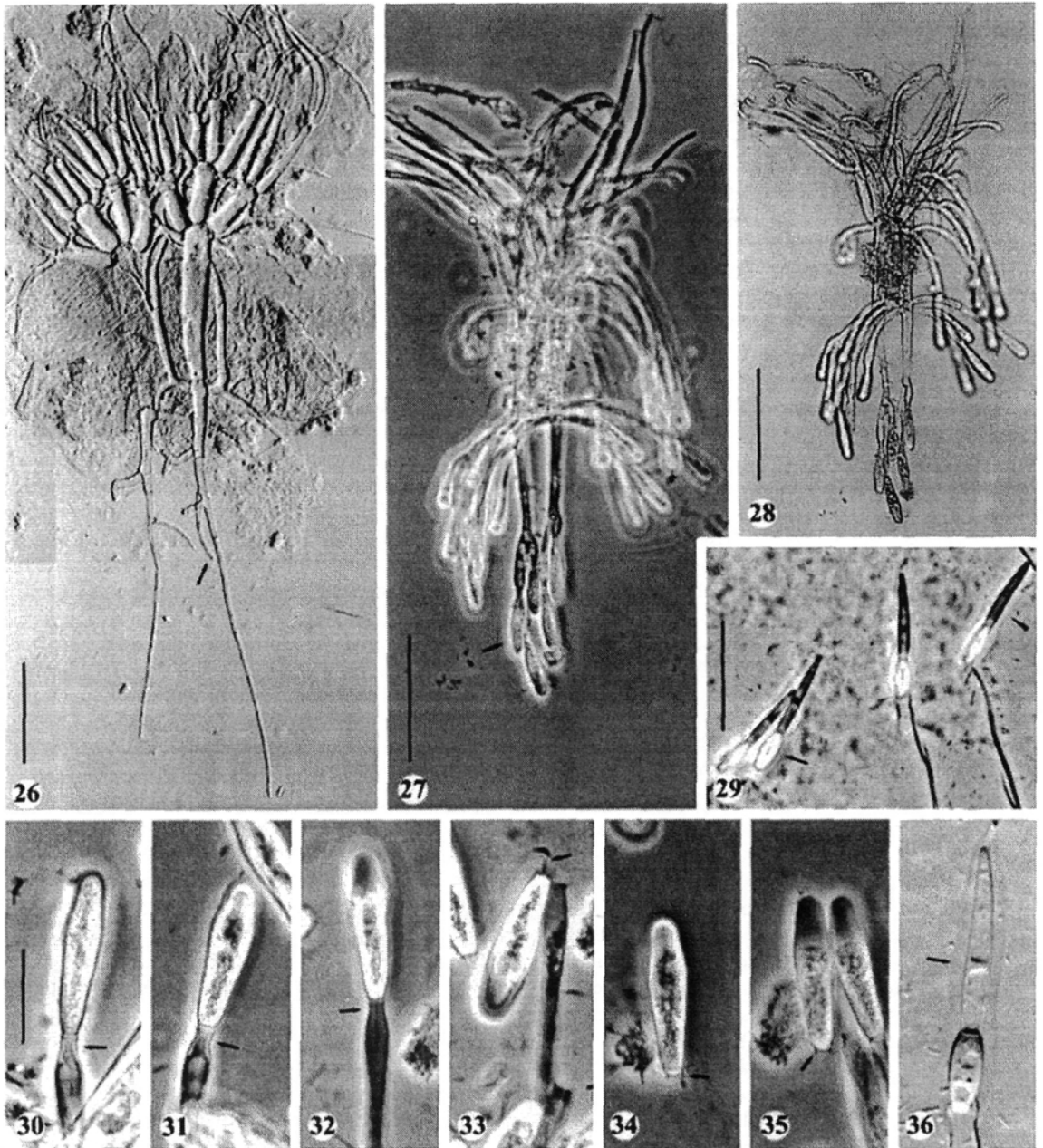
Other characters used to segregate the two species are the presence of rhizoid-like lateral branches in *T.*

leptophlebiidarum (Figs 3–7), not seen in *T. robustus*. These branches are unique amongst *Harpellales*. A main broad or sometimes lobulate basal cell is found only in young thalli of *T. leptophlebiidarum* (Fig. 3). Mature individuals grow so profusely that the presence of a single basal holdfast is probably insufficient to forcefully anchor the fungus to the gut, and therefore, the presence of these lateral branches might improve this role. Most of these rhizoidal branches grow in an evident unilateral ramification pattern (Fig. 4), but those thalli from the single collection from Font Cabrera (Figs 3, 5–6) show dichotomous branching. In the same population, a lobulate basal cell form is also predominant (Figs 8–10). These are assumed to be intraspecific variations.

Mature thalli of *T. robustus* do not show a clearly distinguished holdfast (Fig. 26), and often they appear freely 'floating' when the gut is dissected. As a hypothesis, the long, tapering and commonly coiled terminal branches could help retain this fungus inside the gut lumen. Moreover, an additional unusual phenomenon has been observed in this species: a late, bi-directional growth of the thallus seems to occur, since a napiform hypha develops from the original basal cell towards the opposite direction where the terminal vegetative branches grow. Fertile branches arise laterally and even apically from this napiform hypha (Fig. 26, arrow). Other trichospore-producing branches grow from the upper zone of the axial cells, just below the swelled zone from which distal vegetative branches develop (Figs 27, 28, out of focus), and these trichospore-bearing branches appear to be always orientated towards the base of the thallus. Consequently, although shown in the conventional way, with the base at the bottom, the photographed mature thalli seem to be rotated 180° (Figs 27–28).

The percentage of infected individuals varied in relation to the area studied. In some streams few individuals were infected (approx. 5%), while in others the ratio reached nearly 80%. Amongst the infected mayflies few had both mature trichospores and zygospores. As in other genera of harpellids, when the thalli reproduce sexually, trichospores are not formed, or at least appear in a very low proportion. Within the infected nymph's guts, just one to three thalli of *T. leptophlebiidarum* were observed per individual. More thalli of *T. robustus* can be found inside the gut, and it was easy to find very immature thalli developing from recently attached spores.

Note the extremely thin generative cells in Figs 17 and 19. In Figs 18 and 19, the arrows indicate the refractive contents at the top of generative cells, which disappear just before trichospore release (Fig. 19, arrowhead). Fig. 20. Zygospore arising from a cross-like conjugation area. Figs 21–23. Released zygospores showing the collar (Figs 22–23, arrows) and the mucilaginous appendage-like structure (Fig. 21, arrow). Fig. 24. Zygospores with zygosporephores (arrows) arising from the middle of conjugation bridges. Fig. 25. Scalariform conjugations (arrows). Figs 6, 18–19 from water-mounted slides; remaining Figs from lactophenol cotton-blue mounted slides. Figs 1–2, light transmission optics; Figs 3–25, phase contrast optics. Bar Fig 1 = 100 µm (Figs 1–2); Fig. 5 = 50 µm (Figs 3–7); Fig. 8 = 25 µm (Figs 8–10); Figs 11–12 = 10 µm; Fig. 13 = 50 µm (Figs 13–19, 24–25); Fig. 20 = 25 µm (Figs 20–23).



Figs 26–36. *Tectimyces robustus* (Fig. 26, BCB-Tr1147; Figs 27–28, 30, BCB-Tr1128; Figs 29, 36, BCB-Tr1126; Figs 31–39, BCB-Tr1129). **Fig. 26.** Overall view of two superposed branching thalli. Note the growing branches from which trichospores will develop (arrow). **Figs 27–28.** Two superposed sporulating thalli. Note the trichospore arrangement (arrow) and the backward orientation of trichospore-bearing branches. **Fig. 29.** Sporangiospores (arrow) germinating after its attachment to the hindgut lining, developing a very initial growing thallus (arrowhead). **Figs 30–31.** Trichospore and generative cell. Note the bottleneck-like shape of the generative cell apex and the inner refracting material. **Figs 32–33.** Two successive stages on trichospore release. In Fig. 32, the refractive material disappears just before the trichospore is released (Fig. 33), carrying a tail of cytoplasmic contents (arrow). **Figs 34–35.** Free trichospores showing the extremely short collar (arrows). **Fig. 36.** Extruded sporangiospore showing a mucilaginous skirt (arrow) surrounding all the young structure. All from water-mounted slides. Figs 27 and 29–35, phase contrast optics; Figs 26 and 36, interference contrast optics; Fig. 28, light transmission optics. Bar Figs 26–28 = 100 µm; Fig. 29 = 50 µm; Fig. 30 = 25 µm (Figs 30–36).

A high proportion of the examined specimens of nymphs were also infected with high densities of filamentous bacteria growing on the hindgut and midgut

lining. These prokaryotic organisms grew on and between the *Tectimyces* thalli making their detection and observation difficult. *T. leptophlebiidarum* is very

common but *T. robustus* is more geographically restricted, and the proportion of infested larvae is lower. Some molts were collected and examined without any trace of these fungi.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Robert W. Lichtwardt for critically reading the manuscript and providing helpful suggestions, and to J. Fortes for translating the Latin diagnoses. This work was supported by the DGES project no. PB98-0538-C04-04 (Flora Micológica Ibérica IV).

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ANNEX V

ORDENACIÓ SISTEMÀTICA DELS GÈNERES D'HOSTES QUE APAREIXEN EN LA MEMÒRIA:

HEXAPODA

COLLEMBOLA

Agrenia
Isotoma
Isotomurus

DIPTERA

Chironomidae

Chironominae
Chironomus
Tanytarsus

Tanypodinae

Psectrotanypus

Orthoclaadiinae

Cricotopus
Eukiefferiella
Orthocladus
Psectrocladius
Psectrotanypus

Diamesiinae

Diamesa
Potthastia
Sympotthastia

Culicidae

Aedes
Anopheles
Culex
Culiseta

Simuliidae

Simulium

Thaumaleidae

Thaumalea

EPHEMEROPTERA

Baetidae

Baetis
Cloeon

Caenidae

Caenis

Ephemerellidae

Torleya

Ephemeridae

Ephemera

Heptageniidae

Ecdyonurus
Epeorus

Leptophlebiidae

Habroleptoides
Habrophlebia
Leptophlebia

Siphonuridae

Siphonurus

PLECOPTERA

Capnidae

Capnia
Allocapnia

Nemuridae

Amphinemura
Nemoura
Protonemura
Zapada

Leuctridae

Leuctra

CRUSTACEA

DECAPODA

Atyaephyra
Procambarus

AMPHIPODA

Gammarus
Echinogammarus

ISOPODA

Aselliidae

Asellus
Proasellus

Oniscidae

Ligia
Trichoniscus
Armadillo
Porcellio

MYRIAPODA

DIPLOPODA

Iulidae

Leptiulus

Glomeridae

Glomeris
Loboglomeris

ANNEX VI

LLISTA DELS NOMS DELS HOSTES QUE APAREIXEN EN LA MEMÒRIA:

- Aedes* Meigen, 1818
Agrenia Börner, 1906
Allocaenia granulata Claassen, 1924
Amphinemura Ris, 1902
Anopheles Meigen, 1818
Armadillo Dumeril, 1816
Armadillo vulgaris Latreille, 1804
Asellus aquaticus Linnaeus, 1758
Asellus meridionalis Racovitza, 1919
Atyaephyra desmaresti Millet, 1831
Baetis Leach, 1815
Baetis alpinus Pictet, 1843
Baetis meridionalis Ikinomov, 1954
Baetis nigrescens Navas, 1931
Baetis rhodani Pictet, 1843
Baetis tricaudatus Dodds, 1923
Baetis vardarensis Ikinomov, 1962
Caenis luctuosa Burmeister, 1839
Capnia Pictet, 1841
Capnia bifrons Pictet, 1841
Chironomus plumosus Linnaeus, 1758
Cloeon Leach, 1815
Cloeon inscriptum Bengtsson, 1914
Cricotopus bicinctus Meigen, 1818
Culex Linnaeus, 1758
Culiseta impatiens Walker, 1848
Diamesa Meigen, 1835
Ecdyonurus forcipula Pictet, 1843
Echinogammarus Stebbing, 1899
Echinogammarus berilloni Catta, 1878
Echinogammarus longisetosus Pinkster, 1973
Echinogammarus tarragonensis Pinkster, 1973
Epeorus Eaton, 1881
Ephemera Linnaeus, 1758
Ephemera danica Müller, 1764
Ephemera vulgata Linnaeus, 1746
Eukiefferiella Thienemann, 1926
Gammarus Fabricius, 1775
Gammarus lacustris Sars, 1863
Gammarus pulex Linnaeus, 1758
Glomeris marginata Villers, 1789
Habroleptoides confusa Sartori & Jacob, 1986
Habrophlebia fusca Curtis, 1832
Isotomurus palustris Marlier, 1942
Leptiulus Verhoeff, 1894
Leptophlebia intermedia Traver, 1932
Leuctra Stephens, 1835
Leuctra aurita Navas, 1919
Leuctra ferruginea Walker, 1981
Leuctra fusca Linnaeus, 1758
Ligia exotica Roux, 1828
Ligia italica Fabricius, 1798
Ligia oceanica Linnaeus, 1767
Loboglomeris pyrenaica Latzel, 1886
Nemoura Laterille, 1796
Nemoura cinerea Retzius, 1738
Nemoura erratica Claassen, 1936
Nemoura humeralis Pictet, 1936
Nemoura linguata Navas, 1783
Nemoura uncinata Despax, 1934
Orthocladus Wulp, 1874
Porcellio Letreille, 1804
Porcellio laevis Latreille, 1804
Potthastia Kieffer, 1922
Proasellus coxalis Dollfus, 1892
Procambarus clarkii Girard, 1852
Prosimulium Roubaud, 1906
Protonemura Kempny, 1898
Protonemura meyeri Pictet, 1842
Protonemura pyrenaica Mosely, 1930
Psectrocladius Kieffer, 1906
Psectrotanypus varius Fabricius, 1787
Simulium Latreille, 1802
Simulium ornatum Meigen, 1818
Simulium tuberosum Lundstrat, 1911
Siphonurus lacustris Eaton, 1870
Sympotthastia Pagast, 1947
Tanytarsus Wulp, 1874
Thaumalea Ruthe, 1831
Torleya Lestage, 1917
Torleya major Klapálek, 1905
Trichoniscus pusillus Brandt, 1833
Trichoniscus roseus Koch, 1838
Zapada haysii Ricker, 1951

ANNEX VII

FITXA DE SEGUIMENT DE LA RECOL·LECCIÓ I INFECCIÓ D'HOSTES:

FULL DE MOSTREIG					
Riu/Riera/Torrent					
Entre fullaraca/ Sota pedres/Altres					
Localitat (poble, municipi)					
Província					
Data de recol·lecció					
Tipus de sòl: Calcari <input type="checkbox"/> Silfíc <input type="checkbox"/>					
Altres caràcters del sòl:					
Vegetació de ribera: Sí <input type="checkbox"/> No <input type="checkbox"/>					
Comentaris: (Estat general)					
Amplada aprox. del llit del riu:.....m.					
Profunditat aprox. <input type="checkbox"/> 20 cm <input type="checkbox"/> 20-50 cm <input type="checkbox"/> 50cm.....					
Cabdal constant <input type="checkbox"/> S'asseca durant època seca <input type="checkbox"/>					
Ambient Lèntic <input type="checkbox"/> Ambient Lòtic <input type="checkbox"/>					
Observacions:					
Mostra d'artròpodes recollida:					
	Família	Abundància	Nº Oberts	Nº Infectats	%infectats
Efemeròpters	Baetidae				
	Caenidae				
	Ephemerellidae				
	Ephemeridae				
	Heptageniidae				
	Leptophlebiidae				
	Potamanthidae				
Plecòpters	Leuctridae				
	Nemouridae				
	Capnidae				
	Perlidae				
Tricòpters					
Dípters	Ceratopogonidae				
	Chironomidae				

	Culicidae				
	Dixidae				
	Simuliidae				
	Thaumaleidae				
Coleòpters					
Col·lèmbols					
Isòpodes terrestres	Armadillidae				
	Armadillidiidae				
	Oniscidae				
	Porcellionidae				
Isòpodes aquatics.	Asellidae				
Amfipodes	Gammaridae				
Miriàpodes Diplòpodes	Glomeridae				
	Iulidae				
	Polydesmidae				

Comentaris:

ANNEX VIII

FITXA DE DESCRIPCIÓ DE LES HARPEL·LALS:

ESPÈCIE:.....FAMÍLIA:.....HARPELLALES													
Hoste:.....Midgut <input type="checkbox"/> Hindgut <input type="checkbox"/>													
Localitat:.....													
.....Data:.....Leg:.....													
Nº Preparació(ns) [BCB-Tr]:.....Hoste guardat? <input type="checkbox"/>													
Fotografies:						Dibuixos:							
DESCRIPCIÓ TAL·LUS:													
Mides	Longitud màxima (holdfast/extrem)												
	Amplada mitja hifa												
Tipus ramificació: No ramificada <input type="checkbox"/> Un sol eix des de la base <input type="checkbox"/> Més d'un eix des de la base <input type="checkbox"/>													
Pinnada <input type="checkbox"/> Verticil·lada <input type="checkbox"/> Altres:.....													
Tipus Holdfast:													
Mucil·lag? <input type="checkbox"/> Base verrucosa <input type="checkbox"/> Terminal <input type="checkbox"/> /Lateral <input type="checkbox"/> Penetra en membr. peritr.? <input type="checkbox"/>													
Altres observ.:.....													
DESCRIPCIÓ TRICÒSPORES:													
Mides	L	L	L	L	L	L	L	L	L	L	L	L	
	A	A	A	A	A	A	A	A	A	A	A	A	
Lliures?:													
Eix: Recte <input type="checkbox"/> Corb <input type="checkbox"/> Helicoidal <input type="checkbox"/>													
Forma contorn: Cilíndric <input type="checkbox"/> Ovoide <input type="checkbox"/> Elipsoidal <input type="checkbox"/> → Eixemplament Inf. <input type="checkbox"/> Mig <input type="checkbox"/> Sup. <input type="checkbox"/>													
Obovoid <input type="checkbox"/> Altres:.....													
Presència de collaret <input type="checkbox"/> → Marges convergents <input type="checkbox"/> Marges divergents <input type="checkbox"/>													
Longitud collaret													
Apèndixs.- Nombre: Forma → Filiformes <input type="checkbox"/> Petal·loides <input type="checkbox"/> Base eixamplada <input type="checkbox"/>													
Disposició un cop lliures → Embolicats <input type="checkbox"/> Helicoidals <input type="checkbox"/> Estesos <input type="checkbox"/>													
Disposició dins c. generativa → Helicoidals <input type="checkbox"/> Paral·lels a paret <input type="checkbox"/>													
Longitud relativa:													
Cèls. generatives.-													
Nº Tricòspores/Branca:													
DESCRIPCIÓ ZIGÒSPORES:													
Tipus:				Lliures?:									
Type I	Type II	Type III	Type IV	Mides	L	L	L	L	L	L	L	L	
					D	D	D	D	D	D	D	D	
Apèndixs:													
Observacions Zigòspores:													
OBSERVACIONS													

ANNEX IX

FITXA DE DESCRIPCIÓ DE LES ASELLARIALS:

ESPÈCIE:.....											FAMÍLIA:.....											ASELLARIALES.																	
Hoste:.....																																							
Localitat:.....																																							
Altitud aprox.:..... Data:../../..... Leg.....																																							
Nº de preparació(ns) [BCB-tr]:																						Hoste guardat? <input type="checkbox"/>																	
Fotografies:											Dibuixos:																												
DESCRIPCIÓ TALLUS:																																							
Mides		Longitud màxima																																					
		Amplada mitja hifa																																					
Tipus de ramificació: Un sol eix principal <input type="checkbox"/> Més d'un eix principal <input type="checkbox"/> Verticil.lada <input type="checkbox"/>																																							
Altres:.....																																							
Tipus de Holdfast:																																							
DESCRIPCIÓ ARTRÒSPORES:																																							
Mides		L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Lliures? Tot el tal·lus es fragmenta en artròspores?											n°Nuclis:.....																												
Forma Contorn:																																							
Observacions:																																							

ANNEX X

FITXA DE DESCRIPCIÓ DE LES ECCRINALS:

ESPÈCIE:..... FAMÍLIA..... ECCRINALES													
Hoste:.....Foregut <input type="checkbox"/> Midgut <input type="checkbox"/> hindgut <input type="checkbox"/>													
Localitat.....													
.....													
..... Data:...../...../..... Leg:.....													
.....													
NºPreparació(ns)[BCB-Tr]:.....Hoste guardat? <input type="checkbox"/>													
Fotografies:						Dibuixos:							
DESCRIPCIÓ TAL.LUS:													
Mides	Long. Màx.	L	L	L	L	L	L	L	L	L	L	L	
	Ampada mitja hifa	A	A	A	A	A	A	A	A	A	A	A	
Tipus Holfast:													
Simple <input type="checkbox"/> .- Múltiple <input type="checkbox"/> / Basal <input type="checkbox"/> - Lateral <input type="checkbox"/> / Penetra membrana peritr?: Sí <input type="checkbox"/> - No <input type="checkbox"/> / o <input type="checkbox"/>													
Granular <input type="checkbox"/> - Fibrós <input type="checkbox"/> - Reticulat <input type="checkbox"/> - Mucilaginós <input type="checkbox"/> .- Presència rizoides: Sí <input type="checkbox"/> - No <input type="checkbox"/>													
Altres observacions:													
Ramificacions a la base? <input type="checkbox"/> Tipus de ramificació:													
Observacions tal.lus:													
DESCRIPCIÓ ESPORANGIÓSPORES													
Mides Espores 1ª	L	L	L	L	L	L	L	L	L	L	L	L	
	A	A	A	A	A	A	A	A	A	A	A	A	
Nº Apendixs: 0 <input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> en un pol <input type="checkbox"/> - en dos pols <input type="checkbox"/>													
Nº Nuclis per esporangi: Uninucleat <input type="checkbox"/> / Binucleat <input type="checkbox"/> / Tetrannucleat <input type="checkbox"/> Forma de les esp 1ª: Oval <input type="checkbox"/> El.lipsoidal <input type="checkbox"/>													
Altres:.....													
Observacions espores 1ª:.....													
Mides Espores 2ª	L	L	L	L	L	L	L	L	L	L	L	L	
	A	A	A	A	A	A	A	A	A	A	A	A	
Nº Nuclis per esporangi: 1 <input type="checkbox"/> (<i>Enterobryus</i>)-2 <input type="checkbox"/> - 4 <input type="checkbox"/> - 8 <input type="checkbox"/>													
Sortida d'espores per la part proximal <input type="checkbox"/> / distal <input type="checkbox"/> de l'esporangi- Presència de " mother-cell" terminal? Sí <input type="checkbox"/> No <input type="checkbox"/>													
Forma de les espores 2ª:													
Altres observacions (Presència de cèls. Diferenciades, no reproduct.):.....													
.....													
.....													
.....													
OBSERVACIONS:													

ANNEX XI

FITXA DE SEGUIMENT DE LES PREPARACIONS:

tr. 0376 *Oryphella catalaunica* + rara

s/Leuctridae

bc = basal cell gc = generative cell
 sgc = supporting generative cell
 tc = terminal cell.

tr. 0376

Riera de Gualba. Parc RACC
 Montseny. 30-X-00

veure prep. 0383.
 0346.

p(99, 11)

tr. 0377 *Oryphella catalaunica*

s/Leuctridae
 Plecopter

Riera Gualba.
 Montseny, Parc RACC
 30-X-00

Desenvolupament diferent
 comparat amb prep.
 anterior. 0376

veure prep. 0383

Rev. correcte.

ANNEX XII (PÀGS. 353-356)

Pòster:

WHITE, M. M., LICHTWARDT, R. W, VALLE, L. G., & STRONGMAN, D. 2003. *Orphella*: an unusual fungus associated with stoenflies.

Presentat a:

The Mycological Society of America (MSA) Anual Meeting, Carmel, California.

Orphella: an unusual gut fungus associated with stoneflies



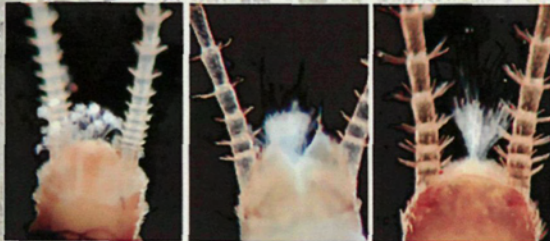
*MERLIN M. WHITE,¹ ROBERT W. LICHTWARDT,¹ LAIA GUARDIA VALLE,² and DOUGLAS STRONGMAN.³

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What are gut fungi?

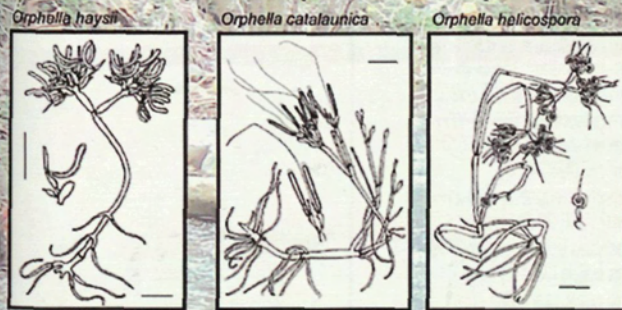
The gut fungi (Trichomycetes) are obligate symbionts of various Arthropoda, worldwide. The traditional, morphologically-based, classification system has included three fungal orders (Eccrinales, Asellariales and Harpellales). Sequence data are providing insights into the natural associations of the members of this class, which will necessitate an eventual reclassification. Members of the Harpellales are unique in the possession of asexual trichospores and conical zygospores (where the sexual process has been observed). Within the Harpellales *Orphella* is among the most unusual of all the genera, in several respects.

Where do you find *Orphella*?



All species of *Orphella* are obligate symbionts of stoneflies (Plecoptera). *Orphella* is one of only three genera of Harpellales that extend beyond the anus of their larval aquatic insect hosts, at maturity.

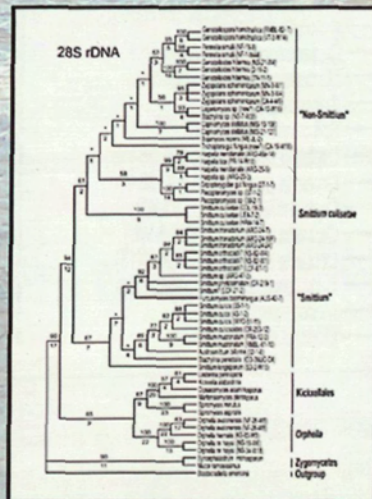
Species of *Orphella* reported from Spain



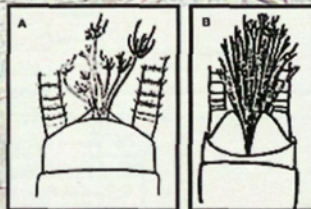
The asexual spores of *Orphella* are released as dissemination units. The dissemination units mature distally on the thallus, as clusters that may be seen as sporulating heads extending beyond the anus of their stonefly (Plecoptera) host. Zygospores have never been reported for *Orphella*. Nonetheless, on a morphological basis, this genus has been recognized as being an unusual harpellid (scale bars approx. 50 micrometers).

Phylogenetic significance of *Orphella*

Based on rDNA sequence data obtained to date, *Orphella* falls outside an otherwise monophyletic clade of Harpellales, more closely allied with the Kickxellales (similarly with 18S rDNA). *Orphella* is an unusual genus that was thought to be highly derived, but consistently appears basal in trees so far generated. This is despite the fact that *Orphella* is unquestionably a natural member of the Harpellales, based on morphological features and habit. Our current efforts have been focused on collecting more samples of *Orphella* (in North America and Europe) and putative closely related taxa to expand the molecular data set used to infer the phylogeny of the group. This tree is a strict consensus of 2 equally most parsimonious trees (1415 steps, CI = 0.391, RI = 0.277) inferred from the 28S rDNA. Numbers above branches indicate bootstrap values (above 50% from 500 replicates; asterisks (*) indicate nodes that collapse in the bootstrap tree) while numbers below are decay indices (from White, 2002).

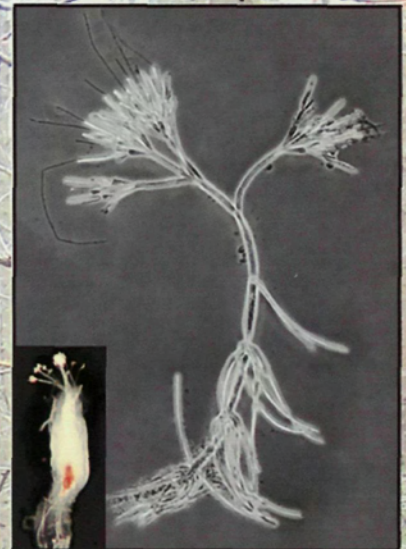


AKA: "anal gills"



Orphella species may be so consistently found among populations of stoneflies that previously they have been used as "delicate" taxonomic characters, called "anal gills" to describe immature stages. A. Fig. 284 of *Leuctra claasseni* from Frison (1935). B. Fig. 8a of *Nemoura asakawae* from Kuhno (1941).

Dissection of Specimens



Intact mature thallus of *Orphella catalaunica* from Norway (composite of several images taken 40X Mag). Inset is entire stonefly hindgut removed from the host; note the food bolus, often pronounced when gut fungi are present. After removal, the gut epithelium is teased opened to reveal the fungal thalli.

Biogeographic patterns and molecular approaches

All *Orphella* species had been reported from three families: Capniidae, Leuctridae, Nemouridae, but we add the Taeniopterygidae. These are all Arctopteran Plecoptera, restricted to the Northern Hemisphere, as are their symbiotic fungi. With one exception, species of stonefly gut fungi in North America (6 genera, 10 species) differ from species in Europe (3 genera, 6 species); Stewart and Stark (1988) suggested that stoneflies speciated following breakage of the last land bridges between the continents (Lower Eocene). We are testing the hypothesis, using rDNA sequence data, that the gut fungi and their hosts have speciated vicariantly and that the fungi may have co-speciated with their hosts.

Table 1: Distribution of species of *Orphella*.

SPECIES	HOST	NORTH AMERICA	EUROPE
<i>Orphella hiemalis</i>	Capniidae	USA (AR, KS, OK)	
<i>Orphella haysii</i> *	Nemouridae	USA (CO)	SPAIN
<i>Orphella avalonensis</i>	Leuctridae	CANADA (NF, NS)	
<i>Orphella nr. haysii</i>	Capniidae	CANADA (NS)	SPAIN
<i>Orphella catalaunica</i>	Leuctridae		SPAIN, NORWAY
<i>Orphella helicospora</i>	Leuctridae		SPAIN, NORWAY
<i>Orphella coronata</i>	Nemouridae		FRANCE, NORWAY

**Orphella haysii* exhibits considerable morphological variation, and perhaps is a species complex. We are currently focused on collecting sequence data to compare it and other species of *Orphella* in N.A. and Europe.

Acknowledgments

Our recent and ongoing studies on gut fungi at KU have been funded the National Science Foundation (DEB-9521811 and DEB-0108110). The DEEB at KU has provided MMW with financial support to help attend meetings and conduct research projects and surveys. Dr. Mike Grose has helped secure the sequence data that has been generated at KU's core facilities (BRSL and NHM DNA laboratories). Dr. Sergi Santamaria, Universitat Autònoma de Barcelona, is gratefully acknowledged for his major contributions to the Harpellales in Europe and for the mentorship and support of LGV's studies. DBS received NSERC support for research and internal travel funds from SMU. Dr. Murray Colbo, Memorial University, has helped collect gut fungi including a *O. avalonensis*, which he also helped describe, from Newfoundland. Dr. Hiroki Sato directed us to the anal gill report from Japan.

→ (Ampliació del cladograma)

28S rDNA

