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Pythium insidiosum sp. nov., the Etiologic Agent of Pythiosis

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***Pythium insidiosum* sp. nov., the etiologic agent of pythiosis, a cosmopolitan disease of horses, cattle, and dogs, is described and illustrated.**

Pythiosis (5) is a cosmopolitan granulomatous disease of horses, cattle, and dogs (10, 16, 17, 18) that is caused by a long-unnamed "phycomycete." (The term pythiosis was proposed in 1980 by Chandler et al. [5] as a more appropriate name for the equine disease variously referred to as bursatii, Florida horse leeches, granular dermatitis, hyphomycosis destruens equi, phycomycosis, phycomycotic granuloma, and swamp cancer.) It is probable that the first reports referring to this disease were those of Smith (20) and Drouin (9), who observed the mycelial nature of the etiologic agent. Although the organism could be cultured, it could not be identified, as it did not sporulate. de Haan and Hoogkamer (7) gave an extensive description of several cases of diseased horses in Indonesia and named the disease hyphomycosis destruens. This name was extended by de Haan (6) to hyphomycosis destruens equi. In a publication by Bridges and Emmons (4), the etiologic agent was called *Hyphomyces destruens*. It was not clear from that publication whether the authors intended to introduce a new binomial or only a provisional name they were anticipating to validate in the future (see reference 21, article 34.1b). The binomial was proposed without a Latin description (21, article 36), and the nomenclatural type was not designated for *H. destruens* (21, article 37). The binomial *H. destruens*, therefore, was in direct violation of articles 34.1b, 36, and 37 of the *International Code of Botanical Nomenclature* (21). Bridges and Emmons considered *H. destruens* to be a phycomycete (zygomycete) on the basis of its morphology in equine tissue as well as its broad, branched, sparsely septate to coenocytic, nonsporulating mycelium in cultures. They could not induce sporulation when *H. destruens* was grown on a wide variety of media. They speculated that the fungus they had studied "may be a species of *Mortierella*."

Austwick and Copland (1) reported that isolates recovered from horses afflicted with swamp cancer in Papua, New Guinea, formed biflagellate zoospores. Zoospore formation occurred when the isolates, grown on Sabouraud dextrose agar, were transferred to a sterilized aqueous medium of rotten maize silage. They concluded that *H. destruens* was a phycomycete belonging to the *Pythiaceae* in the *Peronosporales* and that it could be included in the genus *Pythium* Pringsheim. These investigators also stated that "Further work is in progress to establish whether it is a recognized or new species." However, additional work on the identity of this oomycete of the kingdom *Protoctista* was not published.

In 1980, Ichitani and Amemiya (11) isolated a *Pythium* sp. from a Japanese equine case of pythiosis. Based on the filamentous zoosporangia, smooth oogonia, and aplerotic (oospores not filling the oogonium), smooth oospores that it produced, they equated their isolate with *Pythium gracile* Schenk.

In the course of an intensive 2-year study of pythiosis in Costa Rican horses, numerous isolates of a *Pythium* species were studied at the Centers for Disease Control, Atlanta, Ga. A reliable and sensitive immunodiffusion test for diagnosing pythiosis in horses was developed (14). With the aid of comparative morphologic studies and use of the immunodiffusion and fluorescent-antibody tests, L. Mendoza, L. Kaufman, and P. G. Standard (unpublished data) found that the following represented a previously undescribed *Pythium* species: (i) the *Pythium* isolates from equine, bovine, and canine cases of pythiosis diagnosed in Costa Rica and the United States; (ii) two isolates from human cases of pythiosis in Thailand; (iii) *Pythium* sp. ATCC 28251 studied by Austwick and Copland (1); and (iv) ATCC 46947 (the Japanese isolate, which has been isolated and identified as *P. gracile* by Ichitani and Amemiya [11]). This finding was confirmed by an additional morphologic study at the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, where some isolates developed sexual organs in agar cultures. Zoosporangium formation was observed in water cultures; hence, a complete description of the new species could be prepared.

MATERIALS AND METHODS

Living cultures. The following isolates of *P. insidiosum* were studied.

(i) From horses in Costa Rica. We studied CBS 574.85 = ATCC 58643 = CDC B-4296, the strain from which the holotype was prepared; CBS 573.85 = ATCC 58644 = CDC B-4297; CBS 575.85 = ATCC 58642 = CDC B-4295; CBS 576.85 = ATCC 58641 = CDC B-4294; CBS 577.85 = ATCC 58640 = CDC B-4293; CBS 578.85 = ATCC 58639 = CDC B-4292; CBS 579.85 = ATCC 58638 = CDC B-4291; and CBS 580.85 = ATCC 58637 = CDC B-4290.

(ii) From horses in Papua, New Guinea. We studied ATCC 28251 = CDC B-4298, N.279/3 of Austwick and Copland.

(iii) From horses in the United States. We studied CDC B-4301, 60932 of R. I. Miller (*Pythium* sp.); CDC B-4302, 609224 of R. I. Miller (*Pythium* sp.); and CDC B-4311, A-1 of M. H. Attleberger.

(iv) From horses in Japan. We studied ATCC 46947 = CDC B-4299, K-K-16-77 of Ichitani and Amemiya.

(v) From dogs in the United States. We studied CDC

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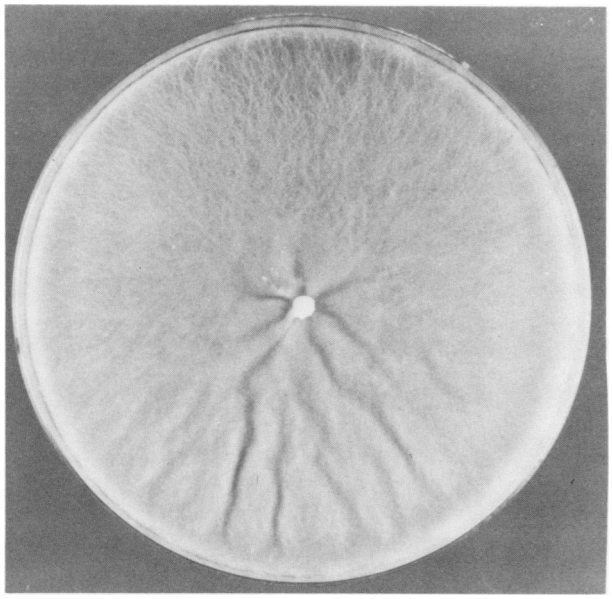

FIG. 1. Four-day-old culture of *P. insidiosum* (CBS 574.85) on Sabouraud dextrose agar.

B-4300, 25156 of R. I. Miller (*Pythium* sp.); and CDC B-4314 from M. H. Attleberger.

In addition, the following cultures of *Pythium* spp. were included in this study for comparison: *P. debaryanum* ATCC 10393 = CDC B-4304; *P. diclinum* CBS 664.79 = CDC B-4303, ex *Beta vulgaris*, The Netherlands; *P. graminicola* ATCC 28458 = CDC B-4306; *P. inflatum* ATCC 38894 = CDC B-4305; *P. monospermum* CBS 158.73 = CDC B-4312; *Lagenidium callinectes* CBS 581.85 = ATCC 24973 = FDL 301, from blue crab eggs, from C. F. Bland; *L. giganteum* CBS 580.85, from mosquito larva in North Carolina.

The isolates were grown on cornmeal agar, 2% malt extract agar, malt-yeast extract agar, Sabouraud dextrose agar, brain heart infusion agar, and several other agar media to study mycelial growth and oogonium production. Mating experiments were carried out on cornmeal agar at 24 and 37°C. Cardinal temperatures were determined on cornmeal and brain heart infusion agars. For studies of sporangium development, water cultures consisting of colonized grass leaves or small pieces of agar cultures in distilled water or soil extract were used. Soil extract was prepared by shaking 400 g of sandy soil in 1 liter of distilled water and then filtering and autoclaving the extract. Water cultures were kept in a warm room (25 to 30°C) or in an incubator at 33°C in the dark.

RESULTS

Comparative morphologic studies showed that the equine, canine, and bovine isolates represented an unnamed *Pythium* species that was distinct from other known *Pythium* and *Lagenidium* species. As a result of these findings, we describe it as a new species.

Pythium insidiosum De Cock, Mendoza, Padhye, Ajello et Kaufman, sp. nov. (Fig. 1 to 6).

= *Pythium* sp. sensu Austwick and Copland, Nature (London) **250**:84, 1974.

= *Pythium gracile* sensu Ichitani and Amemiya, Trans. Mycol. Soc. Jpn. **21**:263–265, 1980 (non-*P. gracile* Schenk, Verh. Phys. Med. Ges. Wurzb. **9**:13–20, 1859).

= *Hyphomyces destruens* Bridges and Emmons, J. Am. Vet. Med. Assoc. **138**:579–589, 1961, nomen nudum (reference 21, article 36).

Coloniae in agaro submersae vel mycelium aerium breve formantes, distincte vel indistincte radiantes, incoloratae vel albae vel dilute flavidae, in agaro Sabouraud-dextrose dicto hyphas radiantes conspicue undulatas formantes. Hyphae principales 4–6 (4–10) μm latae, vix angustatae; rami laterales saepe orthogonales, latitudine hyphis principalibus similies vel angustiores. Septa transversalia numerosa, praecipue in hyphis principalibus, prope quae hyphae saepe in segmenta longitudine variabilia diffranguntur, 50–300 (12–300) μm, plerumque semel vel bis ramosa. Appressoria clavata nonnumquam praesentia. Intumescentiae hypharum globosae intercalares raro formatae, 12–28 μm diam. Zoosporangia in mycelio aqua submerso formantur, filamentosa, ab hyphis vegetivis haud distincta, seu terminalia seu intercalaria; tubi evacuationis tenuitunicati, forma irregulares, 45–700 μm vel longiores, 3–4 μm lati, ad 5–8 μm dilatati. Maturitate plasma in vesiculam terminalem ad 20–60 μm diam extensam transfertur in qua zoosporae biflagellatae, 12–14 × 6–8 μm, formantur, encystatae 8–12 μm diam.

Oogonia in nonnullis culturis 24 vel 30°C formata, intercalaria, nonnumquam subterminalia, incolorata, levia, subglobosa, partem hypharum supportantium untribque includentia, tubo fertilisationis indentata, 23–30 (19–36) μm diam. Antheridia singula vel bina, raro terna, diclina, terminalia, inflate clavata vel tubularia, 11–37 × 6–10 μm, oogonio tota longitudine adfixa, persistentia, tubo fertilisationis crassitunicato, 4–6 μm lato. Oosporae apleroticae, raro quasi pleroticae, plasmate flavido repletae, tubo fertilisationis ad latus repulsae, 20–25 (17–27) μm diam, pariete 1–3 (1–4) μm crasso. Temperatura minima 10°C, optima 34–36°C, maxima 40–45°C.

Typus: Colonia exsiccata CBS 574.85, isolatus ex Equo cavallo morbo "pythiosi" afflicto in Costa Rica.

Colonies on cornmeal agar, 2% malt extract agar, malt-yeast extract agar, and brain heart infusion agar are submerged or have a very short aerial mycelium, with a vague or distinct, finely radiate pattern. Colonies are colorless to white or yellowish white. Colonies on Sabouraud dextrose agar have an undulating, radiate pattern (Fig. 1).

The main hyphae mostly measure 4 to 6 (range, 4 to 10) μm in diameter, do not taper, and have rounded tips. The lateral branches are often perpendicular to and as wide as the main hyphae or are sometimes thinner than the main hyphae and measure 2.5 μm or more in diameter. Cross septa are present in viable plasma-filled hyphae, particularly in the main hyphae. The cross septa are sparse in young hyphae grown on cornmeal agar and in hyphae developed in water cultures. They are relatively abundant in old viable hyphae in colonies grown on cornmeal agar and even in young hyphae in colonies grown on malt-yeast extract agar, Sabouraud dextrose agar, and several other media. The septa are apparently double as the hyphae disarticulate into segments with blunt ends when they are squashed (Fig. 2a). In squashed preparations, hyphal segments of various lengths and with one or more side branches measuring 50 to 300 (range, 12 to 300) μm are more generally observed (Fig. 2b and c). Club-shaped appressoria (Fig. 2d to g) and intercalary, globose hyphal swellings measuring 12 to 28 μm in diameter and presumably representing undeveloped oogonia are also observed (Fig. 2h and i).

The zoosporangia are produced only in water cultures. They are filamentous and are not differentiated from the

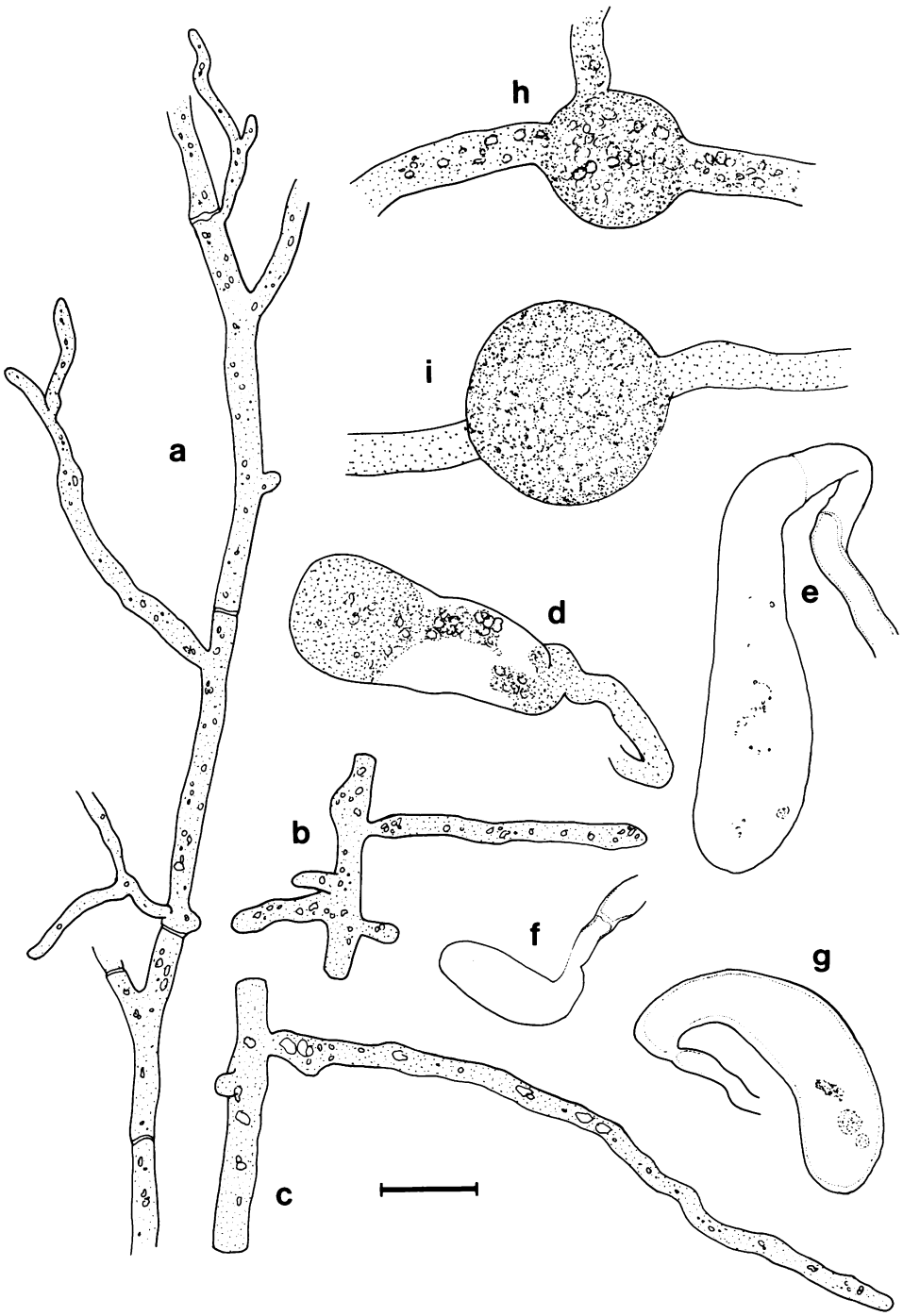

FIG. 2. *P. insidiosum*. (a) Part of septate hypha on malt-yeast extract agar. (b and c) Single segments disarticulated after squashing of a hypha on Sabouraud dextrose agar. (d to g) Appressoria. (h and i) Hyphal swellings. Bar, 25 μm for panels a to c and 10 μm for panels d to i.

vegetative hyphae. Either a terminal part of a sparsely septate hypha or a complete segment of a hypha may function as a sporangium (Fig. 3). The discharge tubes develop at the tip of a hypha or laterally on a segment. They are thin walled, irregular in outline, 45 to 700 μm or more in length, and 3 to 4 μm in diameter and widen at the tip to measure 5 to 8 μm. The transition from a thin hypha into a discharge tube is not always distinct. At maturity, sporangial protoplasm flows through an apical opening in the discharge tube and forms a vesicle. The vesicles are globose to subglobose and hyaline and measure 20 to 60 μm in diameter. Through progressive cleavage, biflagellate zoospores are formed inside the vesicle, and the vesicle wall continues to extend during this process (Fig. 4). The zoospores are released through a break in the vesicle wall. The zoospores have two laterally inserted flagella and measure 12 to 14 by 6 to 8 μm. They are attracted by boiled pieces of grass blades and nonsterile human and canine hair. The encysted

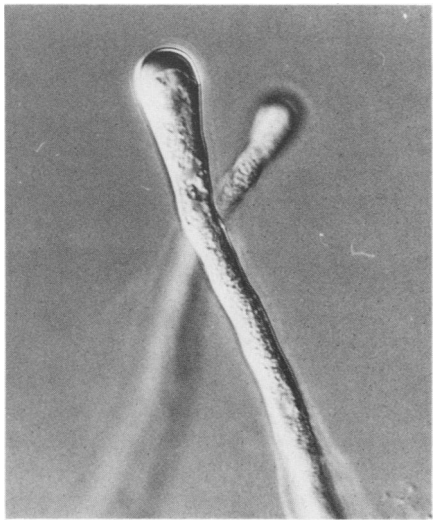

FIG. 3. Early stage in the development of a discharge tube in a grass leaf-water mount. Magnification, ×875.

zoospores are globose and measure 8 to 12 μm in diameter (Fig. 5). They germinate by means of germ tubes.

Oogonia develop occasionally on cornmeal agar at 24 and 30°C and often cluster around the solid parts of the agar. They are intercalary, occasionally subterminal in position, colorless, smooth, subglobose, and include a small part of the subtending hyphae at both ends (Fig. 6a and b). They often appear deformed because of the rigid fertilization tube and measure 23 to 30 (range, 19 to 36) μm in diameter. One, two (Fig. 2a and b), or, rarely, three antheridia per oogonium are observed. They are diclinous (oogonium and its artheridium or antheridia originating from different hyphae), terminal, and inflated clavate to tubular in shape and measure 11 to 37 by 6 to 10 μm. The antheridia are attached over their entire length to the oogonium. The tip of the antheridium produces a fertilization tube that indents the oogonial wall (Fig. 2c and b). The antheridia are persistent and appear slightly shrivelled after fertilization. The fertilization tube is relatively thick walled and measures 4 to 6 μm in diameter (Fig. 6). The oospores are aplerotic (not filling the oogonium completely), occasionally almost plerotic (filling the oogonium), and pressed to one side of the oogonium by the rigid fertilization tube. They measure 20 to 25 (range, 17 to 27) μm in diameter, with a wall 1 to 3 (range, 1 to 4) μm thick. The oospore contents often appear yellowish and finely granular and show a distinct ooplast (Fig. 6).

Cardinal temperatures: minimum, 10°C; optimum, 34 to 36°C; maximum, between 40 and 45°C.

Holotype: CBS 574.85. Living cultures from which the holotype was prepared have been deposited at the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (CBS 574.85) and at the American Type Culture Collection, Rockville, Md. (ATCC 58643).

Daily growth on cornmeal agar is 8 mm at 24°C and 12.5 mm at 34°C; that on brain heart infusion agar is 11 mm at 24°C and 18.5 mm at 34°C.

Aside from CBS 574.85, only two other isolates (CBS 576.85 and CBS 578.85) developed oogonia on cornmeal agar similar to those of the type strain. Stimulation of oogonium production was not observed in our mating experiments. All of the equine, canine, and bovine isolates and two of the human isolates proved to be so similar antigenically that they were considered to represent one species. The results of these studies are being prepared for publication. The isolates differed only slightly in their average hyphal diameter and growth rate.

DISCUSSION

The *Pythium* species of the order *Peronosporales* belong to the phylum *Oomycota* of the kingdom *Protoctista* (13). The oomycetes are eucaryotic and produce zoospores with two flagella, one tinsel and the other whiplash. The nuclei in the vegetative hyphae of members of the *Oomycota* are diploid, and their hyphal cell walls usually contain a celluloselike material, even though some oomycetous fungi have chitin in their hyphal cell walls. All of these characteristics separate the members of the *Oomycota* from the members of the kingdom Fungi. Some of the *Pythium* species are pathogenic for plants and such animals as some fish and crustacea. For more complete details of the life cycle of *Pythium* spp., readers should refer to the relevant literature (12, 13, 19).

P. insidiosum possesses characteristics of *Lagenidium* (order *Lagenidiales*) and *Pythium* (order *Peronosporales*) species. In both genera, zoospores develop inside a vesicle. The genus *Lagenidium* consists of holocarpic oomycetes

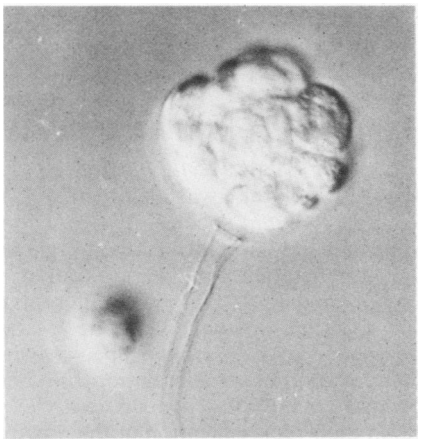

FIG. 4. Differentiation of *P. insidiosum* zoospores in a vesicle containing the protoplasm discharged from a sporangium. Magnification, ×875.

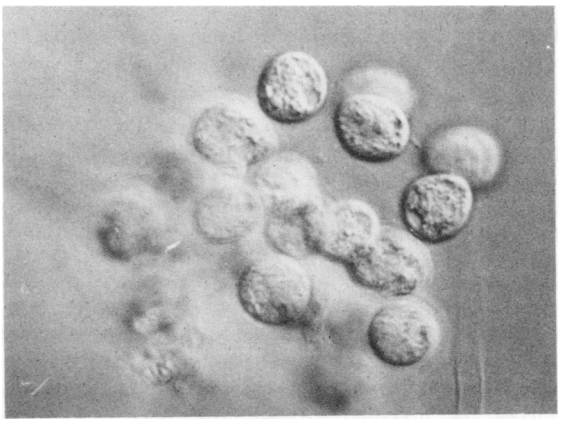

FIG. 5. Group of encysted *P. insidiosum* zoospores. Magnification, ×875.

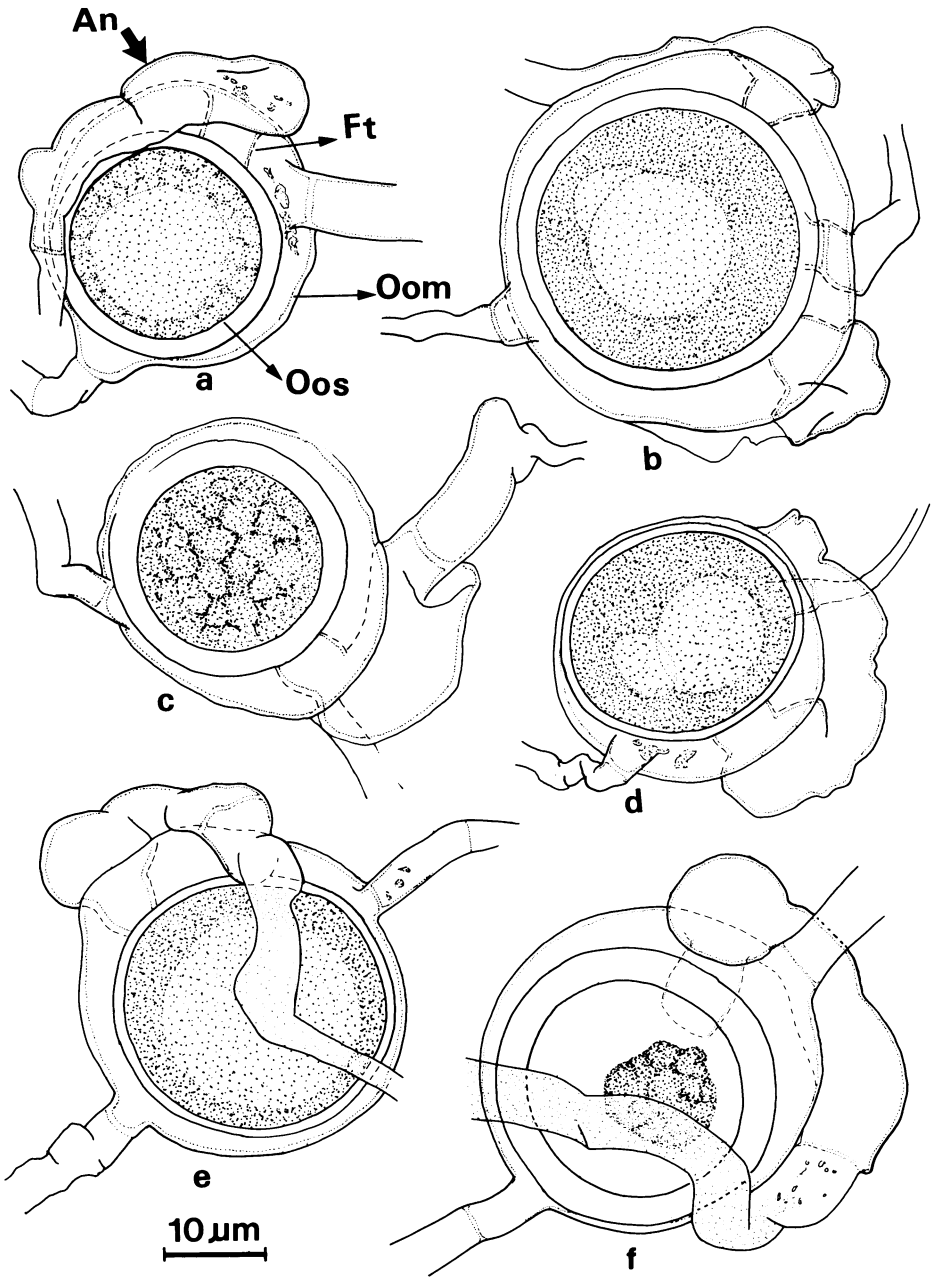

FIG. 6. *P. insidiosum*. (a to f) Oogonium (Oom), oospore (Oos), antheridium (An), fertilization tube (Ft), and diclinous antheridia attached to oogonia as well as to fertilization tubes and aplerotic oospores.

with a one-celled thallus or a mycelium of slight extent, built up of segments. A few species develop an extensive mycelium of septate hyphae, namely, *L. callinectes* Couch, *L. caudatum* Barron, *L. chthalamophilum* Johnson, *L. closterii* De Eildeman, *L. giganteum* Couch, *L. humanum* Karling, and *L. marchalianum* De Wildeman (12). In the genus *Pythium*, cross septa only develop to delimit reproductive organs or to separate empty from viable parts in older hyphae. The septation or segmentation of the thicker, viable hyphae of *P. insidiosum*, as shown on many agar media, is *Lagenidium*-like, that is, the thallus is comparable to that of the mycelial *Lagenidium* species. The sexual structures, as far as known, are less well differentiated in the *Lagenidium* species than in *P. insidiosum*. In this respect, *P. insidiosum* is closer to the *Pythium* species, although a thick-walled fertilization tube was observed in *L. giganteum* (3) and not in any *Pythium* species. The assignment of the equine, canine, and bovine isolates to the genus *Pythium* here was based on the phenotypic nature of the septation or segmentation and on the relatively well-differentiated sexual structures. In fact, *P. insidiosum* connects the mycelial *Lagenidium* species to the *Pythium* species with filamentous, noninflated sporangia. A relationship between the genera *Lagenidium* and *Pythium* was recently expressed by Dick et al. (8) in their reclassification of the downy mildews. They placed both genera in a new order, *Pythiales*.

Most *Lagenidium* species cannot be cultured. They have only been described on the basis of their development on and

in host tissues. Thus, their comparison with *P. insidiosum* is difficult. Most of the mycelial *Lagenidium* species appear to have wider hyphae, less differentiated oogonia and antheridia, or both than does *P. insidiosum*. *P. insidiosum* may be close to *L. caudatum* (2). However, this species, of which the extremely short diagnosis only gives data on its hyphae, sporangia, and zoospores, has thinner hyphae, shorter discharge tubes, and considerably bigger zoospores than does *P. insidiosum*.

P. insidiosum differs from all known *Pythium* species by the septation of the main hyphae, as produced in particular on many agar media, and by the formation of conspicuous, thick-walled fertilization tubes. It may be assigned to the group of *Pythium* species with filamentous, noninflated sporangia. In this group, it is unique because of its large antheridia and high optimum temperature, in addition to the characteristics already mentioned above. Only *P. sulcatum* Pratt and Mitchell has tubular antheridia but differs in almost every other characteristic from *P. insidiosum*. *P. chondricola* De Cock and *P. porphyrae* Takashi and Sasaki are the only species in this group with predominantly intercalary oogonia, but these marine species have thinner hyphae and plerotic oospores.

The identification of the equine isolate from Japan as *P. gracile* by Ichitani and Amemiya (11) added more confusion to the already uncertain taxonomic status of *P. gracile* Schenk. According to Van der Plaats-Niterink (19), *P. gracile* Schenk sensu de Bary is synonymous with *P. monospermum* Pringsheim.On the other hand, *P. gracile* sensu Middleton (15) is synonymous with *P. diclinum* Tokunaga. A morphologic study of the isolate of Ichitani and Amemiya revealed that the vegetative hyphae of this isolate were broader (8 to 10.5 μm) than those of *P. diclinum* (5.6 μm). Its encysted zoospores were also larger (8 to 12 μm) than those of *P. diclinum* (6 to 7 μm). Moreover, in a comparative antigenic study, Mendoza et al. (unpublished data) found the isolate of Ichitani and Amemiya to be identical to all of the equine, canine, and bovine isolates of *P. insidiosum* that we have studied. The *P. insidiosum* isolates were found to differ from *P. debaryanum* Hesse, *P. graminicola* Subramanium, *P. inflatum* Matthews, and *P. monospermum* Pringsheim isolates in their antigenic properties.

P. insidiosum causes pythiosis in equines, canines, and bovines, but we have also confirmed it as an etiologic agent of pythiosis in two human cases of subcutaneous lesions in Thailand. These cases will be reported separately.

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