An undescribed species of *Microascus* from the Cave of Ramioul

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An isolate of a fungus from decaying meat in the Cave of Ramioul, Belgium, is presented as the undescribed species ***Microascus caviariformis*** sp.nov. It grows fastest and reproduces most abundantly on media low in soluble sugars and appears to be strongly adapted to growth on proteinaceous substrata at temperatures below 25°C. Production of a prolific and typical *Scopulariopsis* anamorph and relatively rapid growth on standard media are typical of most *Microascus* species, while its very narrow ascospores and slowly developing ascomatal ostiola are more characteristic of the genus *Pithoascus*. The apparent presence of a germ pore in the ascospores suggests the former genus to be more appropriate.

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Les auteurs présentent l'espèce non décrite ***Microascus caviariformis*** sp.nov. à partir d'un isolat du champignon obtenu de viande en décomposition dans la caverne de Ramioul en Belgique. Il croit et se reproduit plus abondamment sur des milieux faibles en sucres solubles et semble fortement adapté à croître sur des substrats protéïniques à des températures inférieures à 25°C. La plupart des *Microascus* spp. se caractérisent par la production du prolifique anamorphe *Scopulariopsis* et une croissance relativement rapide sur milieux usuels; les ascospores très minces et le lent développement des ostioles ascomatales sont cependant plus typiques du genre *Pithoascus*. La présence apparente d'un pore germinatif sur les ascospores suggère que le genre *Microascus* serait plus approprié.

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Introduction

For a number of years, the junior author has been involved in a detailed study of the Cave of Ramioul (Flemalle, Province de Liège, Belgium). During one of the visits to the cave he discovered a small piece of cooked chicken that had inadvertently been left by a previous visitor and that appeared to be undergoing a striking microbial transformation. To observe this phenomenon more closely Hubart placed some fresh pieces of cooked meat in a more accessible part of the cave and observed the changes that took place. At first the meat appeared to break down in an unremarkable manner, but after about 3 or 4 weeks small black spheres about 0.5 mm in diameter began to appear. After about 8 weeks the meat had disintegrated completely, leaving only a small cluster of the black spheres. The experiment was repeated several times in various parts of the cave, always with the same result. The phenomenon became well known to Hubart and associates, who eventually nicknamed the spheres "Le Caviar de Ramioul."

In 1985 Hubart sent a sample of the black spheres, preserved in formalin, to the senior author, who determined them to be fungal perithecia, probably produced by a member of the ascomycetous family Microascaceae. Because the material was preserved and hence dead, it was not possible to grow the fungus in axenic culture, a step necessary for reliable identification.

In April 1965, during a visit by the senior author to Belgium, we made a visit to the cave, observed the fungus *in situ*, and collected a number of samples for attempts at isolation. Later, in Toronto, perithecia and ascospores were placed on Weitzman and Silva-Hutners agar (Weitzman and Silva-Hutner, 1967), a medium that is especially useful for the isolation and identification of members of the Microascaceae. Although several fungi were isolated, none of these proved to be the desired one. In June, 1985 Hubart sent further collections of the material to Malloch who attempted this time to isolate it on Leonian's agar (Malloch 1981*a*). These attempts were successful, yielding moderately fast-growing colonies producing an abundant *Scopulariopsis* anamorph.

After cultivating the fungus on a number of media as described below we were successful in observing a complete life cycle and concluded that it represented an undescribed species of *Microascus*.

Description

Microascus caviariformis Malloch & Hubart, sp.nov.
Figs. 1–6

Coloniae in agaro "Difco Nutrient Agar" diam aetate 16 dierum 44–54 mm, lanosae, vinaceo-brunnescens, ad margine albae. Ascomata globosa, 409–704 mm crassa, ostiolata vel nonostiolata, nigra, glabra. Asci ad globosi pyriformi, 8.6–12.9 × 6.7–9.4 μm, octospori, evenescentes. Ascosporae asymmetricae, fusoideae, 6.3–8.9 × 2.0–2.8 μm, laeves, unicellares, dextrinoideae, pallide auranticae vel cupriae. Anamorpha *Scopulariopsis*. Cellulae conidiogerae 5.2–10.0 × 2.4–5.2 μm. Conidia obovoidea, ad basin truncata, vinaceo-brunnea, unicellares, catenulata.

HOLOTYPUS: TRTC 50940; in colonia sicca conservata est, ex caverna Ramioulensis, Flemalle, Prov. de Liège, Belgica (TRTC).

Colonies on nutrient agar (Difco) attaining a diameter of 44–54 mm in 16 days at 20°C, white and often appressed to lanose in the central 10–15 mm, with central area surrounded by a thick floccose-lanose zone beyond which it is appressed lanose, conspicuously zonate, with scattered drops of clear exudate but otherwise appearing dry, vinaceous or violaceous brown (Munsell 5R 5/2-4) alternating with nearly white zones,

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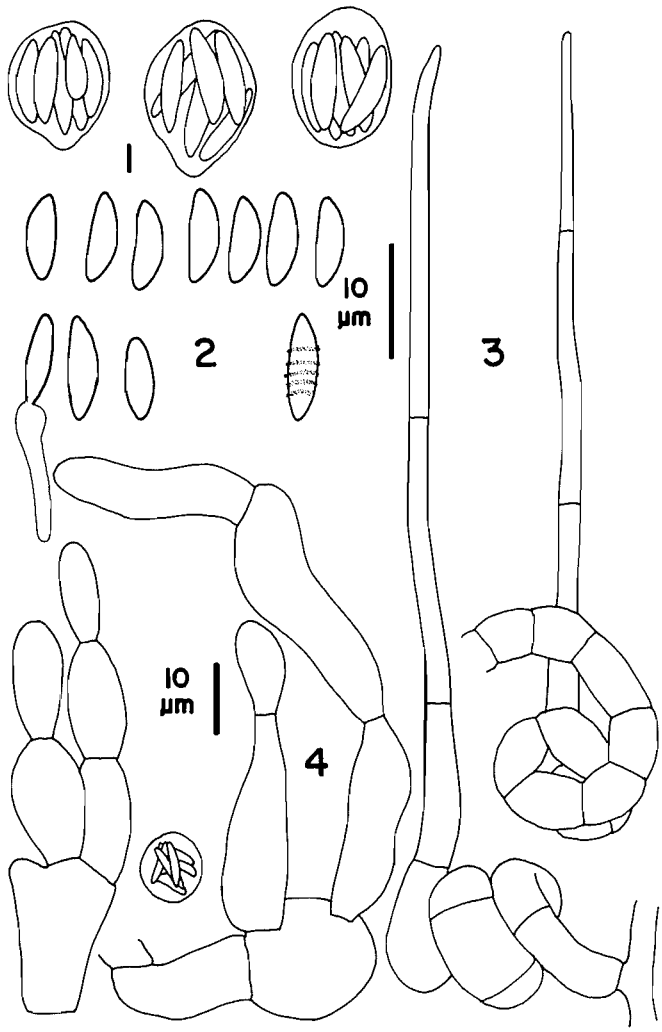


FIGS. 1–4. *Microascus caviariformis*. Fig. 1. Asci. Fig. 2. Ascospores: at lower left germinating after 3 days on nutrient agar; at lower right immature and showing rings of dextrinoid material. Fig. 3. Ascomatal initials. Fig. 4. Paraphysis-like sterile filaments from ascomatal centrum with ascus included to provide a size comparison.

even and appressed at the margin. Colony reverse similar in colour to the obverse but more dilute and tinted with yellow as a result of the colour of the medium. Diffusing pigments not produced.

Ascomatal initials (Fig. 3) produced abundantly within 16 days on nutrient agar and some other media (see below), forming regular helices of 2–5 turns, septate, prolonged at apex into a long filament of apparently indeterminate length, becoming surrounded by proliferating hyphae to form the ascomatal peridium. *Ascomata* spherical to subspherical, 409–704 μm in diameter (mean = 525.7 ± 75.8 μm), developing a small papillate ostiole or remaining cleistothecial, white at first but soon black, glabrous or with a few dark hyphal connections, with a thick pseudoparenchymatous peridium. Cells of the ascomatal peridium forming a *textura angularis* in surface view, forming a tissue 5–7 cells deep in cross section. *Ascomatal centrum* initially pseudoparenchymatous and composed of very large and fragile cells, later becoming filled with radiating moniliform paraphyses (Fig. 4), among which develop the asci. *Asci* (Fig. 1) subglobose to ellipsoidal, 8.6–12.9 × 6.7–9.4 μm (mean = 10.8 ± 0.7 × 7.9 ± 0.2 μm), with a more or less truncate base in some, borne singly at the base of the paraphyses, thin-walled, evanescent, eight-spored. *Ascospores* (Fig. 2) unilaterally flattened-fusoid, 6.3–8.9 × 2.0–2.8 μm (mean = 7.8 ± 0.6 × 2.5 ± 0.2 μm), with length/width ratio of 2.8–3.9 (mean = 3.2 ± 0.3), one-celled, smooth, dextrinoid when young, pale orange to copper-coloured in mass, probably with an apical germ pore, often parallel or nearly so in the ascus. *Anamorph* (Fig. 5) *Scopulariopsis*; composed of simple or irregularly branched conidiophores bearing clustered or solitary metulae, which in turn bear clusters of annellides. *Conidiophores* 8.8–36.5 × 2.7–7.0 μm, septate, arising from undifferentiated hyphae, never synnematous, hyaline, often branched irregularly one to several times. *Metulae* 7.3–12.8 × 3.1–6.6 μm, hyaline, smooth, cylindrical to swollen-ellipsoidal, in clusters of 2 to 5. *Annellides* with swollen base 5.2–10.0 × 2.4–5.2 μm and neck variable in length, with annellations indistinct, in clusters of 3 to 6 at the apex of the metulae. *Conidia* (Fig. 6) 5.4–7.2 × 3.7–4.7 μm (mean = 6.1 ± 0.4 × 4.2 ± 0.2 μm), obovoid to ellipsoidal, truncate at base, smooth, without pores or slits, dry, vinaceous to violaceous brown in mass, unicellular.

HOLOTYPE: TRTC 50940. Belgium, Province de Liège, Flemalle. Isolated from decaying meat in the Cave of Ramioul. J.-M. Hubart, June 1985. Dried culture on Difco nutrient agar. TRTC.

Cultures derived from the holotype have been deposited with the American Type Culture Collection, Rockville, MD, U.S.A. (ATCC), the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (CBS), and the University of Alberta Microfungus Collection and Herbarium, Edmonton, Alta., Canada (UAMH).

Taxonomy

The placement of *M. caviariformis* in *Microascus* is not without some reservations. von Arx (1973*a*) established a new genus *Pithoascus* and later (von Arx 1973*b*) expanded it to include some fungi that are quite similar to *M. caviariformis*. In fact, we initially identified our new isolate as *Pithoascus exsertus* (Skou) von Arx, a species with ascospores similar to those of *M. caviariformis* and frequently nonostiolate perithecia. It is only with later cultural studies that we began to doubt this identification. According to von Arx, species of *Pithoascus* are characterized by (*i*) slow-growing colonies (usually less than 1.5 mm/day), (*ii*) ascomata with a tendency to remain nonostiolate, (*iii*) narrowly fusiform ascospores ($D/d > 2$) without apparent germ pores, and (*iv*) lack of anamorphs. All of the species of *Pithoascus* are segregates from *Microascus*, leaving that genus to contain species with faster growth rates, conspicuously ostiolate ascomata, broad ascospores, and *Scopulariopsis* or *Wardomyces* anamorphs.

Microascus caviariformis resembles species of *Pithoascus* in ascomatal and ascospore characteristics, while resembling those of *Microascus* in its anamorph and growth rate. The reason that we have elected to put it in the latter genus is that the ascospores germinate as though they have an apical pore. The very slow development of the ostiole may not be significant; many species of ascomycetes representing numerous families appear to have lost or suppressed ostiole formation (Malloch 1981*b*).

The apparent intermediacy of *M. caviariformis* between *Microascus* and *Pithoascus* reinforces some doubts about the separation of these two genera. Domsch et al. (1980) and Roberts (1985) reported a *Scopulariopsis* anamorph in *P. intermedius* (Emmons & Dodge) v. Arx and questioned the utility

of maintaining both genera. It appears likely that as additional species are discovered and unusual substrata investigated, recognition of two genera for this group of fungi may become even more difficult. Although we suspect that this generic distinction will eventually become untenable, we hesitate to comment further until the type species of *Pithoascus, P. nidicola* (Massee & Salmon) v. Arx, is studied in detail, particularly on a variety of media and other growth conditions. Whatever alternative mycologists ultimately choose, it seems clear that as Roberts (1985) and Corlett and MacLatchy (1986) pointed out, the family Pithoascaceae Benny & Kimbrough is entirely superfluous.

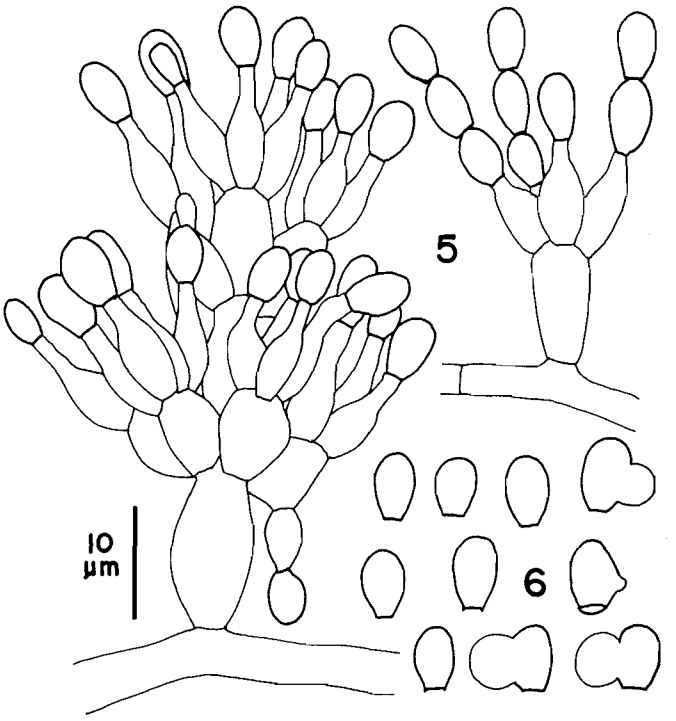
The other genus in which the new species might be sought is *Petriella* Curzi, but all of the species in that genus have *Scedosporium* or *Graphium* anamorphs and ascospores larger, darker, and broader than those of *M. caviariformis. Petriella musispora* Malloch is the only species of *Petriella* having ascospores as narrow as those of *M. caviariformis* but differs not only in having larger and darker ascospores but also in having a *Scedosporium* anamorph. Although Malloch (1970) originally described *P. musispora* as having a *Scopulariopsis* anamorph, he now repents. According to Malloch and Salkin (1984) species of *Scedosporium* produce conidia that collect in wet masses at the apex of the annellides, while those of *Scopulariopsis* species occur in dry chains.

The *Scopulariopsis* anamorph of *M. caviariformis* does not fit any of the described species of *Scopulariopsis* very closely and could be described separately as a new species. However, we do not believe that it is necessary to describe as new the anamorph of a fungus when it is not known to occur unaccompanied by the teleomorph. Since single-conidium cultures produce the teleomorph, it is likely that the species is homothallic.

Morphology

Although *M. caviariformis* is typical of the Microascaceae in most of its characteristics there are a few features that should be discussed. The first of these concerns the ascospores. As discussed previously, ascospores are important in determining the generic placement of an isolate of Microascaceae. Particularly important is the nature of the germ pore. von Arx (1973*a*, 1973*b*, 1975) has stressed the taxonomic importance of the usually prominent germ pores in ascospores of members of the genus *Microascus* and Malloch (1970) generalized that they are present in all Microascaeae. von Arx (1973*a*, 1973*b*, 1975) has pointed out that species of *Pithoascus* appear to lack germ pores, or at least these are not readily observed. *Microascus caviariformis* has ascospores resembling those of *Pithoascus* species in not having germ pores apparent. In many fungi, for example, *Chaetomium* and *Sordaria* species, the presence of germ pores, even when not observed, can be inferred by the way germination occurs. When a spore lacks germ pores, it may swell considerably during germination and then rupture to release the germ tube. If a germ pore is present, a germ tube is usually preceded by the extrusion of cytoplasm through the pore to form a globular "bubble." The "bubble" then goes on to produce a germ tube. Throughout this process the ascospore wall remains relatively unswollen or may even shrink slightly. This phenomenon has been illustrated for many fungi, including Microascaceae (e.g., see Malloch 1970), and was sought in *M. caviariformis*.

Ascospores of *M. caviariformis* were spread out on plates of nutrient agar and allowed to stand for germination. After several such attempts only one ascospore was ever observed to germinate. This ascospore (Fig. 2, bottom left) produced a typical germination "bubble" at one end and did not increase in size, indicating that a germ pore was probably present. This can be contrasted with the germinating conidia (Fig. 6, right side) which show a considerable stretching and distortion of the wall on the side producing the germ tube.



FIGS. 5 and 6. *Microascus caviariformis*. Fig. 5. *Scopulariopsis* anamorph. Fig. 6. Conidia: four at lower right germinating after 24 h on Blakeslee's malt agar.

A second feature of the ascospores that is noteworthy is the presence of rings of dextrinoid wall material when the very young spores are mounted in Melzer's solution (Fig. 2, bottom right). This appears to be a very constant phenomenon in all mounts of young perithecia.

The development of the perithecia appears to be typical of the Microascaceae (Corlett 1966; Malloch 1981*b*). As with all species in the family investigated to date the young centrum becomes filled with radiating paraphysis-like elements. Unlike those species studied by Corlett (1966) the paraphyses in *M. caviariformis* are very large and composed of almost monilioid elements. Corlett (1963, 1966) stressed the point that the asci in *Microascus* species may be terminal, lateral, or intercalary on the ascogenous hyphae, but we were unable to determine this in our isolate. In agreement with Corlett's observation was the presence of a thick pseudoparenchymatous layer at the periphery of the centrum of *M. caviariformis*.

Growth characteristics

Because of initial problems in growing *M. caviariformis* it was inoculated on a number of common as well as unconventional media and incubated at 20°C. Growth rates and perithecial production on these media are presented in Table 1. As noted above Weitzman and Silva-Hutner's agar is usually very good for members of the Microascaceae and it was thus surprising to find that our isolate grew extremely poorly on it. Growth was very slow, appressed, and nearly colourless. Conidiogenesis occurred only around the nutrient agar inocu-

TABLE 1. Growth characteristics of *Microascus caviariformis* on various culture media at 20°C

Medium	Growth rate (mm/day)	Mature perithecia present
Weitzman–Silva-Hutner's agar	1.2	–
Czapek's solution agar	1.7	–
Czapek's solution agar + 40% sucrose	1.4	–
Blakeslee's malt agar	2.5	–
Modified Leonian's agar	2.4	–
Potato dextrose agar (Gibco)	1.7	–
V8 agar	2.5	+
Nutrient agar (Difco)	3.1	+
Nutrient gelatin (Difco)	2.0	–
Soybean agar	3.5	+
Lentil agar	3.0	+
Pea agar	2.8	+
Sunflower agar	2.5	+

lum plug and the conidia were very pale in colour. Perithecia were never formed. Similarly poor growth occurred on Czapek's solution agar (Raper and Fennell 1965) and Czapek's solution agar with 40% sucrose.

Three media relatively high in carbohydrate and low in nitrogen were tried. These media, Blakeslee's malt agar (Raper and Fennell 1965), modified Leonian's agar, and potato dextrose agar (PDA), allowed abundant conidiogenesis but little or no teleomorph production. In all of these the colony margins were rather irregular. Growth on PDA, although restricted, was very thick and lanose and was remarkable for the colour of the conidial masses, which were pink, in contrast with the more violaceous tones on other media.

V8 vegetable juice agar (Miller 1955) allowed rapid growth and lanose colonies, abundant conidiogenesis, and limited perithecial production. Colony margins were somewhat irregular in outline. The relatively good growth on V8 agar was surprising in light of the poor performance on Weitzman and Silva-Hutner's agar. In our experience most fungi behave rather similarly on these two media.

Of all the more conventional media tried, Difco nutrient agar gave the best results. Colonies grew relatively quickly, producing abundant conidia, and thick and lanose growth, with an even spreading margin. Ascomatal initials appeared abundantly after 2 weeks but did not appear to develop rapidly into perithecia. The first evidence of perithecia came after 30 days cultivation. These were at first pure white and very smooth but within 2 days became first mottled and then entirely black. These were scattered throughout the colony and were mostly immersed in the agar. Generally perithecial development commenced when the colonies had reached the edges of the Petri dishes. Although perithecia appeared after 30 days, asci containing morphologically mature ascospores were not found until the 55th day.

Difco nutrient agar consits only of beef extract, peptone (a meat digest), and agar and is thus low in soluble carbohydrates and high in amino acids and proteins. Presumably any fungus that is able to grow on nutrient agar is capable of utilizing amino acids as an energy source and possibly also of hydrolyzing proteins. As a quick test for proteolysis *M. caviariformis* was grown on nutrient gelatin medium (Difco), a medium identical with nutrient agar but containing gelatin (a protein) as a solidifying agent instead of agar. The fungus grew rapidly on this medium and completely liquified it within 2 weeks.

The rapid growth of *M. caviariformis* on nutrient agar and poor growth on carbohydrate media raise the question of whether it is inhibited by the presence of carbohydrates or simply lacks an essential nutrient in these media. To test this we made up batches of nutrient agar containing 0, 1.0, 2.5, 5.0, 10.0, and 20.0 g/L of glucose. At 0, 1.0, and 2.5 g/L, growth and perithecial formation appeared to be unaffected by the glucose; in fact, the plates with 2.5 g/L showed slightly faster growth and heavier perithecium formation. At 5.0 g/L and above, growth was progressively inhibited and colonies were progressively more irregular in appearance. After 6 weeks at these higher concentrations no perithecia were observed. However, after 20 weeks it was found that the plates containing 10 and 20 g/L contained large numbers of rather large perithecia that were more regularly ostiolate and more often superficial on the agar surface. Thus it appears that glucose may inhibit growth but not eventual perithecium formation. The nature of this phenomenon is still under investigation.

The frequent occurrence of species of *Microascus* on stored seeds (Barron et al. 1961) and the apparent proteolytic nature of *M. caviariformis* led us to try one additional series of media. These media were prepared from lentils, peas, soybeans, and sunflower seeds (see Appendix 1 for formulae). It was decided to try sunflower seeds because of the recent isolation of several *Microascus* species from stored sunflower seeds by Roberts et al. (1986). Of the three legume media, soybean medium appeared to be best, allowing the fastest growth rate of all media tried and abundant conidiogenesis. Perithecia appeared abundantly after 22 days, about a week earlier than those on nutrient agar. Neither lentil medium nor pea medium allowed as rapid growth or abundant sporulation as soybean but were nevertheless better than the carbohydrate media tried earlier. It should be pointed out that in contrast to *M. caviariformis* some other unrelated ascomycetes grew and sporulated much better on lentil medium than on soybean medium. For none of the fungi tested was pea agar the best.

Sunflower medium proved to be the best of the seed media. Colonies grew rapidly and produced very abundant conidia and perithecia. Perithecia were produced superficially on the agar more often on this medium than any other. One characteristic of *M. caviariformis* on sunflower medium was the production of a bright green colour reaction that finally dominated the entire medium. This reaction was found to occur with some other fungi as well, but not all of them.

Attempts were made to grow *M. caviariformis* on nutrient agar at 5, 20, 23, 25, and 30°C. At these temperatures colonies reached an average diameter of 25, 73, 57, 21, and 0 mm, respectively, in 23 days. It is noteworthy that the colony at 5°C produced fertile perithecia *before* it reached the edge of the plate and that these were produced mainly at the surface of the medium.

Ecology

It should be emphasized at the outset that there is no clear-cut evidence that *M. caviariformis* is specifically adapted to growing in caves. Although it is morphologically unusual for a *Microascus* species, it has no features that are not common among normal terrestrial fungi. Its tolerance to low temperatures would be an advantage in the cool (10°C) cave environment, but it is capable of growing at temperatures as high as 25°C and would thus have no difficulty with temperatures in

such habitats as forest litter. On the other hand, it appears to be very abundant in the Cave of Ramioul and to be very effective in its dispersal there.

The occurrence of *M. caviariformis* on meat left in the cave and its good performance on proteinaceous culture media suggest that it may be associated with animals in the cave. There are several species of arthropods present in the cave and any of these might serve as a substrate, but at present we have no data to substantiate this.

Species of *Microascus* and *Scopulariopsis* frequently occur in situations where they come in close contact with insects. While evidence that they grow on insects, living or dead, is not available, it is quite possible that insects do acts as agents of dispersal. Several individuals of *Speonumus longicornis* Saulcy (Coleoptera) were collected aseptically in the Cave of Ramioul and sent to the laboratory in Toronto, where they were placed on plates of nutrient agar. Typical colonies of *M. caviariformis* developed from some of these. It appears most likely that these insects were not infected with the fungus but were merely carrying conidia or ascospores on their bodies. The Cave of Ramioul is known to contain about 124 species of animals and a number of these (Collembola, Diptera, and Coleoptera) have been found on the substrata where *M. caviariformis* occurs. Several attempts have been made to isolate *M. caviariformis* from the cave soil but have been unsuccessful. This again suggests that the fungus is associated with specific substrata and has effective vectors.

Although *M. caviariformis* should be able to grow outside the cave environment, it has not yet been discovered there. This may be due to the fact that the Cave of Ramioul provides special conditions favouring its proliferation. The cave appears to support a very limited fungal flora. We have so far isolated only about six or seven species of filamentous fungi and some yeast species from it. Given this relatively low level of competition it is not surprising that situations can be found where *M. caviariformis* appears to dominate. If a piece of meat were placed on soil outside the cave, it would probably be attacked by a much larger number of microorganisms as well as being eaten by a variety of animals. Under these conditions *M. caviariformis* might be difficult to detect. Thus what may be a ubiquitous fungus may be most easily observed in the unique subterranean environment of biologically active caverns such as the Cave of Ramioul.

Acknowledgements

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Appendix 1

Lentil medium

Green lentils	100.0 g
Agar	15.0 g
Distilled water	1.0 L

The lentils are soaked overnight in the water and then ground in a blender until creamy. The resulting mixture is strained through a 1-mm mesh and heated to dissolve the agar.

Pea medium

Prepared in the same way as lentil medium except that yellow split peas are used instead of lentils.

Soybean medium

Prepared in the same way as lentil medium except that soybeans are used instead of lentils.

Sunflower seed medium

Raw, hulled, unsalted sunflower seeds	100.0 g
Agar	12.0 g
Distilled water	1.0 L

The seeds are powdered in a blender and added to the dissolved agar. The solidified agar has a thin layer of oil on the surface.

All of the above media require a longer sterilization period than normal "sugar" media, about 30–45 min at 121°C.