

Bojamyces transfuga sp. nov. and new records of Trichomycetes from mayfly larvae in Spain

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Abstract: The new species *Bojamyces transfuga* is described. It was found growing in the hindgut of mayfly nymphs and in their shed abdominal exoskeleton lumen. *Bojamyces transfuga* shows single appendaged trichospores and zygospores of type I, unlike *Bojamyces repens*, where appendages and zygospores are unknown. The generic description of *Bojamyces* is emended to accommodate this new taxon. *Bojamyces repens*, known only from the United States, has been found for the first time in the Iberian Peninsula on mayfly molts. We also note the presence of *Legeriomyces rarus* in Europe, on the Iberian Peninsula and Balearic Islands. Previously it was known only in Western Australia.

Key words: Ephemeroptera, exuvia, gut fungi, Legeriomycetaceae, scanning electron microscopy, species distribution, taxonomy

INTRODUCTION

The species we describe are included in the order Harpellales, with species having branched or unbranched thalli separated into two families, respectively the Legeriomycetaceae and the Harpellaceae. Legeriomycetaceae develop within the hindgut lining of their larval hosts, mainly flies (Diptera), stoneflies (Plecoptera), mayflies (Ephemeroptera) (Lichtwardt et al 2001) and very rarely, beetles (Coleoptera) (Lichtwardt et al 1999), caddis worms (Trichoptera) and isopods (White 1999). Harpellaceae grow within the peritrophic matrix of Diptera larvae (Lichtwardt et al 2001).

Trichospores provide weighty characteristics used for the classification of genera and species, including their size and shape, the number of appendages and their arrangement on fertile branches. Likewise, zygospores, when found, are very important for estab-

lishing the generic limits. Their relative position on zygosporophore is the basis to designate the zygosporophore type (Moss et al 1975). The shape of the holdfast and the ramification pattern of the thalli are other interesting features to consider in classification (Lichtwardt et al 2001).

Longcore (1989) described the genus *Bojamyces* with the single species *B. repens* from nymphs and molts of *Leptophlebia intermedia* Traver (Ephemeroptera, Leptophlebiidae) in the United States. Lichtwardt and Williams (1992) reported immature thalli from Australasian mayflies that resemble *B. repens*. No further occurrence of the taxon has been reported since.

We have found the new species of *Bojamyces* growing and infecting *Caenis luctuosa* Burmeister (Ephemeroptera, Caenidae) molts and larvae. Both trichospores and zygospores were found, allowing us to study and consider diagnostic characters that are different enough to describe a new taxon. The new records of *Bojamyces repens* have been found in molts of *Habroleptoides confusa* Sartori & Jacobs (Ephemeroptera, Leptophlebiidae) with the typical characters of the species but without zygospores.

Longcore (1989) provided a generic diagnosis based on the single species, *B. repens*, but we believe that the new species must be included also in the genus *Bojamyces* by morphological and ecological traits. The generic description should be emended to account for characters discovered in the new species.

Legeriomyces rarus Lichtw. was known only by its original description from Western Australia in an endemic genus of Caenidae, *Tasmanocoenis* (Williams and Lichtwardt 1993). In Spain, this species was found together with the new *Bojamyces* species.

MATERIALS AND METHODS

The description of the new species is based on material found in localities from Northeast Spain and Balearic Islands. Collections of ephemeropteran nymphs were made by hand picking from the stream substrate with entomological forceps and by dragging aquatic nets over rocks and sand in streams. Nymph's molts were collected between leaves and organic debris on the river shores and transported in a small cooler to the laboratory. Insect nymphs were dissected to extract and clean the guts under a stereomicroscope using fine-pointed forceps and entomological

Accepted for publication April 13, 2004.

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needles. Molts containing the ectodermic hindgut remains also were examined. Fungi were placed on a slide with water as mounting medium and photomicrographed using phase contrast and interference contrast optics (DIC). Slides were fixed and stained with lactophenol cotton blue to be preserved in the herbarium (BCB-Mycotheca, at the institutional address of the authors).

Some Caenidae individuals, collected from the locality where the infestation percentage was greatest (Marganell), were placed in an aquarium until molts were obtained. Some of the living larvae were dissected just before molting.

For SEM, *Bojamyces* material together with the hindgut molt was fixed with 2.5% glutaraldehyde in cacodylate buffer 2M (pH 7). The thalli were washed with the same cacodylate solution, and dehydrated in an ethanol series (15 min in each 10, 20, 40, 60, 80%) to absolute ethanol before the thalli were critical point dried and gold metallized. Samples were examined in a Hitachi S-570.

TAXONOMY

Bojamyces Longcore, *emend.* L.G. Valle & Santam.

Thallus with sparse and indeterminate branching, attached to the host hindgut cuticle. Generative cells scattered on the thallus, interspersed with vegetative cells. Trichospores elongate-ellipsoidal, with or without a single appendage and with a flared collar. Zygosporangia perpendicularly and medially attached to the zygosporophore (type I [Moss et al 1975]), with a collar. In ephemeropteran nymphs.

Typus generis: *Bojamyces repens* Longcore.

Bojamyces transfuga L.G. Valle & Santam., sp. nov.

FIGS. 1–15

Trichosporae (24–)30(–36) × 5–6 μm, collare 1.5–2.5 × 2–2.5 μm et appendicem, conspicuam in cellula genitali, ferentes. Trichosporae post liberatas appendicem confertim plicatam praebentes. Zygosporae (27–)42(–50) × 8–9 μm, basaliter inflato zygosporophoro ad perpendiculum et in medio affixae. Zygosporae post liberatas cum plane campaniformi collari 7–7.5 μm longitudine et filiformi appendice.

Trichospores (24–)30(–36) × 5–6 μm, with a 1.5–2.5 μm long and 2–2.5 μm broad collar and a single appendage (FIG. 6), visible inside the generative cell before the trichospore release (FIG. 1, arrows). After trichospore release, the appendage appears compactly folded (FIG. 7, arrow). Trichospores arise from generative cells without a definite direction. Zygosporangia (27–)42(–50) × 8–9 μm, perpendicularly and medially attached to the zygosporophore, which is inflated at the lower part (FIGS. 10, 11, arrows). Once released, a 7–7.5 μm long flared collar and a single filiform appendage can be observed. Later, typical scalariform conjugation bridges develop (FIGS. 2, arrows, and 3, 5), and zygosporangia grow from the prox-

imities of these bridges, from one of the conjugants (FIGS. 2, and 5, arrows).

Etymology. *L., transfuga* = transgressor, deserter; referring to its habitat, outside the living larval host.

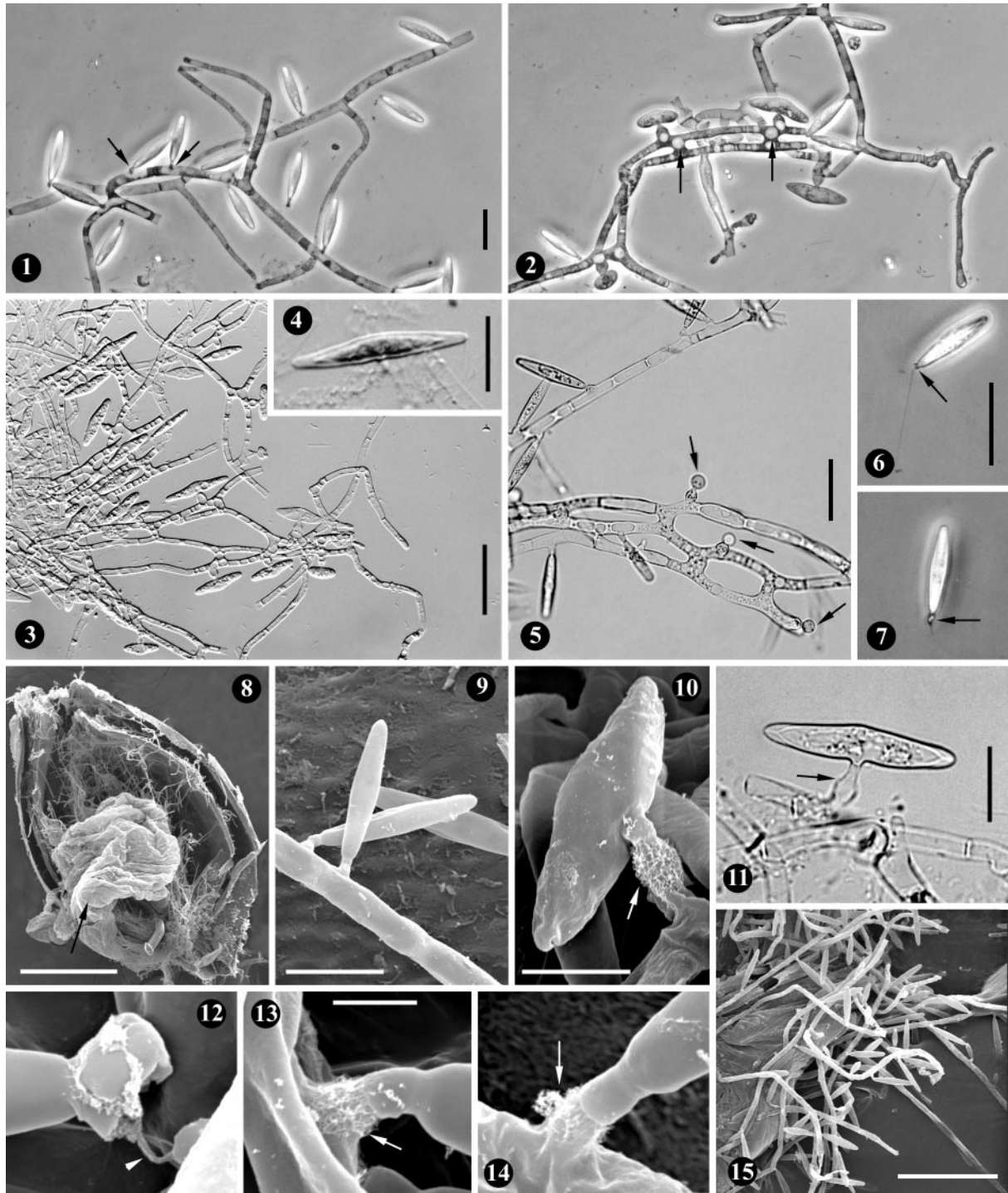
Specimens examined. SPAIN. BARCELONA: Marganell, Riera de Marganell, prepared from *Caenis luctuosa* larvae and molts, 7-V-2001, L. G. Valle, BCB-Tr0729 (HOLOTYPE), BCB-Tr0707 to BCB-Tr0750 (ISOTYPES); same locality, 6-VI-2001, BCB-Tr0830, BCB-Tr0831; Cerdanyola del Vallès, Riera de St. Iscle, prepared from *Caenis luctuosa* larvae and molts, 7-VI-2001, L. G. Valle, BCB-Tr0832–0834.

The genus *Bojamyces* is well-defined by its thallial morphology, development and ecology. Characteristics of appendages in trichospores, important for delimitation of many genera in Harpellales, is considered here as a secondary trait, useful for specific separation purposes. Accordingly, the new species has been placed in this genus because the thallial features, such as the scattered branching, the extremely long hypha which are irregularly septated in generative cells and the disarranged trichospores, are coincident with those described for *Bojamyces repens*, the type species of the genus. Nonetheless, the new species reveals the presence of characteristics not included in the original diagnosis of the genus (Longcore 1989), as trichospores bearing one appendage and the occurrence of zygosporangia, as well as a different ecology in a new host family.

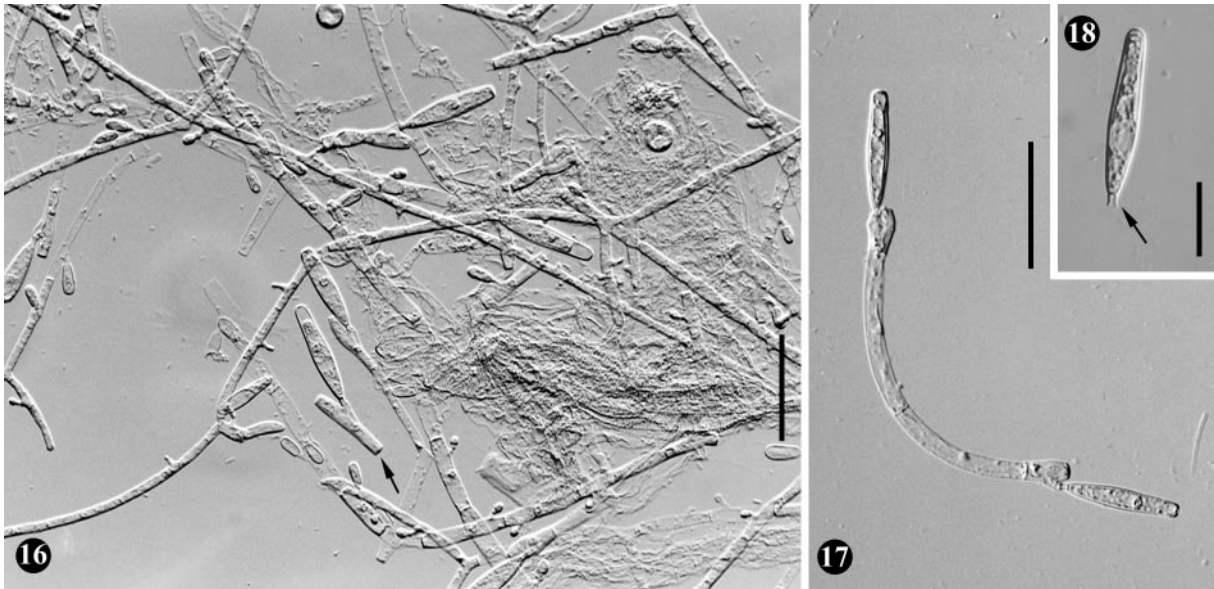
It is not unusual within Harpellales that different species of the same genus live in different insect genera, even belonging to unrelated insect families. Moreover, Longcore (1989) reported the presence of a *Bojamyces*-like fungus in *Siphonurus* sp. (Ephemeroptera, Siphonuridae). Several genera of Harpellales display, at least, narrow generic host specificity, but some are not so specific, like those found mostly in Dipteran larvae such as *Smittium*, *Stachylina* and *Simuliomyces*, which also include species in Plecopteran nymphs (Lichtwardt et al 2001). Species of *Zygopolaris* grow also in different Ephemeropteran families (Lichtwardt and Williams 1984, Moss et al 1975). Therefore, it is not so unusual that a species of *Bojamyces* could be present in Caenidae rather than Leptophlebiidae or Siphonuridae.

Trichospores of *B. transfuga* are smaller and narrower than those of *B. repens*, and show an appendage (FIGS. 6, 7) that can be seen even inside the generative cell (FIG. 1, arrow) and when just released, it is folded compactly (FIG. 7, arrow). It is not easy to observe the appendage completely unfolded after trichospore release.

The use of SEM has revealed the presence of a net-like skirt of an unknown substance covering the lower part of the zygosporangia (FIG. 10, arrow), and also located just at the lower margins of the tricho-



FIGS. 1–15. *Bojamyces transfuga*. 1. Fertile branches with intercalary trichospores showing the appendage sac inside the generative cell (arrows) and free trichospores [BCB-Tr1832]. 2–3. Conjugating thalli with bridges (arrows in FIG. 2), zygospores (immature in FIG. 2, immature and mature in FIG. 3) and few trichospores [BCB-Tr1832]. 4, 11. Mature zygospores of type I and zygosporophore (arrow in FIG. 11) [BCB-Tr0744]. 5. Thalli with young zygospores (arrows) and trichospores [BCB-Tr0745]. 6–7. Free trichospores with a diminutive collar (arrow in FIG. 6), an unfolded appendage in FIG. 6, partially folded in FIG. 7 (arrow) [BCB-Tr1832]. 8. Exuvia showing the abdominal exoskeleton lumen with hyphal filaments expanding throughout the internal area, and the hindgut molt in the center (arrow). 9. Trichospores showing a smooth surface. 10. Zygospore and zygosporophore with a net-like skirt covering it (arrow). 12. Basal segment of a released trichospore with the broken collar and the appendage (arrowhead). 13–14. Basal segment of a trichospore, with the collar carrying the net-like skirt (arrow), just before being released. 15. Hyphal filaments from the outside of the hindgut molt with intercalary



FIGS. 16–18. *Bojamyces repens* [BCB-Tr0770]. 16. Overview of a thallus with intercalary trichospores and a disarticulated segment with a trichospore (arrow). 17. A disarticulated segment with two trichospores. 18. Released trichospore with the collar (arrow) and no appendage. All FIGS. photographed from lactophenol cotton-blue mounted slides using DIC. Scale bars: FIGS. 16, 17 = 25 μm ; FIG. 18 = 10 μm .

spore collars (FIGS. 13, 14, arrows). This structure may be related with the dispersion and adhesion of the spores until being consumed by the hosts. The surface of the spores does not show any ornamentation (FIGS. 9, 10). A similar structure, covering both trichospore surfaces and collars, was observed, also with SEM, in *Smittium culicis* Manier (Valle and Santamaria, 2004, FIGS. 62, 63, in press). SEM images have shown the possible three-dimensional view of *B. transfuga* inside the host, revealing its expansion outside the intestinal lumen to occupy the abdominal cavity of the molt (FIG. 8).

Bojamyces transfuga was found in high densities in Caenidae molt hindgut lumens and in lesser proportion inside the nymph's hindguts, where thalli were mostly immature. Trichospores and zygospores also were found exclusively in molts. We could get thalli of *B. transfuga* from the Caenid larvae molts stored in the aquarium. Nearly 100% of these molts were infected with the gut fungi.

Bojamyces repens Longcore FIGS. 16–18

This species has been found in the hindgut molts of *Habroleptoides confusa* Sartori & Jacob, from a

small calcareous mountain stream. The material examined matches the original description based on fungi collected in the American *Leptoblebia intermedia* Traver (Longcore 1989), with unappendaged trichospores measuring $(43\text{--})47\text{--}(51) \times 7\text{--}8 \mu\text{m}$, and with a collar of $2\text{--}3.5 \times 2\text{--}3 \mu\text{m}$ (FIG. 18, arrow). The thallus disarticulates in small portions containing each 2–4 generative cells with their respective trichospores (FIGS. 16, 17). No zygospores were found.

Specimens examined. SPAIN. TARRAGONA: Ports de Be-seit, Torrent del Mas de la Franqueta, prepared from *Habroleptoides confusa* (Leptophlebiidae) larvae molts, 24-V-2001, L.G. Valle, BCB-Tr0770–0771.

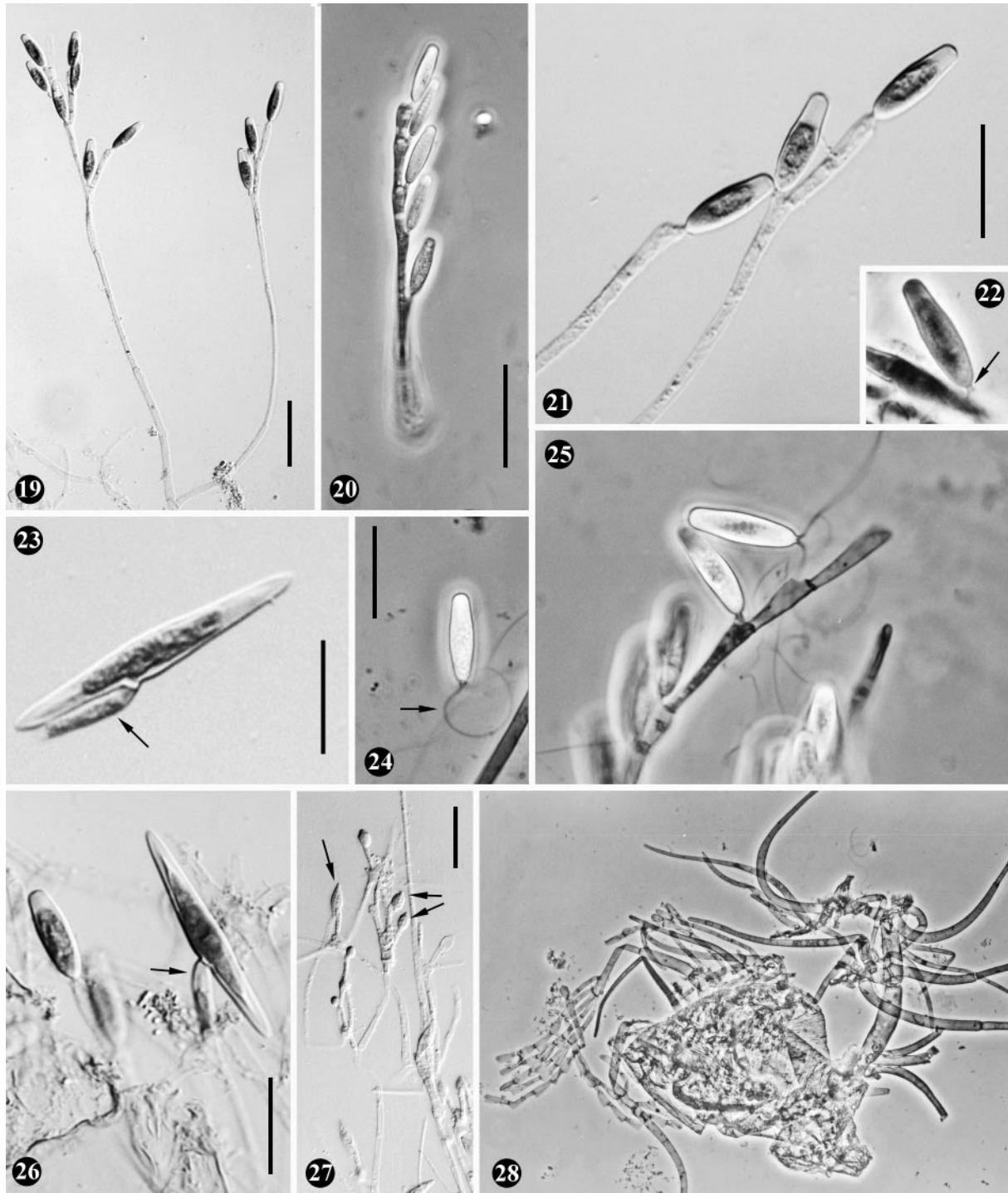
Legeriomyces rarus Lichtw. & M.C. Williams

FIGS. 19–28

Species found in the hindgut lining of *Caenis luc-tuosa* larvae, as well as in hindgut molts, where the thalli do not grow, but show some capacity to persist a period of time before they degrade completely, with only the trichospores remaining. The basal area of the mature thallus is a complex of rhizoid-like hypha, often pluricellular (2–3 cells) and occasionally ramified (FIG. 28). These basal branches vary in number.

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trichospores. All FIGS. photographed from water mounted slides, except FIG. 4 from lactophenol cotton-blue mounted slide, and SEM images. FIGS. 3–4 by DIC, FIGS. 1–2, 6–7 by phase contrast, FIGS. 5, 11 by bright field. FIGS. 8–10, 12–15 are SEM. Scale bars: FIGS. 1 (use for FIGS. 1 and 2), 4–5, 6 (use for FIGS. 6 and 7) and 11 = 25 μm ; FIG. 3 = 50 μm ; FIG. 8 = 0.3 mm; FIG. 9 = 14 μm ; FIG. 10 = 9 μm ; FIG. 13 = 3 μm (use for FIGS. 12, 14).



FIGS. 19–28. *Legeriomyces varus*. FIGS. 19–21. Fertile terminal branches with trichospores [BCB-Tr1813]. 22. Released trichospore with the short-lived collar (arrow) [BCB-Tr1813]. 23. Released zygospore with collar (arrow) [BCB-Tr1813]. 24. Released trichospore with two fine appendages (arrow) [BCB-Tr1303]. 25. Fertile branch with trichospores attached to generative cells, and a released trichospore with two appendages [BCB-Tr1303]. 26. Zygospore with the zygosporophore (arrow), and a trichospore in focus [BCB-Tr1813]. 27. Young developing zygospores (arrows) [BCB-Tr1809]. 28. Overview of an immature thallus [BCB-Tr1303]. FIGS. 19, 21–23, 26, 27 photographed from lactophenol cotton-blue mounted slides. FIGS. 20, 24, 25, 28, photographed from water mounted slides. FIGS. 19, 21, 23, 26–27 by DIC. FIGS. 20, 24–25 by phase contrast. FIGS. 22, 28 by bright field. Scale bars: FIGS. 19–20, 27 (use for FIGS. 27, 28) = 50 μ m; FIG. 21 (use for FIGS. 21, 22, 25), 23, 24 and 26 = 25 μ m.

In young, and more rarely in mature thalli, a discoid holdfast is observed. The Iberian individuals of this species are identical to those described from Australia (Williams and Lichtwardt 1993). The trichospores of our samples measure $21.5\text{--}30 \times 7.5\text{--}9 \mu\text{m}$, without a definite collar, although some deciduous remains ($1.5\text{--}2 \mu\text{m}$ long) from the generative cells can be observed as a short-lived collar in some trichospores (FIG. 22, arrow). We also have observed the typical pair of appendages in free trichospores (FIGS. 24, arrow, and 25). We have not noticed the presence of sexual processes nor zygospores in the peninsular individuals, but zygospores measuring $40\text{--}53(-62) \times 7\text{--}9 \mu\text{m}$ and zygosporophore of $14.5\text{--}19 \times 3\text{--}5 \mu\text{m}$ (FIGS. 23, 26, arrows) have been reported from nearly all the examined Majorcan specimens. The conjugation tube is a broad structure between the two conjugants and the zygosporophore arises from the center of these conjugations, which can be present in a variable number between the same two conjugant hyphae. Released zygospores show a collar as long as the respective zygosporophore (FIGS. 23, 26, arrows).

Specimens examined. SPAIN. BARCELONA: Marganell, Riera de Marganell, prepared from *Caenis luctuosa* larvae, 10-IX-2001, L.G. Valle, BCB-Tr1178; 13-I-2002, L.G. Valle, BCB-Tr1201; 6-III-2002, L.G. Valle, BCB-Tr1303–1306, Tr1321, Tr1323. BALEARIC ISLANDS (MALLORCA): Sóller, Barranc de Biniraix (Cases de L'Ofre), prepared from *Caenis luctuosa* larvae, 27-V-03, L.G. Valle, BCB-Tr1807–1810; Valldemossa, Torrent de Valldemossa, prepared from *Caenis luctuosa* larvae, 30-V-03, L. Sáez & L.G. Valle, BCB-Tr1813–1816.

DISCUSSION

After Longcore (1989) described the genus *Bojamyces* with *B. repens*, only Lichtwardt and Williams (1992) reported immature thalli from Tasmanian leptophlebid nymphs, resembling *B. repens* by the absence of appendages in trichospores, but remained unnamed because of unsuitable material to provide an appropriate description. However, the authors mentioned the absence of thallial disarticulation in their specimens, which coincides with observations of *B. transfuga* in Spanish samples. To answer the posed question by Lichtwardt and Williams (1992) about the generic or specific condition of the thallial disarticulation character, we consider it a specific attribute, as was considered already by Longcore (1989).

Infested Caenidae molts show long hyphal filaments of *B. transfuga* expanding within the shed exoskeleton (FIG. 8). Longcore (1989) also remarked on the prevalence of *B. repens* in molts, but did not report its expansion toward the outside limits of the internal gut lining. The ecological significance of this

phenomenon is not well understood, but development of the thalli and spores within the molt might improve fungal dispersion. It has been observed that other species of trichomycetes easily disintegrate (at least hyphal filaments, whereas zygospores and trichospores can persist) when present in insect exuviae, as happens with *Legeriomyces rarus*. It does not seem possible for these trichomycetes to grow outside the larval body. In the case of *Bojamyces*, trichospores germinate inside the larval gut lumen, but sporulation and hyphal mass is greater in exuviae, where the thallus extrudes from the hindgut molt and extends throughout the lumen of the exoskeleton abdomen. The studied thalli from the hindgut of living hosts have never been observed to penetrate and trespass the inner gut membrane. Moreover, within larval hindguts, no mature thalli with zygospores have been noticed. After these observations, the only convincing explanation for the presence of great amounts of hyphal filaments with spores outside the hindgut lining and between both the old outer cuticle and hindgut of almost all observed host exuviae, is by means of a fungal growth after molting. The free extreme of the hindgut molt, opened toward the external digestive environment, might allow the gut fungi to expand its hyphal filaments and produce large amounts of trichospores (FIG. 8). If this growth capacity outside the living host may be by means of proper energetical reserves or by a major nutritional and ecological plasticity is not clear. Unfortunately, we have no support for this hypothesis of a saprophytic growth and we have not been able to axenically culture this fungi, at least with brain-heart infusion agar (following culture methods of Lichtwardt et al 2001).

It will be interesting to determine if there are some behavioral patterns in host feeding which match the dispersal ecology of *B. transfuga*. It has been observed that during the molting stages of *Caenis*, large quantities of exuviae accumulate at the river shore, among leaves, aquatic herbs or other debris. Because the species was found in a fast-flowing zone, the presence of trichospores in molts represents a means for their increasing propagation in a quiet sheltered place.

No less surprising than the discovery of a new species, is the observation of another species that only has been reported from a very different and distant geographic territory and within a different host. This is the case of *L. rarus*, described from a western Australian river, inhabiting larvae of the endemic genus *Tasmanocoenis* (Caenidae) (Williams and Lichtwardt 1993). The absence of any new record of this species from other countries is remarkable, especially in Europe, where Caenidae are distributed widely. In our opinion, the presence of *L. rarus* in Iberian Caenidae

may be interpreted to be a consequence of its cosmopolitan distribution, although not yet reported from other unexplored countries. Another possible explanation for the presence of this species in such disjunct territories could be related to the effect of the climatic domain, since the southwestern Australian region, where the *Tasmanocoenis* hosts were collected, has a Mediterranean climate. The effect of the climate on trichomycete distribution is not well studied, since the principal ecological restriction is logically, the presence of the host, which is most affected by climatic conditions. In any case, this hypothesis only could explain the actual distribution and implies that in the past *L. rarus* occupied a wider area, being restricted to these territories, since the Mediterranean climates were established relatively late in geologic history, after the continents reached their current position and after the main groups of animals and plants (mammals, birds, reptiles, insects and angiosperms) had evolved (Stebbins 1989). In this case, these areas play the role of climatic refugia and the species would be relict. With the purpose of clarifying which of the hypothesis is true, we must look for this species in other countries.

The example of *L. rarus*, with its broad distribution and host range, adequately supports the hypothesis of an ancient trichomycete-arthropod coevolutionary relationship, in which the symbiosis was established within an ancestral host and persisted throughout its speciation (Cafaro 2000, White et al 2001).

Another surprising observation, is the restricted distribution of *L. rarus* throughout the examined Iberian territory, having been collected in one particular peninsular locality (with an infection ratio of 18% among a sample of 205 individuals), and in two insular (Balearic) streams. As mentioned above, species of *Caenis* are not uncommon in the Mediterranean area and they have been collected from several streams where *Bojamyces transfuga* has been collected occasionally, accompanied by *L. rarus* only once. In any case, although possibly distributed worldwide, *L. rarus* is not common. The Balearic Caenidae were infested in higher proportion (with an infection ratio of 65% among a sample of only 19 individuals collected), probably because of the more Mediterranean stationary character of the watercourses, with periods of low flow that could improve the spore dispersion within the caenid host population. There are no morphological differences between the two disjunct populations in the Spanish territories.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to RW Lichtwardt, for helpful comments and suggestions to the manuscript, to J. Fortes for translating the Latin diagnosis. To E. Descals and colleagues ("Institut Mediterrani d'Estudis Avançats", CSIC-UIB, Majorca) for their kind welcome during a research sojourn in their laboratory and to the personal 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, project No. REN2002-04068-C02-02 ("Flora Micológica Ibérica V").

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