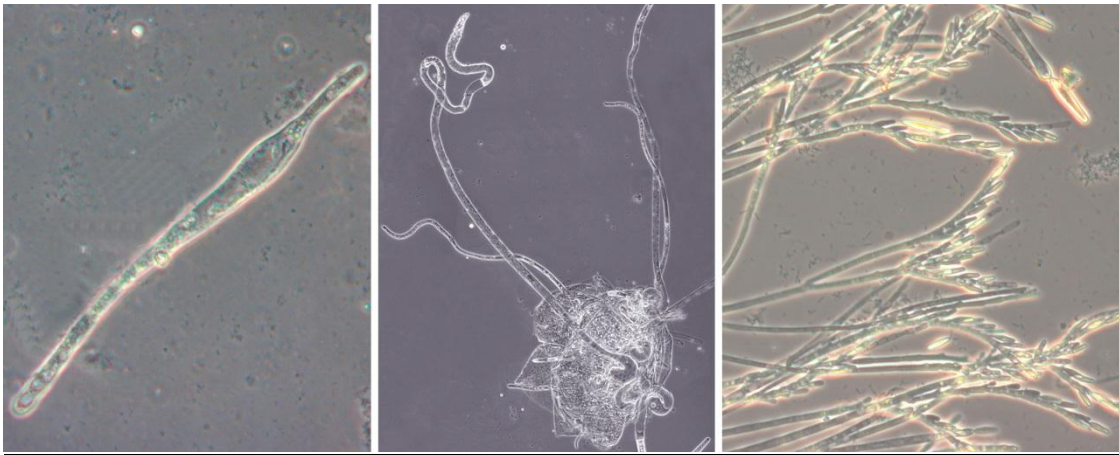


New species and records of Harpellales and Amoebidiales from the North-eastern Iberian Peninsula



Author sign:

Tutor sign:

Author: **Laia Busquets Casas**

Tutor: **Laia Guàrdia Valle**

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Unitat de Botànica. Departament de Biologia Animal, Biologia Vegetal i d'Ecologia.

Facultat de Ciències. Universitat Autònoma de Barcelona

Justification

Knowing the biodiversity of the natural ecosystems is a key aspect for habitats management in order to improve their ecological quality or prevent future perturbations. Among the least studied habitats, we find the aquatic ecosystems (McAllister et al. 1998) so that the knowledge of the biodiversity in these habitats is highly incomplete, especially regarding free-living and symbiotic microorganisms (Cafaro 2002).

The present project is focused on knowing a little more about the biodiversity present in the river systems of Ripollès, Garrotxa and Osona, three demarcations in Northern Catalonia. Specifically, the purpose of this study is to know about the biodiversity of fresh-water arthropod-gut-symbionts (trichomycetes) in these habitats, because they are relatively unknown cryptic organisms that most likely play an important ecological role in arthropod populations, so the knowledge of these microorganisms could give the necessary information for the maintenance of arthropods and thus for the integrity of the system (Valle and Arranz 2014).

The present study was achieved through field and laboratory tasks in which I provided my active participation, with the help of the tutor and in some instances also with the help of another final project (TFM) student. Collections were performed from February 2016 to June 2016 in different rivers and streams of the Ripollès, Garrotxa and Osona landscape. Initially, we collected samples in nearby sites to practice the techniques of micro-dissection to attain the needed ability for sample processing before the incursion in the area of study.

The days after sample collection, I extracted and processed endosymbiont trichomycete thalli from the aquatic arthropods in the laboratory of mycology, at *Universitat Autònoma de Barcelona*. Then, fungal specimens were identified with the help of the tutor and bibliographic resources. Finally, interesting samples were preserved in microscopy slides at the BCB-mycotheca (UAB, BABVE, Unit of Botany).

The descriptions of new taxa and new geographic records were made by myself and revised by the tutor. Furthermore, I revised samples of previous collections prepared by the tutor, in order to have more information for the descriptions.

This entire project will be presented in the format of a scientific article (*Mycological Progress*). First of all, an introduction will provide the lecturer with general information about the microorganisms and the area of the study. In the next section, the techniques and materials used will be explained in detail. Then, descriptions of the new species and records will be provided in a taxonomic section, the main body of the study. Finally, the discussion will remark the most important results extracted, and will provide a discussion about possible taxonomic conflicts.

Abstract

New data about trichomycetes from Iberian Peninsula, concretely in the demarcations of Garrotxa, Ripollès and Osona, are reported, including the description of two new Amoebidiales and a new Harpellales: *Paramoebidium contortum* from Baetidae and Ephemerellidae (Ephemeroptera) nymphs, *Paramoebidium lateralis* from Capniidae and Leuctridae nymphs and *Ejectosporus chimera* from Capniidae nymphs. *P. contortum* is characterized by its particular coiled long thalli and *P. lateralis* is identified by a lateral holdfast. But the most important aspect to highlight of *P. lateralis* is the observation, in one specimen, of a thallus originated from the fusion of what seems to be two cystospores. It could be an important addition in the study of Amoebidiales, since sexuality is unknown for this order. The new species of Harpellales, *Ejectosporus chimera*, is a rare dimorphic species with a narrow distribution and has got similarities with other genera, like *Genistelloides* and *Lancisporomyces*, so the identification of this species is complex. Some additional geographical records for Iberian Peninsula are also provided, including *Paramoebidium corpulentum*, *Paramoebidium hamatum* (Amoebidiales) and *Glotzia centroptili* (Harpellales). New observations are provided for species previously known from this region: *Tectomyces leptophlebiidarum*, with unusual large zygosporangia and *Legeriomyces ramosus* with chlamidospore-like structures. Another species remain unidentified: *Capniomyces* sp., a very rare genus within stonefly nymphs. Comments are provided for each of the species as well as photographs to illustrate each taxon.

Key Words: *Ejectosporus*, fresh-water arthropods, gut fungi, *Paramoebidium*, trichomycetes.

Introduction

Fresh-water ecosystems are very vulnerable to human activities, and aquatic species have suffered higher extinction rates per decade than species in terrestrial ecosystems (Strayer and Dudgeon 2010). Despite of this worrying data, aquatic environments are among the least studied habitats (McAllister et al. 1998). Therefore, it would be interesting to focus future studies on aquatic ecosystems, among other fragile and threatened habitats, which may deserve the attention of conservationists and scientists.

In spite of the predicted high diversity of fungi on earth, with roughly estimations pointing out 1.5 million of possible species (Hyde et al. 2007), only about 3000 species are currently known to occur in aquatic habitats (Shearer et al. 2007). However, because aquatic environments comprise a huge diversity of habitats suitable to be colonized by fungi, it might be assumed that the potential number of fungal taxa developing in fresh-water ecosystems is much higher than the number recorded nowadays (Wurzbacher et al. 2011). Among the potential habitats to be

colonized by symbiont fungi and related microorganisms, we find the macroinvertebrates, a high diverse and specialized group of freshwater inhabitants at the base of food webs. Some of these symbionts, the so called trichomycetes, have evolved towards an endosymbiotic life-style, growing attached within the guts of aquatic arthropods. The trichomycetes are a polyphyletic grouping, thus they are represented in lower case, and include two fungal orders: Harpellales and Asellariales (Kickxellomycotina), and two orders of the protist clade Mesomycetozoea, at the early divergence of animals and fungi: Eccrinales and Amoebidiales (Valle 2014a). The Harpellales and the Amoebidiales are the most representative groups of trichomycetes and both are included in this study.

In the case of the ecological group trichomycetes, fungi (Harpellales) and protozoa (Amoebidiales) show convergent characteristics, even when they belong to different kingdoms (Hibbett et al. 2007). This prompted their historical classification within the taxonomic Class Trichomycetes (Zygomycota), actually not accepted as a natural group. They are studied together because both grow obligatory in the digestive tracts of arthropods that live in freshwater, marine or terrestrial habitats, sharing the same ecology and biology, which has shaped their similar evolutionary traits. Moreover it is interesting to study these symbiont microorganisms of arthropods because often, unexpected changes in inland water systems are due to alterations in the complex connections among sediment-dwelling macroinvertebrates and associated trophic webs (Goedkoop and Johnson 1996; Lodge et al. 1998; Stockley et al. 1998) so trichomycetes surely have an ecological role in the integrity of the system too, because they take part in macroinvertebrates' biology.

The bodies of trichomycetes (thalli) are firmly attached to the host's gut lining and absorb nutrients from transiting particles, which allow them growing, metabolizing and reproducing (Lichtwardt et al. 2001). These gut fungi do not usually affect their hosts adversely or beneficially in any obvious way, so they are often called commensalisms. Nevertheless, there are several reports evidencing that at least one species of the fungal genus *Smittium* (Harpellales) can cause high mortality to mosquito larvae cultured in the laboratory (Dubitskii 1978; Sweeney 1981; López-Lastra 1990). However, other studies have demonstrated a possible mutualism between the mosquito larvae and a species of *Smittium* in poor conditions of vitamins (Lichtwardt and Horn 1981; McCreadie et al. 2005). This indicates that there may be a range of symbiotic relationships between trichomycetes and their arthropod hosts, as a result of a long coevolutionary process, because these basal clades of fungi and protists are associated with some of the most ancient insect groups (Lichtwardt 1995). However, this important biological question has not been addressed to the extent it should be, in part, because only about 6% of the recognized species of trichomycetes have been axenically cultured (Lichtwardt et al. 2001).

The Harpellales is the most diversified order within trichomycetes; reside in the chitinous guts of the larval and nymphal stages of Diptera, Ephemeroptera and Plecoptera and more rarely in the immature stages of Coleoptera, Trichoptera and freshwater Isopoda (Valle and Arranz 2014). The thalli of Harpellales are septate and can be either branched or unbranched. They attach to the gut by a cellular fixation element or an acellular mucilaginous secretion (holdfast), or a combination of both (Valle 2009). The asexual reproduction of Harpellales is carried out by the trichospores, which are unispored sporangia formed by a generative cell. Some of them have one or more appendixes, which facilitate the adhesion of the spores in the substrate (Valle 2009). The sexual reproduction is by means of the zygosporangia, which are biconical or conical resistant spores. Zygosporangium formation is often associated with the molting cycle of their host (Lichtwardt 2001) as a way to ensure fungal survival and propagation after ecdysis (Valle 2010).

The order Amoebidiales include two genera, *Amoebidium*, with ectosymbiotic species, and *Paramoebidium*, with endosymbiotic species, which are present in a wide diversity of hosts. This last genus includes filiform and sack-like microorganism, with a simple and unicellular thallus attached by a noncellular holdfast in the digestive system of immature aquatic insects, including Dipterans, Ephemeropterans and Plecopterans (Valle 2014a).

These species, like the Harpellales, have adapted to attach, nourish and reproduce on the hindgut lining, habitually in synchrony with the cycle of the host (Valle 2014a; Whisler 1966; Lichtwardt 2001). During the ecdysis, these endosymbiont microorganisms will be shed together with the exoskeleton. Then, amoeboid cells will be released from the thallus in order to move towards a refuge before encystment and cystospore production, that are presumably released to the environment to be ingested by a new host (Valle 2014a).

The specificity between *Paramoebidium* species and their hosts is a question to resolve because there are only 16 accepted species and it seems likely that many more species remain to be discovered, considering the variety of potential hosts that may bear them. The order Amoebidiales is poorly known from the Iberian territory, with only 5 species previously reported (Valle 2009; Valle 2014a; Valle 2014b).

We add here some new species and new records from the Iberian Peninsula found in Ripollès, Garrotxa and Osona, three demarcations of Catalonia that are situated in the pre-Pyrenees. This territory is characterized to have a large river system. In these areas it were found two new species of Amoebidiales (*Paramoebidium*) and a new of Harpellales (*Ejectosporus*), moreover new observations and new geographical records have been reported from species already described.

Material and methods

Area of study

All species reported herein were collected from diverse rivers and streams of Catalonia, concretely at the Ripollès, Garrotxa and Osona demarcations during the years 2001-2016 (Table 1).

Table 1 Collection sites with reference numbers and descriptions. Only those sites with trichomycetes recorded.

Site	Date	Site description	Latitude/ Longitude
1	1 Mar 01	SPAIN. Catalonia. Osona. Rupit, Coll de Condreu. Om River. 982 m.	42°02'45.49"N 2°29'27.56"E
2	28 Jan 03	SPAIN. Catalonia. Garrotxa. Falgars d'en Bas. Pujolriu Stream. 916 m.	42°04'53.60"N 2°26'26.51"E
3	29 May 13	SPAIN. Catalonia. Barcelona. Cànoves i Samalús. Vallforners Stream, above the dam. 884 m. 12°C.	42°46'08.3"N 1°17'05.05"E
4	26 June 13	SPAIN. Catalonia. Garrotxa. Falgars d'en Bas. Puig stream. 864 m.	42°04'27.59"N 2°26'55.41" E
5	10 July 14	SPAIN. Catalonia. Osona. Manlleu. Ter River. 443 m.	41°59'46.95"N 2°16'15.02" E
6	3 Mar 15	SPAIN. Catalonia. Garrotxa. Falgars d'en Bas. Pujolriu Stream. 916 m.	42°04'53.60"N 2°26'26.51"E
7	3 Mar 15	SPAIN. Catalonia. Osona. Rupit. Om River. 985 m.	42°02'57.85"N 2°28'57.70" E
8	3 Mar 15	SPAIN. Catalonia. Osona. Rupit. Escaions Stream. 980 m.	42°02'48.5"N 2°29'29.2"E
9	29 Mar 16	SPAIN. Catalonia. Ripollès. Vallfogona de Ripollès. Vallfogona del Ripollès Stream. 885 m. 7°C.	42°11'36.6"N 2°18'13.4"E
10	29 Mar 16	SPAIN. Catalonia. Ripollès. Sant Joan de les Abadesses, Malatosca Stream. 820 m. 12°C.	42°14'45.2"N 2°17'22.6"E
11	18 Apr 16	SPAIN. Catalonia. Ripollès. Camprodon, Colònia Estabanell, La Molina Stream. 1039 m. 9°C.	42°17'14.31"N 2°20'46.71"E
12	3 May 16	SPAIN. Catalonia. Garrotxa. Sant Feliu de Pallerols. Brugent River. 475 m.	42°04'44.4"N 2°30'09.0"E
13	11 May 16	SPAIN. Catalonia. Ripollès. Camprodon, Colònia Estabanell, La Molina Stream. 1039 m.	42°17'14.31"N 2°20'46.71"E
14	24 May 16	SPAIN. Catalonia. Ripollès. Camprodon. Ritort River. 990 m.	42°19'42.0"N 2°23'52.3"E

Experimental methodology

Collection of the potential aquatic hosts of trichomycetes, like larvae, nymphs and insect exuviae from the streams (Fig. 1) was made by dipping aquatic nets with fine mesh into the water current while disturbing the substrate, rocks, pebbles and vegetation, which made that the arthropods were incorporated into the flow and could be retained by the net (Valle 2014a; Lichtwardt et al. 2001).

The potential aquatic hosts were selected after placing the net content into a white tray with stream water by using plastic pipettes. The alive hosts were transported to the laboratory in tupperware-like plastic recipients with little stream water, maintaining the temperature low inside a transportable ice-cooler (Valle and Arranz 2014).

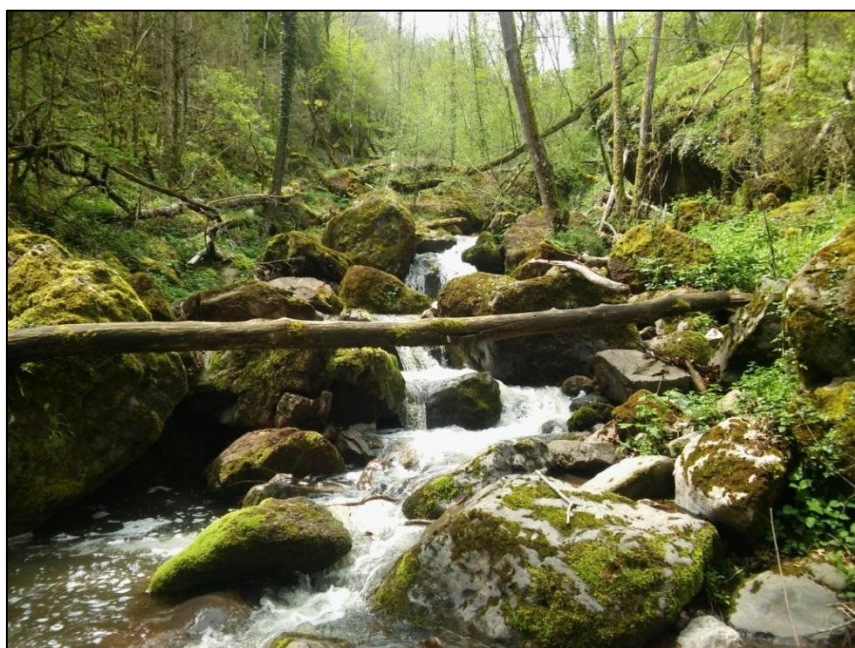


Fig. 1 La Molina stream in Camprodon (Ripollès, Catalonia), one of the collection sites of this study.

Once in the lab (UAB), each insect was identified with the help of bibliographic resources (Tachet 2000) and then was placed in a clean drop of water on a microscope slide. The hindgut and also midgut (in the case of the dipteran hosts) were removed and cleaned with the aid of a stereomicroscope, ultrafine forceps and entomological needles, to detect and isolate gut fungi from the gut. When endosymbionts were present, these were placed in the midpoint of another drop of clean water with the help of the ultrafine forceps. A cover-slide was carefully placed on the drop. Then the preparation was ready to be observed under the compound microscope at 100, 200 and 400x, under phase contrast or interference contrast optics. Lactophenol cotton-blue was used as final semi-permanent media to preserve thalli of trichomycetes. Finally, cover slides were sealed with clear fingernail polish (Valle 2007). Samples were deposited in the

BCB-Mycotheca, at the institutional address of the tutor. Trichomycetes were microphotographed with a Zeiss Axioscope compound scope equipped Jenoptik ProGresC3 digital camera.

In the case of *Paramoebidium* species, the slides with water were maintained in moist chambers to allow amoebogenesis and cyst development. Some of the mayfly and stonefly nymphs were maintained alive in stream water (with the naturally present microalgae and organic detritus) up to 2 months in appropriate trays, covered with a screen to avoid the escape of flying adults, and kept at 9–10°C in a climate chamber allowing the incidence of natural light. Hosts were retained there until molting, so that *Paramoebidium* cysts could be recovered from exuviae. Also, the exuviae with cysts were maintained in Petri dishes with a layer of filtered stream water under the same conditions and periodically examined to follow the maturation of cysts and cystospore formation.

Additional specimens of previous samplings from the BCB-Mycotheca were observed for comparison and morphometric analyses.

Results

New taxa

Ejectosporus chimera L.G. Valle & L. Busquets, *nom. prov.*

Figs. 2-10

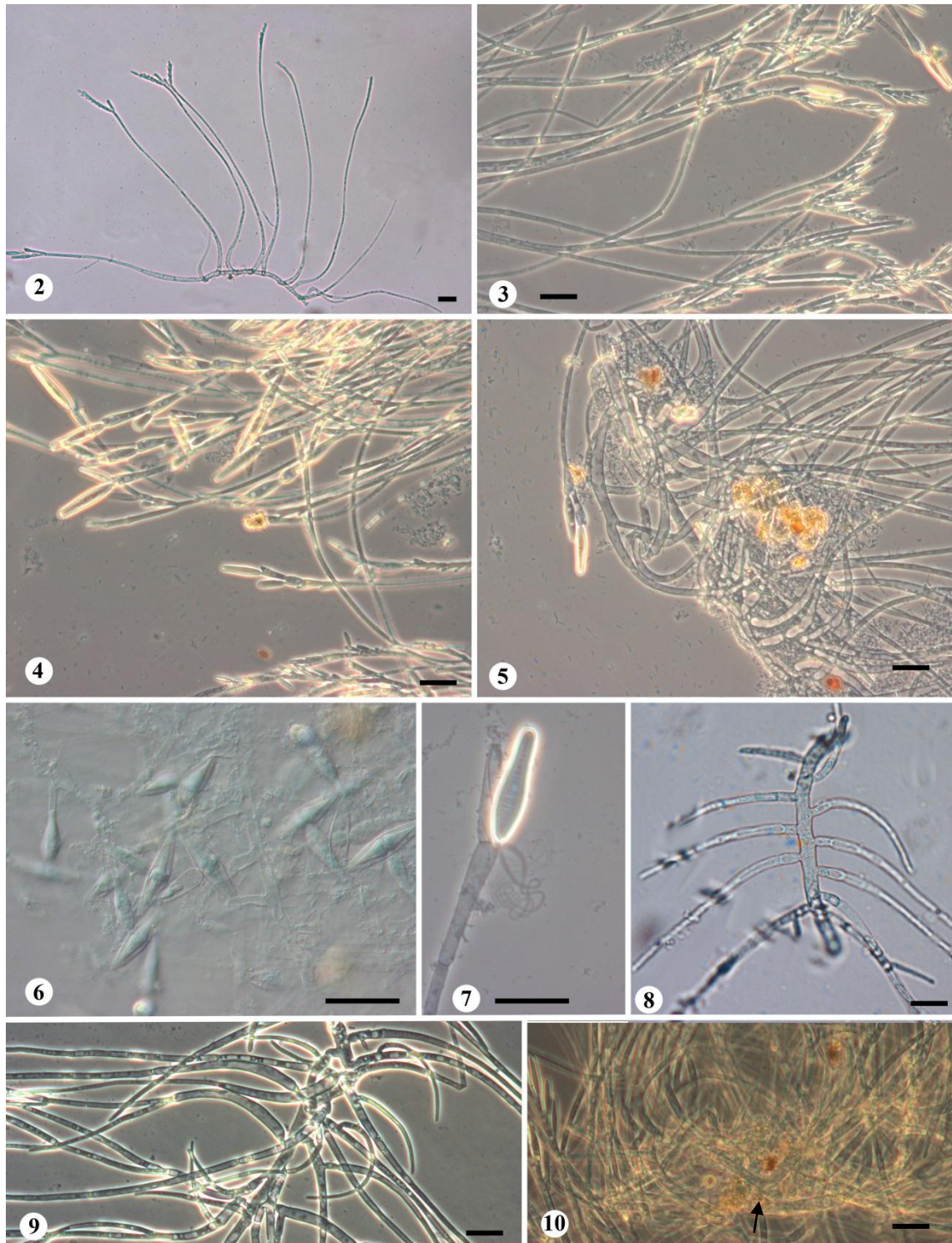
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Thallus prostrate at maturity, with a main axis pinnately branched and sparse secondary branches (Figs. 1, 2, 8, 9). Transverse septa absent in the main axis, present in the basal section of lateral branches, which are often sinuous or slightly bend at their base and thin (Fig 8). Holdfast discoid in young thalli, inconspicuous in mature specimens (Fig. 10). Trichospores ovate-elliptical, with two differentiate types according to their size: spores type α measuring 9-13 x 2.5-4 μm , arranged in series of 10-25 short generative cells at distal ends of thin fertile branches or lateral branches (Fig. 3). Trichospores type β measuring 30-38 x 7-9 μm , arranged in shorter series of 2-10 generative cells at the tips of fertile branches (Figs. 4, 5). Both trichospore types have 2 thin appendages, helically coiled inside the generative cells, and rapidly uncoiled after released (Fig. 7). Zygosporophore biconical, type I, measuring 28-33 x 7-8 μm and distal tips of the zygosporophore straight (Fig. 6). Zygosporophore 18-21 x 7-9 μm , wider at the upper section (Fig. 6). Undeciduous, vegetative sporangia not observed. In the hindgut of aquatic Capniidae nymphs.

Etymology. *chimera*, for the presence of thallial and spore characters that resemble those of different genera of Harpellales.

Specimens examined. SPAIN. Catalonia. Girona. Falgars d'en Bas, Pujolriu stream, 42°04'53.60 N, 2°26'26.51 E. 916 m. 28 Jan 2003. Microscope slide BCB-Tr1699, prepared from the hindgut lining of *Capnia bifrons* Newman nymph (HOLOTYPE), from Site 2; Site 1: Microscope slides BCB-Tr0570-0572, Tr0574-0575, Tr0579-0581, Tr0583-0584, Tr0587, Tr1899-1903, Tr1910, all prepared from *Capnia bifrons* nymphs; Site 9: BCB-Tr2349 prepared from *Capnia bifrons*.

Notes. The genus *Ejectosporus* S.W. Peterson, Lichtw. & M.C. Williams was described from stoneflies *Allocapnia* (Capniidae), in the USA (Lichtwardt et al. 1991). Later, *Simuliomyces spica* S.W. Peterson & Lichtw., was revealed to be the trichosporic stage of the type species *Ejectosporus magnus* S.W. Peterson et al., whilst this later species corresponded to the vegetative spore stage, with characteristic undeciduous sporangia extruding the sporangiospore content within the same gut (Strongman 2005). Our new species has dimorphic trichospores (deciduous asexual sporangia with a sporangiospore), but undeciduous vegetative sporangia have not been observed. This genus was previously unknown from the Iberian Peninsula. The species here described shares some characteristics with the type species *E. spica* (S.W. Peterson & Lichtw.) Strongman. Trichospores type α of *E. chimera* overlap in size with the trichospores of *E. spica* (12-14[-16] x 2 μm , according to Strongman 2005). However, trichospores type β in *E. chimera* are not comparable with those of *E. spica*, the latter showing undeciduous vegetative sporangia measuring 50-85 x 8-10 μm (Strongman 2005) which are also differentiable by their shape from normal trichospores. Additionally, zygospores in *E. chimera* are smaller than those of *E. spica* (36-43 x 7-10 μm , in Strongman 2005). The other species of the genus, *E. trisporus* Strongman, recorded from Canada, has also trichospores resembling those of the new species, although being slightly longer (12-18.5 x 2-4.5, in Strongman 2005). More obviously, the Canadian species can be differentiated by the presence of markedly larger zygospores (54-71 x 9-12.5[-14] μm , in Strongman 2005). This species also had deciduous vegetative sporangia, similar to those reported for *E. spica*. Trichospores type α and β of *E. chimera* grow from the same thalli but usually from different fertile branches. While many individuals produced both trichospore types, other presented only trichospores type α , and other (fewer) exclusively trichospores type β . The mucilaginous substance imbedding the axial cells always contains large quantities of associated bacteria. *Ejectosporus chimera* has characteristics also shared by members of the genus *Genistelloides* S.W. Peterson, Lichtw. & B.W. Horn (1981), as the presence of two appendages, and type I zygospores. Nonetheless, appendage characteristics more closely resemble those of *Ejectosporus*, being more discrete, not so stout at the proximal end, and not so compactly folded. Thallial characteristics resemble *Lancisporomyces* in some cases because of the pinnately branched and sparse secondary branches, but in general the thallus resembles most the genus *Ejectosporus*.



Figs. 2-10 *Ejectosporus chimera* from *Capnia bifrons* nymphs. 2. Overview of *Ejectosporus chimera* thallus. 3. Fertile branches with intercalary trichospores of type α and one trichospore of type β . 4, 5. Fertile branches with trichospores of type β . 6. Zygosporophore and zygospores of *Ejectosporus chimera*. 7. Trichospore showing the two appendages. 8. Young thallus of *Ejectosporus chimera*. 9, 10. Detail of the thallus and the holdfast (arrow) of *Ejectosporus chimera*. Bars = 25 μm .

Paramoebidium contortum L.G. Valle & L. Busquets, *nom. prov.*

Figs. 11-21

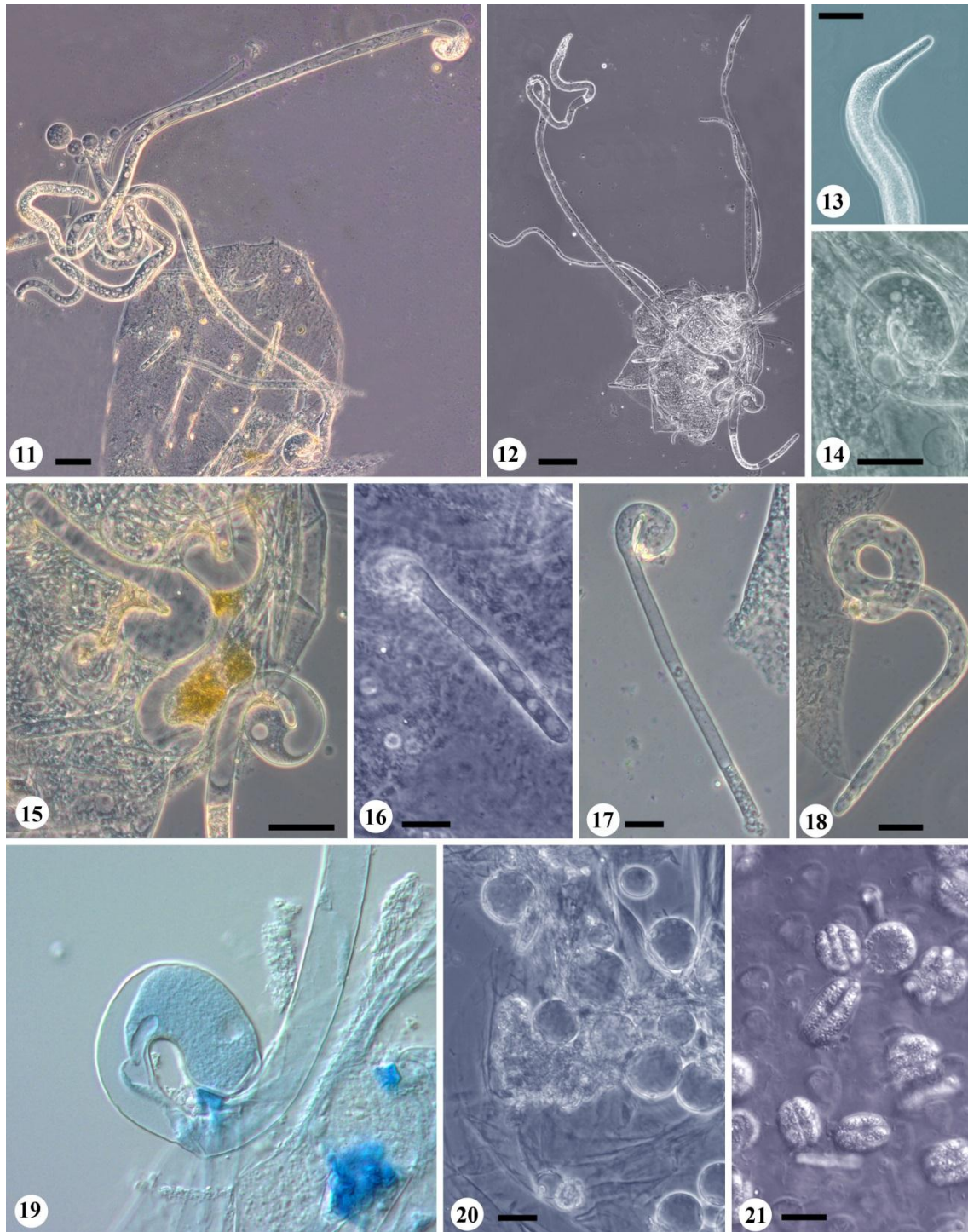
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Thalli 1000-1400 x 20–25 µm, sinuous and twisting, especially at the distal sections, and curved and swallowed at the holdfast region (27–38 µm diam) (Figs. 11, 12). Middle section more straight and isodiametric. Holdfast slightly tapering towards the base, and of minor diameter (9–15 µm) than the corresponding thallus (Figs. 14, 18, 19). Amoebae not observed. Cysts spherical, 22–35 µm diam (Fig. 20). Cystospores elongate and somewhat curved, 25–30 x 5.5-7 µm (Fig. 21). Attached to the hindgut lining of Baetidae and Ephemerellidae (Ephemeroptera) nymphs.

Etymology. *contortum*, for the characteristic contorted thallus.

Specimens examined: SPAIN. Catalonia. Barcelona. Cànoves i Samalús. Vallforners stream (above the dam). 1°17'05.05" E, 42°46'08.30" N. 884 m. 29 May 2013. Microscope slide BCB-Tr2239. Prepared from the hindgut lining of *Baetis* sp. (HOLOTYPE), from Site 3. Site 3: BCB-Tr2240, Tr2241. Prepared from the hindgut lining of *Baetis* sp.; Site 12: BCB-Tr2394. Prepared from the hindgut lining of *Ephemerellidae*.

Notes. This species of *Paramoebidium* is clearly identifiable by the coiled long thalli with a curved swallowed base where the holdfast is produced. It would somewhat resemble the basal region of *P. curvum* Lichtw., but with a very different thallial structure, because *P. curvum* is much shorter and stout, and appears on or near the anal gills of Simuliidae larvae (Dang and Lichtwardt 1979). Unfortunately we could not observe the amoeboid cells, and thus it has not been possible to corroborate whether if the adhesive uroidal filament observed in other *Paramoebidium* species (Valle 2013) also applies for *P. contortum*. Cysts and cystospores were observed in shed exuviae. The cystospores were formed after two weeks of the initial cyst observation, on unsealed slides with water as mounting media kept in moist chambers. There are other species of *Paramoebidium* described from Baetidae nymphs, but all of them have different features. *Paramoebidium argentinense* Mazzucchelli, López Lastra & Lichtw., have a terminal papilla, and a more simple thallial disposition within the gut, however, this species also has a bend basal section, but the curvature is not as basally and robust as in the new species (Lichtwardt 2000). *Paramoebidium hamatum* Bench & M.M. White is another species recently recorded from Baetidae, Ameletidae and Chironomidae hosts in the USA (Bench and White 2012). The species has an elongated thalli hooked basally that somewhat resembles *P. contortum*, although *P. hamatum* has a straight terminal portion and a basal section not as swallowed as in *P. contortum* and it has smaller cysts too (Bench and White 2012).



Figs. 11-21 *Paramoebidium contortum* from *Baetidae* nymphs. 11, 12. Overview of a mature thalli, with the typical sinuous and twisting thallus of this species and young short thalli. 13. Detail of the tip of the *P. contortum*, with a thin apex. 14, 15. Detail of the curved and swallowed holdfast. 16. Initial stages in thallus attachment and growth. 17. Detached immature thallus, with the typical curved and swallowed holdfast. 18, 19. Details of the holdfast of *P. contortum* attached to the hindgut of *Baetidae*. 20. Cysts. 21. Cysts with cystospores inside. Bars 11, 14, 15 = 50µm, Bars 12 = 100 µm, Bars 13, 16, 17, 18, 19, 20, 21 = 25 µm.

Paramoebidium lateralis L. Busquets & L.G. Valle, *nom. prov.*

Figs. 22-29

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Thalli 300-500(-1100) x 10-45 μm , with a forceps-like holdfast (Fig. 27), globose at maturity and measuring 7-8 μm diameter (Fig. 26, 29), located laterally, at about the middle zone of mature thalli (Figs. 22, 23, 25 arrows). Overall thallus shape straight or slightly curved (Fig. 28) with one arm of the thallus (at one side of the holdfast) usually wider and more convex than the other (Figs. 22, 23). Amoebae and cysts not observed. Attached to the hindgut lining of Plecoptera: Capniidae and Leuctridae nymphs.

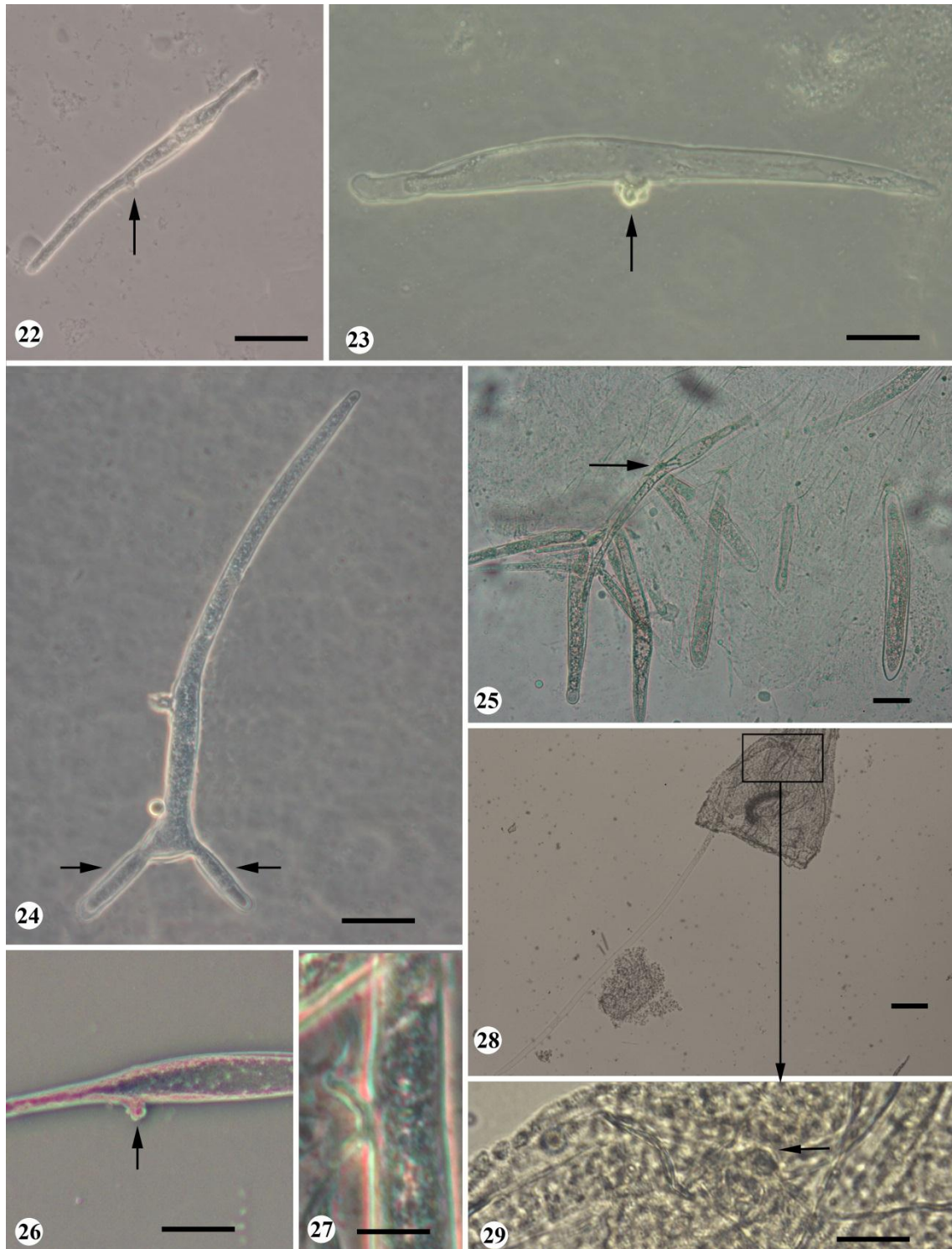
Etymology. *lateralis*, for the lateral holdfast.

Specimens examined. SPAIN. Catalonia. Girona. Vallfogona de Ripollès. Vallfogona del Ripollès stream. 885 m. 29 Mar 16. 42°11'36.6"N, 2°18'13.4"E. Microscope slide BCB-Tr2349. Prepared from the hindgut lining of *Capnia bifrons* (HOLOTYPE), from Site 9. Site 6: BCB-Tr2314, Tr2315, Tr2317. Prepared from the hindgut lining of *Capnia bifrons*.; Site 7: BCB-Tr2321. Prepared from the hindgut lining of *Capnia bifrons*; Site 8: BCB-Tr2332, Tr2333. Prepared from the hindgut lining of *Capnia bifrons*; Site 10: BCB-Tr2357. Prepared from the hindgut lining of *Leuctra* sp.; Site 14: BCB-Tr2404. Prepared from the hindgut lining of *Leuctra* sp.

Notes. *Paramoebidium lateralis* is mainly characterized by having a lateral holdfast, among other features. This trait is not exclusive for *P. lateralis*; other species such as *P. avitruviense* L.G. Valle and *P. bibrachium* M.C. Williams & Lichtw. also show a lateral attachment. However, *P. avitruviense* species is easily identifiable by its characteristic four-branched thallus, observable at maturity. This species also presents a different holdfast structure, being more complex and larger, with a constriction above the cementing holdfast material set in a wider structure (see Valle 2014a). Regarding its ecology, *P. avitruviense* has a high specificity for hosts in the genus *Siphonoperla* (Chloroperlidae) and have not been recorded within either Capniidae or Leuctridae (Plecoptera) nymphs (Valle 2014a, 2016).

Paramoebidium bibrachium, like the new species here described, presents an asymmetric thallus. The lateral holdfast and the straight shape of the thallus of *P. bibrachium* resemble that of *P. lateralis*, although the thallus is larger (up to 2300 μm x up to 180 μm , in Williams and Lichtwardt 1990). Finally, it is interesting to remark that the hosts of *P. bibrachium* are Ephemeroptera nymphs, not Plecoptera as in *P. lateralis*.

The most surprising observation regarding *P. lateralis* may be three-branched thallial structure observed in Figure 24, which seems to be the result of a fusion between two cysts or mother cells (arrows), which later may have originated a central thallus. Both cells (cysts) have the same morphology. This exciting observation is inedit among Amoebidiales, and will be discussed in more detail elsewhere in this paper.



Figs. 22-29 *Paramoebidium lateralis* from *Capniidae* and *Leuctridae* nymphs. 22, 23. Thalli overview, with the typical lateral holdfast (arrows). 24. Thallial structure resulting from the fusion of two germinating cists (arrows). 25. Thalli of *P. lateralis* attached to the hindgut of Plecoptera. 26. Detail of the globose holdfast of *P. lateralis* at maturity. 27. Detail of forceps-like holdfast. 28. Thalli overview of *P. lateralis*. 29. Detail of the lateral holdfast. Bars 22, 23, 25, 26, 27, 28 = 50 μ m, bars 24, 29= 25 μ m.

New geographic records for the Iberian Peninsula

Paramoebidium corpulentum Lichtw. & M.C. Williams (Fig. 30)

This species inhabits the hindguts of *Allocapnia* spp., *Protonemura nitida* and *Nemoura* sp. (Nemouridae, Plecoptera). Its known distribution, in addition to USA and Canada, includes Italy (Valle et al. 2014). This is the first report of the species for the Iberian Peninsula. *P. corpulentum* was found in *Protonemura nitida* (BCB-Tr2397), from the collection Site 13. The species was identified by the size and shape of the thallus, which is curved in the base, up to 300 µm long and a diameter between 30-90 µm.

Paramoebidium hamatum Bench & M.M. White (Fig. 31)

This species, described originally from USA in Chironomidae, Ameletidae and Baetidae (Ephemeroptera), has been found in the Site 12 (BCB-Tr2393, Tr2395) from Baetidae nymphs. So this is the first record of this species from the Iberian Peninsula. In addition, this is first known occurrence of this species in *Ephemerella ignita* from the Site 19 (BCB-Tr2392). The species was identified by the curved portion at the basal one-eighth to one-third of the thallial length.

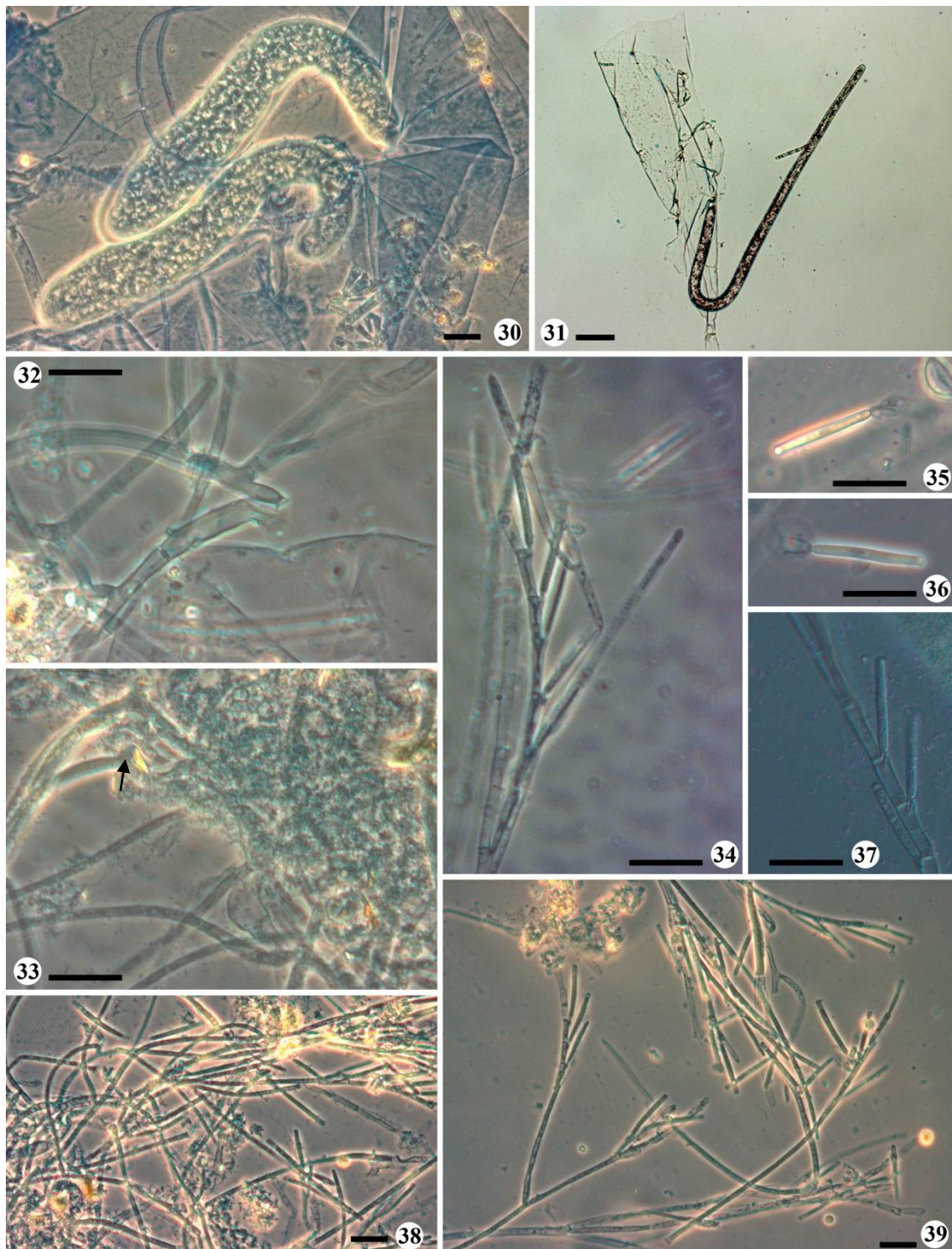
Glotzia centroptili Gauthier ex Manier (Fig. 32-39)

This species was described in France from *Centroptilum luteolum* nymphs by Gauthier (1936). However, in the original description of this species, there were not any photos; the author included only a drawing of just one thallus and some details of the spores and basal section with holdfast (Gauthier 1936). We provide here the first photographic images of *G. centroptili* (Figs 32-39), and add new data to complete the description of this taxon. This is the first record of this species in Iberian Peninsula, collected from Site 4 (BCB-Tr2304, Tr2305, Tr2306) and the Site 5 (BCB-Tr2253, Tr2254). In fact, the species had never been recovered since its original description in year 1936.

The species was identified by the long fine appendage initially spiralled around two short appendages in the trichospores (Fig. 35-37), and by the characteristic refractive cap at the apex of the trichospore. The size of the trichospores in the original description was 40 x 4 µm (Gauthier 1936). Provably few specimens were examined in that occasion, and the intraspecific variability of spores and other structures was not recorded. Here we provide a more real size range that includes the variability of the observed trichospores (35-43 x 4-6 µm). The number of the generative cells per thallus described is 6-7, according to Gauthier, which correspond with the observed Iberian specimens (Fig. 34).

Gauthier (1936) described a lateral holdfast with 2 or 3 branches growing in the base (Fig. 33), although it has been observed that some thalli present a terminal holdfast too (Fig. 32). In

mature specimens, the basal sections show conspicuous swollen hyphae which may be profusely ramified. In upper sections dichotomous branching is evident. Our specimens were obtained also from *Centroptilum* nymphs.



Figs. 30-39 30. Overview of two *Paramoebidium corpulentum* from *Protonemura nitida* nymph. 31. Overview of *P. hamatum* from *Ephemerella ignita*. 32-39. *Glotzia centroptili* from *Centroptilum luteolum* nymphs. 32, 33. Detail of the holdfast of *G. centroptili*. 34. Detail of the fertile branch. 35, 36. Trichospores with long fine appendage initially spiralled around two short

appendages. 37. Detail of the trichospores in the fertile branch. 38, 39. Thalli overview of *G. centroptili*. Bars 30, 32, 33, 34, 35, 36, 37, 38, 39= 25µm. Bars 31= 100 µm.

Unusual observations on previously known taxa

Legeriomyces ramosus Pouzar (Fig. 40)

Legeriomyces ramosus has been found in Site 9 (BCB-Tr2346, Tr2363, Tr2368) from Baetidae nymphs. Concretely the sample BCB-Tr2368 presents a structure like a chlamyospore, which is a thick-walled hyphal cell that functions as a spore (Fig. 34). These structures are a result of unfavourable conditions, so it seems that the formation of trichospores or zygospores were not sufficient. It is worth saying that similar structures can be observed just prior to the conjugation, as a result of a sexual-stimulus, probably triggered by a yet unknown molecule. *Legeriomyces ramosus* was morphologically identified because the trichospores are characteristically bottle-shaped and have got two appendages, which commonly attenuate to the distal end. Also, this species has a characteristic the disc-like holdfast and thallial disperse branching. Zygospores were also observed, being type II and had the typical a flared-inward collar (Pouzar 1932).

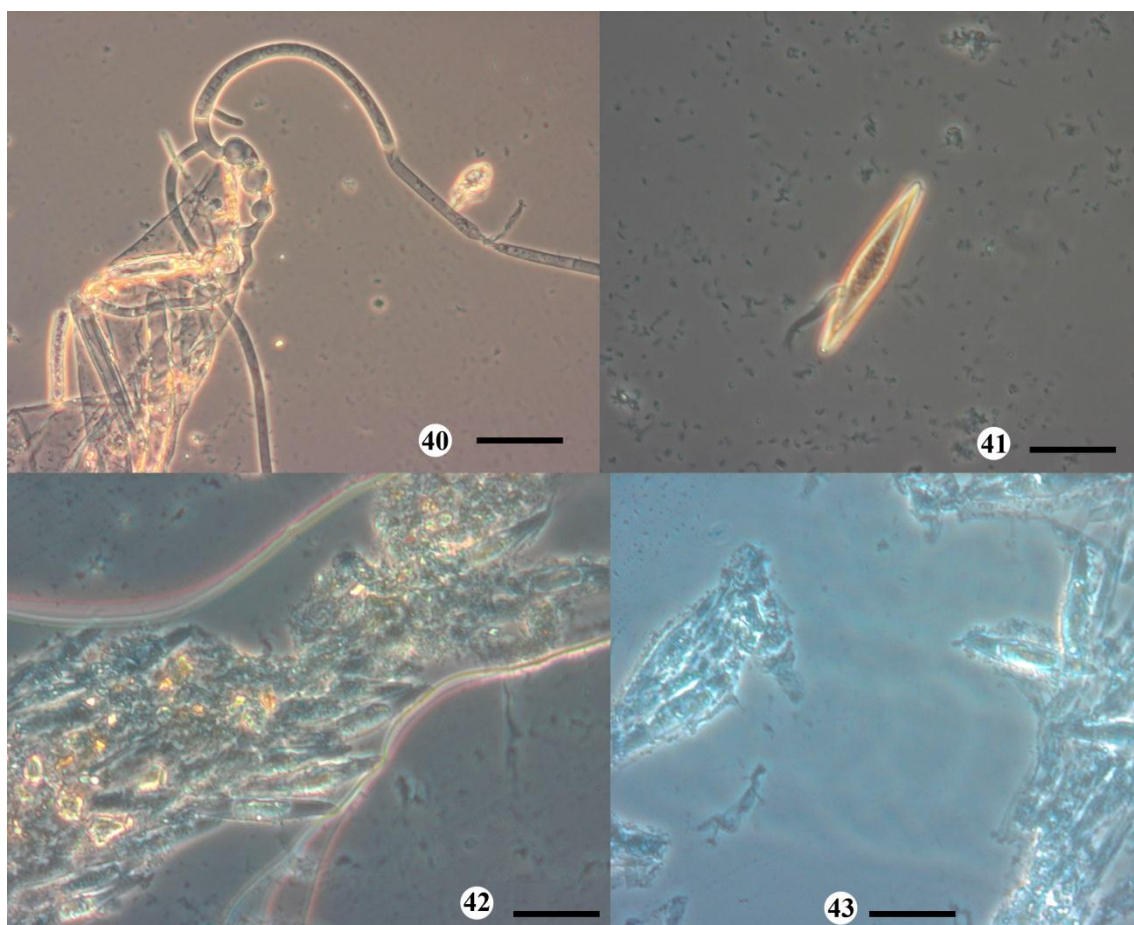
Tectiomyces leptophlebiidarum L.G. Valle & Santam. (Fig. 41)

This species described from *Habroleptoides confusa* (*Leptophlebiidae*) nymphs was found in the collection Site 9 (BCB-Tr2343B). The zygospores of this sample presents sizes of 51-56 x 7-9 µm which are larger than those previously described (41-47 x 6-8 µm, according to Valle and Santamaria 2002). However, only one sample was found so it is not sufficient to make a modification of the description, because it could be an exception or a new variety of this species.

Unidentified taxa

Capniomyces sp. (Figs. 42, 43)

This species, observed within a unique infested host of *Nemouridae* nymphs, was identified as *Capniomyces* sp. because of the shape of zygospores (Fig. 30, 31). These zygospores are type II, biconical, obliquely and submedially attached to the zygosporophore (Peterson and Lichtwardt 1983). The genus *Capniomyces* Peterson & Lichtwardt comprises three species: *C. celatus* L.G. Valle, *C. stellatus* Peterson & Lichtwardt and *C. sasquachoides* White & Bench. The zygospores observed in the collection Site 11 (BCB-Tr2386) resemble most those of *C. celatus* (35–38 x 5–6 µm, in Valle 2007), although being slightly bigger (37-44 x 5-8 µm). Unfortunately, in this sample only the zygospores were present, but were not the trichospores, so the identification of the species could not be completed.



Figs. 40, 43. 40. Structures like a chlamydospore or structures observed just prior to the conjugation of *Legeriomyces ramosus* from *Baetidae* nymphs. 41. Zygospores of *Tectiomyces leptophlebiidarum* from *Habroleptoides confusa*. 42, 43. Zygospores of *Capniomyces* sp. detached from *Nemouridae* nymphs. Bars = 25 μ m.

Discussion

The knowledge of trichomycetes from the Iberian Peninsula is relatively acceptable in terms of diversity, with 63 species reported for this geographic region (Santamaría and Girbal 1997; Santamaria and Girbal 1998; Valle 2007; Valle 2014a; Valle 2014b, Valle 2016). The Harpellales are the best-known group of trichomycetes in this zone, and new species are being described, as *Ejectosporus chimera* from our collections in Ripollès, Garrotxa and Osona, three demarcations of Catalonia. Asellariales, Eccrinales and Amoebidiales are not as well documented as the Harpellales in the Iberian Peninsula, in spite of the recent publications reporting new data of Amoebidiales (Valle 2014a, 2014b, 2016), because this order of trichomycetes appears to be much more diversified than previously thought (Reynolds et al 2015), our attention has been turned towards the endosymbiont species in the genus *Paramoebidium*, and two species with unique characteristics have been here described.

Ejectosporus chimera is a rare dimorphic species with a narrow distribution described, which has been found living in the hindguts of plecoptera nymphs (Capniidae). This species has similarities with different genus like: *Ejectosporus* S.W. Peterson, Lichtw. & M.C. Williams, *Genistelloides* S.W. Peterson, Lichtw. & Horn and *Lanciosporomyces* Santam. The shape of the thallus is similar with that of *Lanciosporomyces*; the shape of the trichospores is similar with those of *Ejectosporus*, and the zygosporangia are similar with those of *Genistelloides*. However, the species here described resembles most the species described within the genus *Ejectosporus*. The specific epithet “*chimera*” reflects this combination of features. It is important to note that the genus *Ejectosporus* has had a complicated taxonomic history. Initially, *Simuliomyces spica* S.W. Peterson & Lichtw. and *Ejectosporus magnus* S.W Peterson, Lichtw. & M.C. Williams were considered different species, but in 2005 Strongman noticed that both species were in fact the same taxon and were combined as *Ejectosporus spica*, while *Simuliomyces spica*, was revealed to be the trichosporic stage of the type species *Ejectosporus magnus* (Strongman 2005). Despite having the accurate and complete description for *E. chimera*, a future molecular study would be necessary to verify its taxonomic position, and also its phylogenetic associations. However, we could not gather enough fresh material to be preserved for DNA extraction and amplification, and this is our next task to develop, because we already know the season and the site where there are these trichomycetes.

Ejectosporus chimera was found exclusively in *Capnia bifrons* nymphs, so it seems to be a very narrow-specific species. *Capnia bifrons* is not very common in the area and has a nymphal phase with an activity period (in our collection sites) ranging from October to April or May. The production of the vegetative undeciduous spores seems to be restricted to the initial cycle of the host (Strongman, pers. com.), but we have not been able to observe this important characteristic phase of the cycle of *Ejectosporus*. This caused our major concern about the identity of the species. In conclusion, to determine this new species it is necessary to have the thallus, the zygosporangia and the trichospores, other way, it could be confused with some of the other similar Harpellales sharing the same host.

The study of Amoebidiales is complex because the lack of clear characters, so sometimes the taxonomy based on morphological traits is insufficient (Valle 2014a) and because, for example, many coiled, looped or hairpin hyphal shapes appear hooked when immature (Reynolds et al 2015), so only the mature thalli have to be used to do the description. However, in this case the distinct characteristics have allowed the description of two new species: *Paramoebidium lateralis* and *Paramoebidium contortum*.

Paramoebidium lateralis has been described exclusively on the basis of its thallial characteristics, since the cysts and cystospores and the amoeboid stage, were not found. These structures sometimes provide an additional information, but often different species have similar

cystic structures and thus may not provide, a conclusive diagnosis for classification (Valle 2014a). The most important characteristic of this species is the lateral attachment of the holdfast, which is a not very common feature. This new species has been found in Capniidae and Leuctridae nymphs, with are hosts located in cold waters. The distribution of *P. lateralis* is similar with that of *Ejectosporus chimera*, because in some cases both have been found together in the same host, *Capnia bifrons*.

The most interesting observation for this new *Paramoebidium* is the structure observed in Figure 24, which seems to be a fusion of two cists. Fusion of cists or other vegetative structures has not been observed before. So if this were the case, this would be the first evidence of sexual reproduction in Amoebidiales. Since the host *C. bifrons* starts the imaginal flying phase just at the time of our samplings, we only accomplished to capture the nymphs at the first sampling in just one host individual. Consequently, we have targeted this species for future collections, in the appropriate season to reach more nymphs, and fortunately, more material to corroborate a possible sexual state, since it could be an important advance in the study of Amoebidiales. Moreover it would be interesting to obtain cystospores and amoebae to provide a more detailed description.

The other new species of *Paramoebidium* described here, *P. contortum*, has clear diagnostic characters because it has a particular coiled long thalli. It was found in some host of Baetidae but in the final collections it was found also in Ephemerellidae nymph, so it will be interesting to get further material of the species in this later host.

The presence of one species of *Paramoebidium* is related with the ecology of the host, for example, it has been observed that the prevalence of *P. avitruviense* depends on the cycle of its host, *Siphonoperla torrentium* (Valle and Tierno de Figueroa 2015). So, it is worth studying both organisms, trichomycetes and host, together because it will provide more information about the ecology of both organisms than studied alone.

Among our samplings, we get also new geographic records of *Amoebidiales*, concretely two species of *Paramoebidium*. *Paramoebidium corpulentum* was described in 1991, but this is the first record for the Iberian Peninsula and the second in Europe, because it was found in Italy two years ago (Valle et al 2014). The thick shape of the thallus and the curved base were the determinant characteristics. *Paramoebidium hamatum* was described originally from the USA (Bench and White 2012) this being a new addition for the European mycoflora. Interestingly, this species was observed in Ephemerellidae, a new host for *P. hamatum*. This indicates that this endosymbiont is not very specific, and further hosts could be explored to increase the knowledge about the ecology of this recently described species.

Glotzia centropitili is the most interesting new record of the Iberian Peninsula in this report, because the original description of this species was not complete and did not provide photographs. We have provided here new images, providing further details of the spores and thallial features. This information is going to be very useful for future identifications of this species. The zygospores were not observed in this occasion, so it would be interesting to do perform future studies to get sexual spores and thus complete the original description, if necessary.

The large size of the zygospores found in our samples of *Tectimyces leptophlebiidarum* could indicate that the conditions were optimal, so that the zygospores could grow up to its plenitude. Differences could simply indicate an intraspecific geographical variation. So it would be interesting to find more zygospores of the species in the concrete site, to determinate if the large size of zygospores is a consistent characteristic. In contrast, in the collection of *Legeriomyces ramosus* it could seem that chlamydospores were formed so these indicate unfavourable conditions. Chlamydospores have been found before in Asellariales (Lichtwardt and Moss 1984) and in the genus *Smittium* (Valle 2007), which might aid the dispersion of those species. Despite of this, it could be that these structures observed in *Legeriomyces ramosus* are the beginning of the conjugation bridges, so it would be interesting to find more structures like this to determine what the reason of this structure was.

In the case of *Capniomyces* sp. it is important to highlight the need for continued collection to identify the species and better understanding its distribution. Since only the zygospores were observed, we could not provide an appropriate description, so the species remain unnamed. Again, this is another targeted species that deserves further study. In this case we can collect enough fresh material; a molecular study would be convenient to resolve its identity, and clarify whereas this is a new species of *Capniomyces* or is the previously known *Capniomyces celatus*. It is noteworthy to say that *Capniomyces celatus* was reported just in one occasion and has not been documented again, after its habitat was destroyed by a fire (Valle 2007). The species in the locus classicus was also very rare, so it seems to be extremely rare species with a very low infestation ratio, so it will be the second record of Europe. On the other hand, if it is a new species of *Capniomyces* it will be the fourth specie described of this genus, in which only *C. celatus* has got the same host, *Nemouridae* nymphs, all other species where described in Capniidae (Lichtwardt et al. 2001).

Clearly, a combination of characters is required for any adequate identification of new or reported species, but those used to date should be reevaluated and supplemented with methods that include genomic samples for molecular study, to generate a more robust taxonomic

framework (Reynolds et al 2015). So molecular studies it will be one of the most important axis for the future studies of trichomycetes.

In conclusion, if new studies of trichomycetes were focused in those countries where there are interesting fluvial systems, but where trichomycetes have never been studied, new data and new species will be found. And consequently this information can change the study of arthropods and thus for the integrity of the water system (Valle and Arranz 2014), because knowing the biodiversity of the natural ecosystems is a key aspect for habitats management in order to improve their ecological quality or prevent future disturbances.

Conclusions

- Three new species of trichomycetes have been found in this study: two Amoebidiales: *Paramoebidium contrortum*, from Baetidae and Ephemerellidae characterized by its particular coiled long thalli and *Paramoebidium lateralis*, from Capniidae and Leuctridae identified by a lateral holdfast, and one Harpellales: *Ejectosporus chimera*, from *Capnia bifrons* and with similarities with other genus.

- Three new geographical records for Iberian Peninsula have been also provided, including: *Paramoebidium corpulentum*, *Paramoebidium hamatum* and *Glotzia centroptili*.

- New observations are provided about *Tectimyces leptophlebiidarum*, *Legeriomyces ramosus* and *Capniomyces* sp.

- Futures studies of trichomycetes have to be performed, because knowing the biodiversity of the natural ecosystem is a key aspect for habitats management.

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