



Evolution of the Sterol Biosynthetic Pathway of *Pythium insidiosum* and Related Oomycetes Contributes to Antifungal Drug Resistance

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ABSTRACT Pythiosis is a life-threatening infectious disease caused by the oomycete *Pythium insidiosum*. Direct exposure to *Py. insidiosum* zoospores can initiate infections of the eye, limb, gastrointestinal tract, or skin/subcutaneous tissue. Treatments for pythiosis have mostly relied on surgery. Antifungal drugs are generally ineffective against *Py. insidiosum*. However, one patient with an invasive *Py. insidiosum* infection recovered completely following treatment with terbinafine and itraconazole. Additionally, the drug target sterol biosynthetic enzymes have been identified in the oomycete *Aphanomyces euteiches*. It remains an open question whether *Py. insidiosum* is susceptible to the antifungal drugs and harbors any of the known drug target enzymes. Here, we determined the *in vitro* susceptibilities of terbinafine and itraconazole against 30 isolates of *Py. insidiosum*. We also analyzed endogenous sterols and searched for genes encoding the sterol biosynthetic enzymes in the genomes of *Py. insidiosum* and related oomycetes. The susceptibility assay showed that the growth of each of the *Py. insidiosum* isolates was inhibited by the antifungal agents, but only at difficult-to-achieve concentrations, which explains the clinical resistance of the drugs in the treatment of pythiosis patients. Genome searches of *Py. insidiosum* and related oomycetes demonstrated that these organisms contained an incomplete set of sterol biosynthetic enzymes. Gas chromatographic mass spectrometry did not detect any sterol end products in *Py. insidiosum*. In conclusion, *Py. insidiosum* possesses an incomplete sterol biosynthetic pathway. Resistance to antifungal drugs targeting enzymes in the ergosterol biosynthetic pathway in *Py. insidiosum* was due to modifications or losses of some of the genes encoding the drug target enzymes.

KEYWORDS *Pythium insidiosum*, pythiosis, sterol biosynthetic enzyme, *in vitro* susceptibility, evolution, antifungal drug

Pythiosis is a life-threatening infectious disease and has been increasingly reported from tropical, subtropical, and temperate countries (1–4). The etiologic agent is the fungus-like organism *Pythium insidiosum* (1). The microscopic morphology of *Py. insidiosum* is similar to that of filamentous fungi (1, 3). However, based on phylogenetic analyses, *Py. insidiosum* belongs to the oomycetes, a unique group of microorganisms in several genera, such as *Pythium*, *Phytophthora*, *Hyaloperonospora*, *Albugo*, *Saprolegnia*, and *Aphanomyces* (5, 6). Unlike most other pathogenic oomycetes, which infect plants, *Py. insidiosum* infects humans and animals (i.e., horses and dogs) (1–3, 6). In the environment, *Py. insidiosum* presents as mycelia and motile zoospores (7). *Py. insidiosum* infection occurs when a patient contacts or ingests pathogen-contaminated water.

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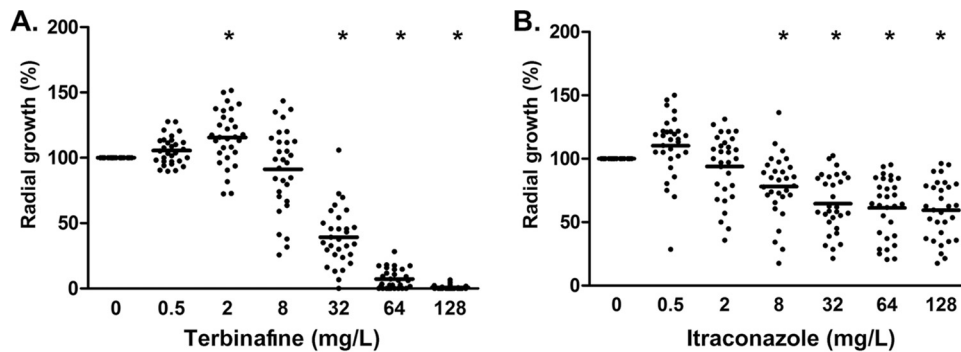


FIG 1 *In vitro* drug susceptibility assay of 30 *Py. insidiosum* isolates against antifungal drugs. The drug concentrations of terbinafine (A) and itraconazole (B) used in the *in vitro* susceptibility assay are 0.5, 2, 8, 32, 64, and 128 mg/liter. A dot represents the percentage of radial growth of an individual isolate in relation to the growth of the same isolate exposed to no drug (control). *, statistical significance compared to the no-drug control.

Common sites of the *Py. insidiosum* infection include the skin, eye, artery, and gastrointestinal tract (1–3).

Due to the aggressive nature of the infections, patients or animals with pythiosis often die if proper management is not provided. The treatment of choice for pythiosis has mostly relied on extensive surgery to remove the infected organ (2, 3). Use of an immunotherapeutic vaccine (which is a crude protein extract of *Py. insidiosum*) exhibits various efficacies, depending on the affected host, with 97% favorable responses in cattle, 60 to 85% in horses, 56% in humans, and only 33% in dogs (8, 9). Conventional antifungal drugs, inhibiting the production of fungal ergosterol, have been used for treatment of pythiosis, but drug resistance is usually observed (2, 4, 10). The oomycetes, including *Phytophthora* and *Pythium* species, are sterol auxotrophs and thus require exogenous sterols for basic metabolism (11–13). Lack of the drug target sterol biosynthetic enzymes in these organisms explains treatment failure of the antifungal drugs (12, 14).

Contrary to the above findings, Shenep et al. reported on successful medical treatment of a patient with invasive *Py. insidiosum* infection using a combination of two antifungal drugs: terbinafine and itraconazole (15). In addition, Madoui et al. analyzed the transcriptomic data of *Aphanomyces euteiches* (16, 17) and showed that this oomycete microorganism contains several sterol biosynthetic enzymes responsible for production of endogenous sterols (12). Such discordant results raise questions about *Py. insidiosum*'s susceptibility to the antifungal drugs and whether the pathogen produces the drug target enzymes in the sterol biosynthetic pathway.

A better understanding of the drug resistance mechanism(s) of *Py. insidiosum* could lead to the development of more efficient agents for the control of this and related pathogens. Recently, genome sequences and transcriptomic data of many oomycetes (i.e., *Py. insidiosum* [18–21], non-*insidiosum* *Pythium* species [22, 23], *Phytophthora* species [14, 24, 25], *Hyaloperonospora* species [26], *Albugo* species [27], *Saprolegnia* species [28], and *Aphanomyces* species [17]) have been made publicly available. These genetic resources were used to explore the sterol biosynthetic pathway in *Py. insidiosum* and related oomycetes. In this study, we aimed to demonstrate the *in vitro* growth inhibitory effects of two key antifungal drugs (terbinafine and itraconazole) against clinical isolates of *Py. insidiosum*. We also sought to identify endogenous sterols and search for genes encoding sterol biosynthetic enzymes in this pathogen.

RESULTS

Antifungal drug susceptibility of 30 *Py. insidiosum* isolates. Two key antifungal drugs, terbinafine and itraconazole (concentrations of 0 [no drug], 0.5, 2, 8, 32, 64, and 128 mg/liter), were tested against 30 isolates of *Py. insidiosum*. In relation to the no-drug control, terbinafine at 2 mg/liter modestly, but significantly, increased the average growth of *Py. insidiosum* to 116% (Fig. 1A). In contrast, at the concentrations 32,

64, and 128 mg/liter, terbinafine significantly reduced the average growth of *Py. insidiosum* to 39%, 7%, and 0.7%, respectively (Fig. 1A). Terbinafine at concentrations of 32, 64, and 128 mg/liter completely inhibited the growth of 1, 8, and 13 of the *Py. insidiosum* isolates, respectively.

When *Py. insidiosum* was tested against 0.5 mg/liter itraconazole, the average growth was modestly, but not significantly, increased to 110% of that of the no-drug control (Fig. 1B). However, when the organism was tested against the higher drug concentrations, the average growth of *Py. insidiosum* was significantly decreased to 78% (at 8 mg/liter itraconazole), 65% (32 mg/liter), 61% (64 mg/liter), or 59% (128 mg/liter) of that of the no-drug control (Fig. 1B). None of the *Py. insidiosum* isolates was completely inhibited by itraconazole.

Identification of *Py. insidiosum* sterols. The sterol extract of *Py. insidiosum* (strain Pi-5) was analyzed for the presence of the sterol end products, such as cholesterol, stigmasterol, ergosterol, and fucosterol. Commercially available sterol standards (cholesterol, stigmasterol, ergosterol, and fucosterol) were used to generate the reference gas chromatography-mass spectrometry (GC-MS) peaks. The sterol extract from *Candida albicans* strain ATCC 90028 was also prepared and analyzed in parallel with the extract from *Py. insidiosum*. To serve as the internal control, 5 α -cholestane was added to the sterol extracts of *Py. insidiosum* and *Ca. albicans*. Two hundred milligrams of lyophilized cells from either *Py. insidiosum* or *Ca. albicans* was derivatized for GC-MS analysis. The retention times of the reference sterols were 15.34 min for 5 α -cholestane (peak 1), 25.80 min for cholesterol (peak 2), 29.26 min for ergosterol (peak 3), 31.56 min for stigmasterol (peak 4), and 33.88 min for fucosterol (peak 5) (Fig. 2A). Two GC-MS peaks, corresponding to 5 α -cholestane (peak 1) and ergosterol (peak 3), were identified in *Ca. albicans* (Fig. 2B), whereas only one GC-MS peak, corresponding to the internal control 5 α -cholestane, was detected in *Py. insidiosum* (data not shown). Even when the amount of lyophilized cells from *Py. insidiosum* was increased 7-fold (1,400 mg), only the 5 α -cholestane peak was observed (Fig. 2C).

Identification of enzymes involved in sterol biosynthesis. The full-length deduced protein sequences of 22 enzymes involved in the mevalonate pathway (HMG1, HMG2, ERG8, ERG10, ERG12, ERG13, ERG19, and IDI1) and the sterol biosynthetic pathway (ERG1 to -7, ERG9, ERG11, ERG20, and ERG24 to -27) of the model organism *Saccharomyces cerevisiae* (Fig. 3; Tables 1 to 3), were subject to BLAST search against the translated genomes or transcriptomes of 17 oomycetes (including *Py. insidiosum*), three algae, two fungi, and one diatom (Table 4). Both fungi (*Ca. albicans* and *Aspergillus fumigatus*), two algae (*Aurantiochytrium limacinum* and *Ectocarpus siliculosus*), and only one oomycete (*Saprolegnia parasitica*) harbored the complete set of 8 enzymes in the mevalonate pathway (Table 2). Only the fungi possessed the complete set of 14 enzymes in the sterol biosynthetic pathway (Table 3). ERG5, ERG10, ERG11, and ERG26 homologs were identified in all species included in this study. ERG2 homologs were absent in all nonfungal microorganisms.

Regarding the oomycetes, two enzymes in the mevalonate pathway (HMG1 and HMG2) and three enzymes in the sterol biosynthetic pathway (ERG1, ERG7, and ERG9) were absent in all organisms of the non-Saprolegniales subgroups (i.e., Pythiales, Peronosporales, and Albuginales) but present in all organisms of the Saprolegniales subgroup (Tables 2 and 3). *Py. insidiosum* contained 5 out of 8 enzymes in the mevalonate pathway, including ERG8, ERG10, ERG13, ERG19, and IDI1 (Table 2). In addition, *Py. insidiosum* harbored genes for only 6 out of 14 sterol biosynthetic enzymes: ERG3, ERG5, ERG11, ERG20, ERG24, and ERG26 (Table 3).

Phylogenetic analysis of ERG11. ERG11-encoding sequences of all microorganisms included in this study were retrieved for phylogenetic analysis (Table 3). The resulting tree showed two major phylogenetic groups (Fig. 4): group 1 included 3 fungi (*S. cerevisiae*, *Ca. albicans*, and *Aspergillus fumigatus*), 3 algae (*Au. limacinum*, *Aureococcus anophagefferens*, and *Ec. siliculosus*), 2 oomycetes (*Aphanomyces euteiches*, and *Sa. parasitica*), and one diatom (*Phaeodactylum tricornutum*), and group 2 contained the 15

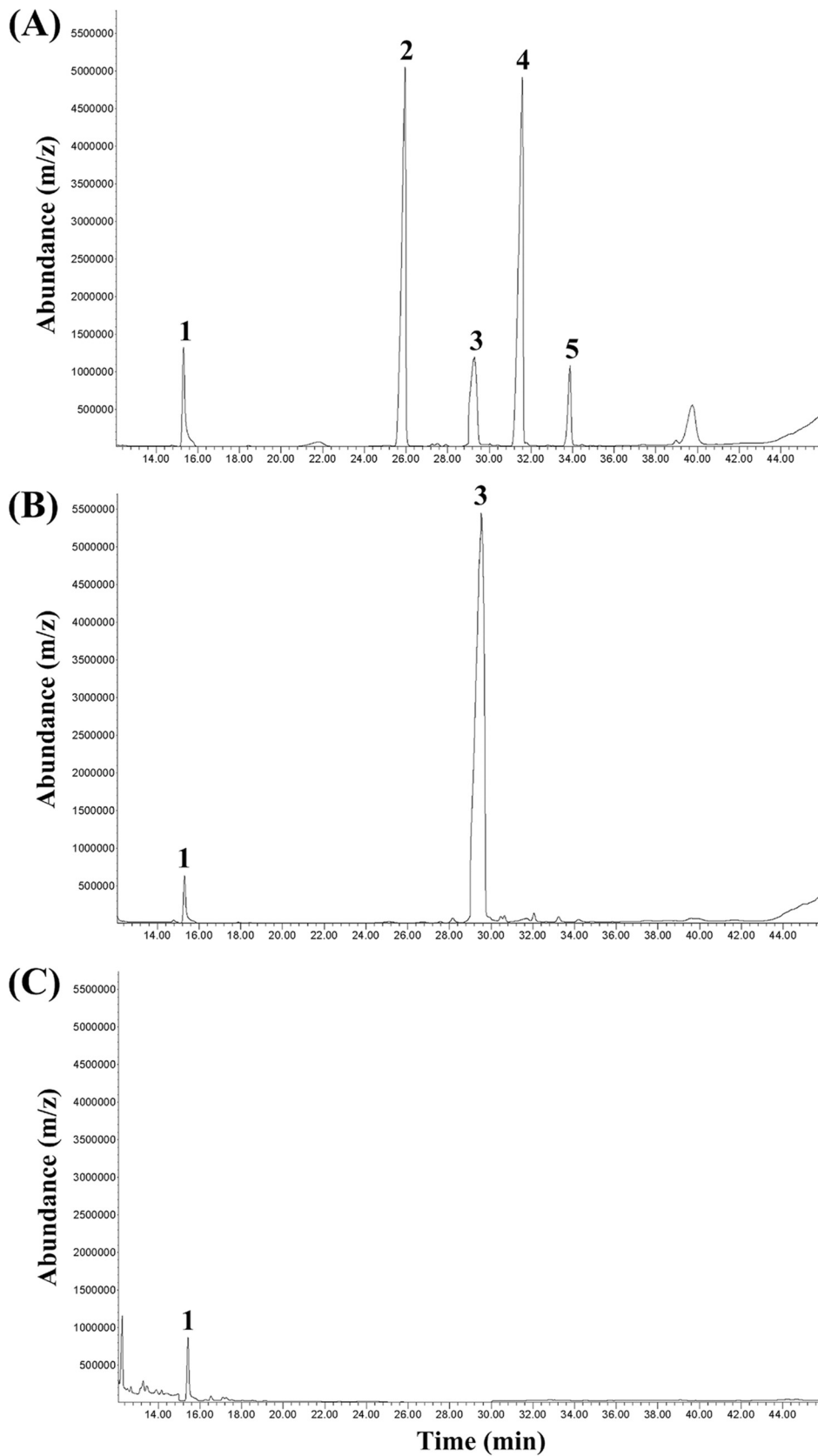


FIG 2 Total ion chromatogram of the sterol extracts generated by GC-MS analysis: (A) Standard mixture of sterol end products, (B) sterol extract from the *Ca. albicans* strain ATCC 90028 (200 mg lyophilized cells), and (C) sterol extract from *Py. insidiosum* strain Pi-S (1,400 mg lyophilized cells). Peaks 1 to 5 indicate 5α -cholestane (internal control), cholesterol, ergosterol, stigmasterol, and fucosterol, respectively.

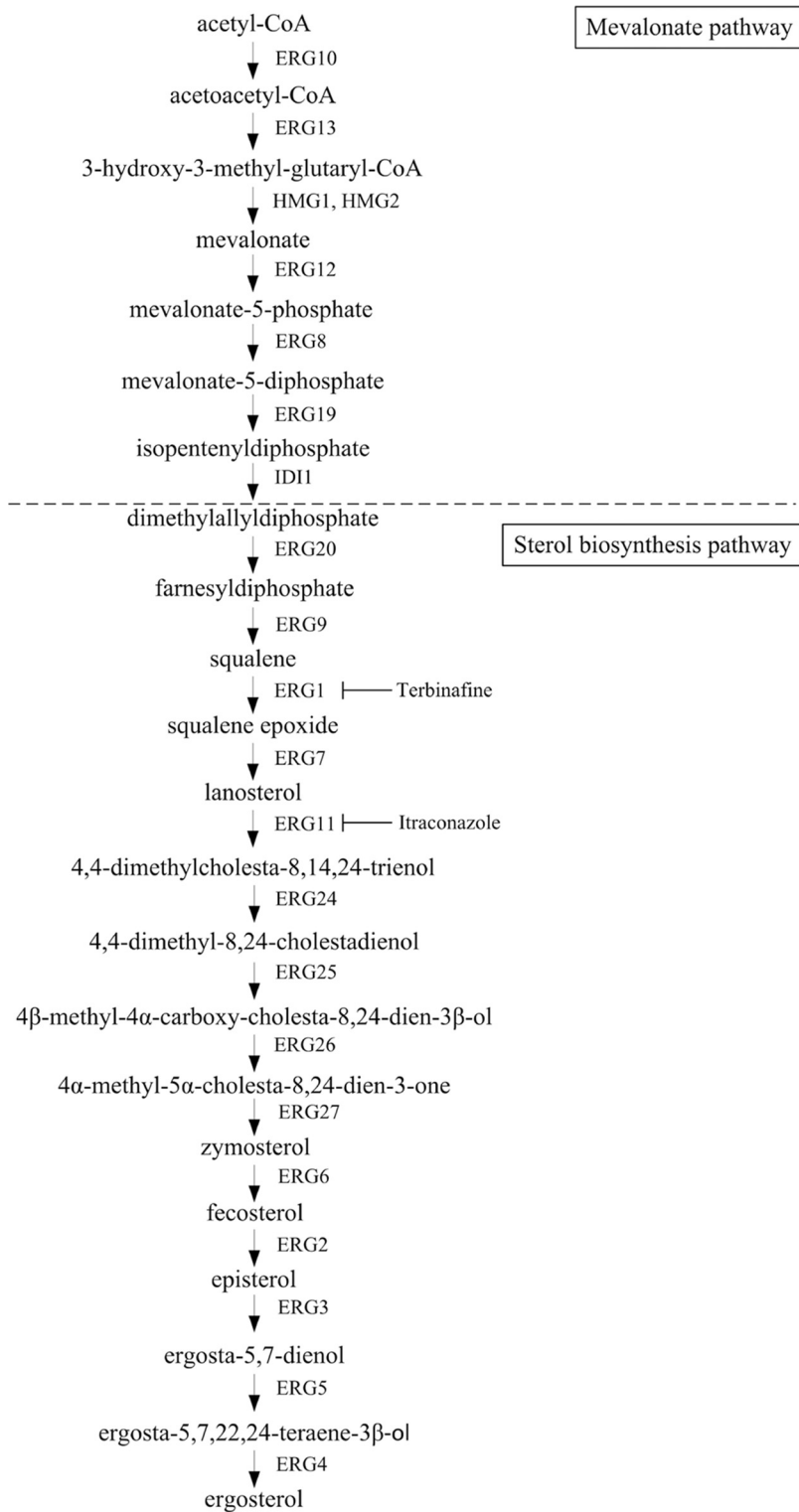


FIG 3 Schematic diagram showing enzymes and substrates in the mevalonate (upper part) and sterol biosynthesis (lower part) pathways. ERG1 and ERG11 are the drug targets of terbinafine and itraconazole, respectively.

remaining oomycetes (7 *Pythium* species [*Py. insidiosum*, *Py. ultimum*, *Py. aphanidermatum*, *Py. arrhenomanes*, *Py. irregulare*, *Py. iwayamai*, and *Py. vexans*], 6 *Phytophthora* species [*Ph. capsici*, *Ph. infestans*, *Ph. sojae*, *Ph. ramorum*, *Ph. parasitica*, and *Ph. cinnamomi*], *Hyaloperonospora arabidopsis*, and *Albugo laibachii*).

TABLE 1 The best BLAST hits and accession numbers of orthologous enzymes in the mevalonate and sterol biosynthetic pathways presented in the *Py. insidiosum* genome^a

Protein	Enzyme ^b	<i>S. cerevisiae</i> enzyme accession no.	<i>Py. insidiosum</i> genome		
			Protein ID	Accession no.	E value
ERG1	Squalene epoxidase	NP_011691			
ERG2	C-8 sterol isomerase	NP_013929			
ERG3	C-5 sterol desaturase	NP_013157	PINS02380006C	GAQ12368.1	3.0E−63
ERG4	C-24(28) sterol reductase	NP_011503			
ERG5	C-22 sterol desaturase	NP_013728	PINS02520001C	GAQ12369.1	2.0E−17
ERG6	Sterol 24-C-methyltransferase	NP_013706			
ERG7	Lanosterol synthase	NP_011939			
ERG8	Phosphomevalonate kinase	NP_013947	PINS00060035C	GAQ12361.1	6.0E−54
ERG9	Squalene synthase	NP_012060			
ERG10	Acetyl-CoA acyltransferase	NP_015297	PINS00050004C	GAQ12360.1	4.0E−133
ERG11	Lanosterol 14- α -demethylase	NP_011871	PINS02520001C	GAQ12369.1	2.0E−11
ERG12	Mevalonate kinase	NP_013935			
ERG13	HMG-CoA synthase	NP_013580	PINS00120058C	GAQ12362.1	2.0E−120
ERG19	Diphosphomevalonate decarboxylase	NP_014441	PINS01150035C	GAQ12364.1	2.0E−92
ERG20	Farnesyl diphosphate synthase	NP_012368	PINS01980016C	GAQ12366.1	6.0E−102
ERG24	Delta14-sterol reductase	NP_014119	PINS01230016C	GAQ12365.1	2.0E−38
ERG25	Methylsterol monooxygenase	NP_011574			
ERG26	Sterol-4- α -carboxylate 3-dehydrogenase	NP_011514	PINS00200056A	GAQ12363.1	3.0E−16
ERG27	3-Keto-steroid reductase	NP_013201			
HMG1	HMG-CoA reductase 1	NP_013636			
HMG2	HMG-CoA reductase 2	NP_013555			
IDI1	Isopentenyl-diphosphate delta-isomerase	NP_015208	PINS02140012C	GAQ12367.1	1.0E−49

^aThe best BLAST hits were defined as having a cutoff E value of less than -6 . Shown are accession numbers of orthologous enzymes in the mevalonate and sterol biosynthetic pathways presented in the genome of *Py. insidiosum* strain Pi-S, isolated from a human patient with vascular pythiosis (19). The query sequences were derived from *Saccharomyces cerevisiae*.

^bCoA, coenzyme A; HMG, hydroxymethylglutaryl.

DISCUSSION

Inconsistent effects of antifungal drugs in the treatment of pythiosis patients have been reported (2, 12, 15). We have sought to determine whether 30 isolates of *Py. insidiosum* are susceptible to the antifungal agents and whether the organism harbors the drug target enzymes of the sterol biosynthetic pathway. There is no standardized antibiotic susceptibility test for *Py. insidiosum*. In our preliminary work to develop such a test, we considered using zoospores or hyphal suspensions as inocula (29, 30). However, in our hands, generation of zoospores from Thai strains is difficult and labor-intensive and did not provide enough zoospores for the susceptibility testing. Likewise, hyphal suspensions, prepared as described by other investigators (15, 30, 31) were not reproducibly uniform in our hands (data not shown). As an alternative, we have used the method of Brown and coworkers (32), starting with agar plugs of active hyphal growth and measuring radial growth of the hyphae. They demonstrated that the radial growth assay was a simple and reproducible method for susceptibility testing of *Py. insidiosum* and argued that the submerged-growth nature of *Py. insidiosum* in agar is an advantage in that the organism is kept in direct contact with the antifungal drug. We have found that this assay is simple, reliable, and reproducible. To control for differences in the growth of the different *Py. insidiosum* isolates, we calculated the percentage of radial growth of each isolate as the growth with exposure to the antifungal drug divided by growth of the same isolate with no drug $\times 100$.

Although RPMI is a commonly used medium for the *in vitro* susceptibility testing of *Py. insidiosum* (30), we have chosen to use Sabouraud dextrose (SD) agar for these assays. Since a particular medium could interfere with antifungal drug activity, this may be a concern. However, Stopiglia et al. compared the performances of RPMI and SD agar for *in vitro* susceptibility testing of a pathogenic fungus against various antifungal drugs (33). They reported no significant differences between the MICs obtained in the two media for many antifungal drugs tested (including itraconazole and terbinafine), with one exception—i.e., amphotericin B. Thus, in the present study, we used SD agar

TABLE 2 The best BLAST hits of orthologous enzymes in the mevalonate pathway presented in the genomes of 17 oomycetes, 3 algae, 2 fungi, and a diatom^a

Organism	Group	Subgroup	E value for enzyme in the mevalonate pathway							
			ERG10	ERG13	HMG1	HMG2	ERG12	ERG8	ERG19	IDI1
<i>Ca. albicans</i>	Fungi	Ascomycota	2.0E-154	0.0E+00	0.0E+00	0.0E+00	8.0E-88	6.0E-81	6.0E-135	1.0E-85
<i>As. fumigatus</i>	Fungi	Ascomycota	1.0E-137	1.0E-164	0.0E+00	0.0E+00	8.0E-68	3.0E-53	1.0E-115	9.0E-73
<i>Ec. siliculosus</i>	Algae	PX clade	3.0E-136	2.0E-125	9.0E-98	4.0E-101	4.0E-45	4.0E-45	1.0E-100	6.0E-52
<i>Au. limacinum</i>	Algae	Labyrinthulida	2.0E-118	8.0E-119	3.0E-139	1.0E-136	2.0E-15	3.0E-36	2.0E-116	3.0E-62
<i>Au. anophagefferens</i>	Algae	Pelagophyceae	3.0E-114	3.0E-109			6.0E-09		5.0E-41	3.0E-53
<i>Ph. tricorutum</i>	Diatoms	Bacillariophyta	3.0E-136	6.0E-105	5.0E-131	2.0E-127	8.0E-44			7.0E-57
<i>Ap. euteiche</i> ^b	Oomycetes	Saprolegniales	1.0E-101		1.0E-117	1.0E-101		9.0E-30		
<i>Sa. parasitica</i>	Oomycetes	Saprolegniales	7.0E-137	2.0E-119	1.0E-134	2.0E-133	4.0E-34	1.0E-45	2.0E-95	2.0E-59
<i>Py. insidiosum</i> ^c	Oomycetes	Pythiales	4.0E-133	2.0E-120				6.0E-54	2.0E-92	1.0E-49
<i>Py. ultimum</i>	Oomycetes	Pythiales	2.0E-133				2.0E-40	4.0E-60	4.0E-99	3.0E-55
<i>Py. arrhenomanes</i>	Oomycetes	Pythiales	1.0E-133	3.0E-118			2.0E-35	6.0E-07	2.0E-106	2.0E-52
<i>Py. irregulare</i>	Oomycetes	Pythiales	1.0E-126	6.0E-120			4.0E-34	7.0E-48	7.0E-93	1.0E-54
<i>Py. iwayamai</i>	Oomycete	Pythiales	1.0E-120	3.0E-75			7.0E-39	3.0E-57	6.0E-26	4.0E-55
<i>Py. vexans</i>	Oomycetes	Pythiales	4.0E-85	3.0E-121			2.0E-43	2.0E-36	2.0E-77	1.0E-52
<i>Py. aphanidermatum</i>	Oomycetes	Pythiales	5.0E-133	8.0E-121			6.0E-42	8.0E-14	6.0E-89	1.0E-51
<i>Ph. sojae</i>	Oomycetes	Peronosporales	9.0E-128	1.0E-119				7.0E-40	7.0E-103	1.0E-50
<i>Ph. ramorum</i>	Oomycetes	Peronosporales	1.0E-128	9.0E-120			2.0E-40	7.0E-37	6.0E-104	5.0E-49
<i>Ph. parasitica</i>	Oomycetes	Peronosporales	5.0E-135	1.0E-120			5.0E-41	2.0E-56	6.0E-34	1.0E-56
<i>Ph. capsici</i>	Oomycetes	Peronosporales	8.0E-132	1.0E-123			1.0E-43	4.0E-39	7.0E-105	2.0E-57
<i>Ph. cinnamomi</i>	Oomycetes	Peronosporales	9.0E-129	5.0E-117			1.0E-38	9.0E-38	1.0E-100	3.0E-56
<i>Ph. infestans</i>	Oomycetes	Peronosporales	4.0E-127	3.0E-117			9.0E-42	3.0E-36	1.0E-103	2.0E-53
<i>Hy. arabidopsis</i>	Oomycetes	Peronosporales	2.0E-127	3.0E-110			1.0E-37		1.0E-104	8.0E-54
<i>Al. laibachii</i>	Oomycetes	Albuginales	1.0E-124	6.0E-119			6.0E-17	4.0E-51	2.0E-92	3.0E-50

^aThe best BLAST hits of orthologous enzymes in the mevalonate pathway are defined as having a cutoff E value of less than -6 . The query sequences were derived from *Saccharomyces cerevisiae*.

^bOnly transcriptome data were available and used for analysis.

^c*Py. insidiosum* (strain Pi-S) was isolated from a human patient with vascular pythiosis (19).

(which is lower cost and easier to prepare) as the medium for *in vitro* susceptibility testing of *Py. insidiosum* against itraconazole and terbinafine.

As shown here, two key antifungal drugs (terbinafine and itraconazole) had different impacts on growth of *Py. insidiosum*. At relatively low concentrations (0.5 to 2.0 mg/liter), both drugs paradoxically slightly enhanced the growth in the majority of *Py. insidiosum* isolates (Fig. 1). At higher concentrations (≥ 8 mg/liter), both drugs inhibited the pathogen in a dose-dependent manner (Fig. 1). Terbinafine was more inhibitory than itraconazole against the organism, which is consistent with the published results of Argenta et al. (34) and Fonseca et al. (30). Both of these groups performed comprehensive *in vitro* susceptibility testing of terbinafine and itraconazole against 30 and 22 Brazilian isolates of *Py. insidiosum*, respectively. To completely inhibit the growth of 50% of the isolates tested required at least 16 mg/liter of itraconazole and 4 mg/liter of terbinafine in the studies by Argenta et al. (34) and at least 64 mg/liter of itraconazole and 8 mg/liter of terbinafine in those by Fonseca et al. (30), while our group found that more than 128 mg/liter of both drugs was required. The different inhibitory effects of the two drugs against *Py. insidiosum* may be due to the differences in the strains of *Py. insidiosum* tested (i.e., Thai versus Brazilian strains) or the differences in the susceptibility methods used. (There is no standardized susceptibility assay available for the organism.)

In the present study, complete growth inhibition of some *Py. insidiosum* isolates was observed only with terbinafine at extremely high concentrations (64 to 128 mg/liter [Fig. 1A]). Although *Py. insidiosum* can be inhibited using the high drug concentrations, such concentrations are difficult to achieve *in vivo*. Kovarik and coworkers studied the pharmacokinetics and tissue distribution of terbinafine and showed that the peak plasma concentration of the drug is ~ 1.7 mg/liter, while the peak tissue concentrations in sebum, hair, nail, and skin are ~ 20 , ~ 10 , ~ 1 , and ~ 1 mg/liter, respectively (35). These plasma and tissue concentrations (especially for the skin, which is a target site of *Py. insidiosum* infection) are lower than the minimal drug concentration required to

TABLE 3 The best BLAST hits of orthologous enzymes in the sterol biosynthetic pathway presented in the genomes of 17 oomycetes, 3 algae, 2 fungi, and a diatom^a

Organism	Group	Subgroup	E value for enzyme in the sterol biosynthetic pathway																
			ERG20	ERG9	ERG1	ERG7	ERG11	ERG24	ERG25	ERG26	ERG27	ERG6	ERG2	ERG3	ERG5	ERG4			
<i>Ca. albicans</i>	Fungi	Ascomycota	5.0E-155	1.0E-138	4.0E-143	0.0E+00	0.0E+00	3.0E-78	2.0E-118	1.0E-139	4.0E-111	8.0E-144	5.0E-66	6.0E-118	0.0E+00	7.0E-166			
<i>As. fumigatus</i>	Fungi	Ascomycota	2.0E-118	8.0E-107	2.0E-89	0.0E+00	5.0E-147	2.0E-146	2.0E-100	2.0E-56	5.0E-36	1.0E-116	8.0E-52	3.0E-91	2.0E-171	2.0E-145			
<i>Au. limacinum</i>	Algae	Labyrinthulida	3.0E-99	5.0E-65	4.0E-138	4.0E-138	1.0E-73	4.0E-103	4.0E-17	9.0E-34	1.0E-08	5.0E-68	1.0E-62	6.0E-33	4.0E-53	4.0E-53			
<i>Au. anophagefferens</i>	Algae	Pelaogophyceae	6.0E-94	1.0E-70	1.0E-129	6.0E-58	1.0E-73	1.0E-73	1.0E-21	3.0E-25	4.0E-10	4.0E-83	4.0E-10	1.0E-10	3.0E-102	3.0E-102			
<i>Ec. siliculosus</i>	Algae	PX clade	1.0E-118	8.0E-91	4.0E-65	4.0E-79	2.0E-66	2.0E-100	5.0E-09	1.0E-37	1.0E-99	1.0E-99	1.0E-16	2.0E-17	3.0E-55	3.0E-55			
<i>Ph. tricornutum</i>	Diatom	Bacillariophyta	7.0E-115	1.0E-70	5.0E-126	3.0E-69	1.0E-100	6.0E-10	6.0E-10	1.0E-33	2.0E-81	2.0E-81	1.0E-19	1.0E-26	1.0E-52	1.0E-52			
<i>Ap. euteiche^b</i>	Oomycete	Saprolegniales	3.0E-94	2.0E-69	4.0E-56	4.0E-66	8.0E-68	2.0E-73	1.0E-61	2.0E-25	2.0E-78	2.0E-78	6.0E-16	1.0E-38	1.0E-38	1.0E-38			
<i>Sa. parasitica</i>	Oomycete	Saprolegniales	9.0E-118	3.0E-79	4.0E-65	2.0E-158	1.0E-80	2.0E-84	2.0E-62	2.0E-50	5.0E-84	5.0E-84	1.0E-62	1.0E-17	3.0E-50	3.0E-50			
<i>Py. insidiosum^c</i>	Oomycete	Pythiales	6.0E-102	1.0E-119	1.0E-119	1.0E-119	2.0E-11	2.0E-38	8.0E-08	3.0E-16	2.0E-10	2.0E-10	3.0E-63	2.0E-17	3.0E-17	3.0E-17			
<i>Py. ultimum</i>	Oomycete	Pythiales	1.0E-119	1.0E-119	1.0E-119	1.0E-119	6.0E-12	3.0E-36	8.0E-08	6.0E-18	7.0E-10	7.0E-10	3.0E-17	3.0E-17	3.0E-17	3.0E-17			
<i>Py. arrhenomanes</i>	Oomycete	Pythiales	6.0E-105	4.0E-123	4.0E-123	4.0E-123	3.0E-14	1.0E-32	1.0E-08	2.0E-19	9.0E-12	9.0E-12	4.0E-81	3.0E-16	1.0E-29	1.0E-29			
<i>Py. irregulare</i>	Oomycete	Pythiales	4.0E-123	2.0E-120	2.0E-120	2.0E-120	4.0E-15	4.0E-40	1.0E-08	1.0E-14	3.0E-10	3.0E-10	1.0E-71	1.0E-15	4.0E-22	4.0E-22			
<i>Py. iwayamai</i>	Oomycete	Pythiales	2.0E-120	5.0E-95	5.0E-95	5.0E-95	6.0E-17	4.0E-31	4.0E-07	5.0E-14	5.0E-12	5.0E-12	1.0E-78	1.0E-14	1.0E-26	1.0E-26			
<i>Py. vexans</i>	Oomycete	Pythiales	5.0E-95	1.0E-118	1.0E-118	1.0E-118	1.0E-11	2.0E-36	6.0E-11	4.0E-14	6.0E-14	6.0E-14	2.0E-81	9.0E-16	4.0E-31	4.0E-31			
<i>Py. aphanidermatum</i>	Oomycete	Pythiales	1.0E-118	1.0E-118	1.0E-118	1.0E-118	6.0E-15	5.0E-35	6.0E-11	2.0E-19	2.0E-19	2.0E-19	2.0E-82	2.0E-16	6.0E-29	6.0E-29			
<i>Ph. sojae</i>	Oomycete	Peronosporales	1.0E-118	1.0E-118	1.0E-118	1.0E-118	3.0E-13	4.0E-38	4.0E-07	4.0E-12	9.0E-12	9.0E-12	7.0E-79	2.0E-16	6.0E-29	6.0E-29			
<i>Ph. ramorum</i>	Oomycete	Peronosporales	4.0E-119	4.0E-119	4.0E-119	4.0E-119	2.0E-14	6.0E-38	4.0E-07	3.0E-13	3.0E-13	3.0E-13	4.0E-79	3.0E-16	5.0E-26	5.0E-26			
<i>Ph. parasitica</i>	Oomycete	Peronosporales	1.0E-115	1.0E-115	1.0E-115	1.0E-115	4.0E-16	1.0E-39	2.0E-07	3.0E-14	4.0E-14	4.0E-14	8.0E-79	3.0E-16	2.0E-26	2.0E-26			
<i>Ph. capsici</i>	Oomycete	Peronosporales	1.0E-117	1.0E-117	1.0E-117	1.0E-117	7.0E-12	8.0E-38	2.0E-08	9.0E-16	4.0E-14	4.0E-14	8.0E-79	7.0E-14	1.0E-27	1.0E-27			
<i>Ph. cinnamomi</i>	Oomycete	Peronosporales	4.0E-117	4.0E-117	4.0E-117	4.0E-117	5.0E-16	4.0E-37	8.0E-07	7.0E-13	6.0E-10	6.0E-10	3.0E-80	5.0E-18	2.0E-27	2.0E-27			
<i>Ph. infestans</i>	Oomycete	Peronosporales	1.0E-115	1.0E-115	1.0E-115	1.0E-115	2.0E-13	4.0E-39	9.0E-08	1.0E-14	2.0E-13	2.0E-13	1.0E-74	3.0E-14	7.0E-28	7.0E-28			
<i>Hy. arabidopsis</i>	Oomycete	Peronosporales	3.0E-105	2.0E-14	2.0E-14	2.0E-14	2.0E-14	4.0E-39	9.0E-08	1.0E-12	4.0E-11	4.0E-11	5.0E-14	5.0E-14	5.0E-14	5.0E-14			
<i>Al. laibachii</i>	Oomycete	Albuginales	2.0E-107	2.0E-107	2.0E-107	2.0E-107	2.0E-10	6.0E-16	6.0E-16	6.0E-16	6.0E-16	4.0E-08	2.0E-11	2.0E-11	2.0E-11	2.0E-11			

^aThe best BLAST hits of orthologous enzymes in the sterol biosynthetic pathway are defined as having a cutoff E value of less than -6. The query sequences were derived from *Saccharomyces cerevisiae*.

^bOnly transcriptome data were available and used for analysis.

^c*Py. insidiosum* (strain Pi-5) was isolated from a human patient with vascular pythiosis (19).

TABLE 4 Results from genome and transcriptome databases for 17 oomycetes, 3 algae, 2 fungi, and a diatom used for the sterol biosynthetic enzyme search

Organism	Group	Genome size (Mb)	Accession no. or source
<i>Pythium insidiosum</i> ^a	Oomycetes	53.24	BBXB01000001 to BBXB01001192
<i>Pythium ultimum</i>	Oomycetes	42.8	http://pythium.plantbiology.msu.edu/
<i>Pythium aphanidermatum</i>	Oomycetes	35.88	http://pythium.plantbiology.msu.edu/
<i>Pythium arrhenomanes</i>	Oomycetes	44.67	http://pythium.plantbiology.msu.edu/
<i>Pythium irregulare</i>	Oomycetes	42.97	http://pythium.plantbiology.msu.edu/
<i>Pythium iwayamai</i>	Oomycetes	43.2	http://pythium.plantbiology.msu.edu/
<i>Pythium vexans</i>	Oomycetes	33.84	http://pythium.plantbiology.msu.edu/
<i>Phytophthora sojae</i>	Oomycetes	82.6	http://www.broadinstitute.org/
<i>Phytophthora ramorum</i>	Oomycetes	66.65	http://www.broadinstitute.org/
<i>Phytophthora parasitica</i>	Oomycetes	82.39	http://www.broadinstitute.org/
<i>Phytophthora capsici</i>	Oomycetes	56.04	http://genome.jgi.doe.gov/
<i>Phytophthora cinnamomi</i>	Oomycetes	77.97	http://genome.jgi.doe.gov/
<i>Phytophthora infestans</i>	Oomycetes	228.54	http://www.broadinstitute.org/
<i>Hyaloperonospora arabidopsis</i>	Oomycetes	73.89	http://protists.ensembl.org/
<i>Albugo laibachii</i>	Oomycetes	32.76	http://protists.ensembl.org/
<i>Aphanomyces euteiches</i> ^b	Oomycetes		http://www.polebio.lrsv.ups-tlse.fr/aphano/
<i>Saprolegnia parasitica</i>	Oomycetes	53.13	http://www.broadinstitute.org/
<i>Phaeodactylum tricornutum</i>	Diatoms	25.05	http://genome.jgi.doe.gov/
<i>Aureococcus anophagefferens</i>	Brown tide algae	56.66	http://genome.jgi.doe.gov/
<i>Ectocarpus siliculosus</i>	Brown algae	391.72	http://bioinformatics.psb.ugent.be/genomes/
<i>Aurantiochytrium limacinum</i>	Microalgae	60.93	http://genome.jgi.doe.gov/
<i>Candida albicans</i>	Fungi	27.56	http://www.candidagenome.org/
<i>Aspergillus fumigatus</i>	Fungi	29.39	http://www.aspgd.org/

^a*Py. insidiosum* (strain Pi-S) was isolated from a human patient with vascular pythiosis (19).

^bOnly transcriptome data were available and used for analysis.

inhibit the organism in our study. Our results explain why *Py. insidiosum* clinically resists this antifungal drug as observed in most pythiosis patients (2).

In fungi, there are 14 enzymes in the sterol biosynthetic pathway (Fig. 3). The mechanisms of action of terbinafine and itraconazole rely on interfering with the synthesis of sterol at the ERG1 step (converting squalene to squalene epoxide) and

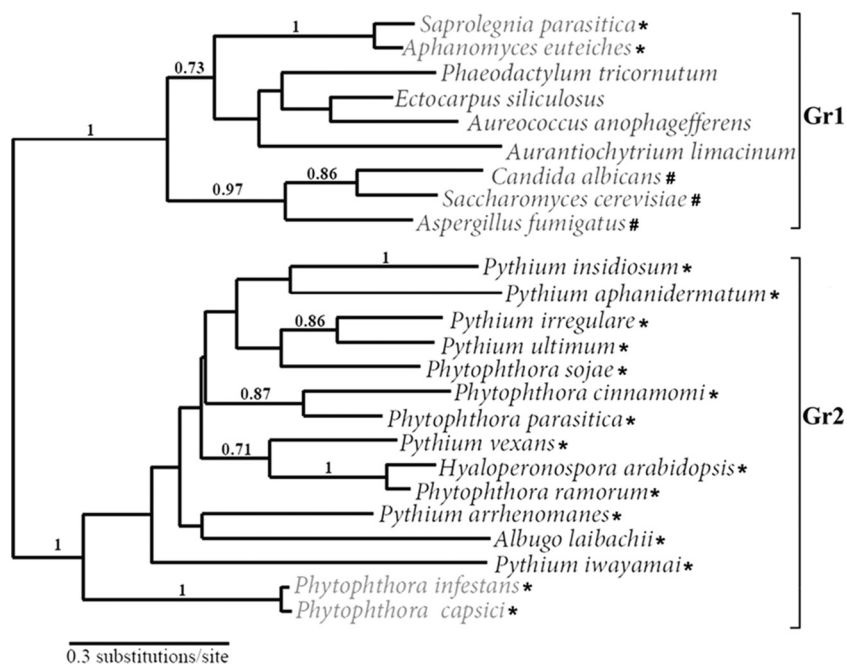


FIG 4 Phylogenetic analysis of *ERG11* orthologs of 17 oomycetes, 3 fungi, 3 algae, and a diatom. A phylogenetic tree was reconstructed based on the neighbor-joining algorithm (see Materials and Methods). The branch support values of at least 70% are shown at corresponding nodes. *, oomycete; #, fungus.

the ERG11 step (catalyzing lanosterol to dimethylcholestatrienol), respectively. Based on the genome search, *Py. insidiosum* contained only six enzymes (ERG3, ERG5, ERG11, ERG20, ERG24, and ERG26) (Table 3) in the sterol biosynthetic pathway. The presence of the drug target ERG11, with an E value of -11 , may explain the limited inhibitory effect of itraconazole against *Py. insidiosum* (Fig. 1B). However, the lack of the drug target ERG1 in the *Py. insidiosum* genome contradicts the fact that the organism was notably sensitive to terbinafine. There might be another target protein responsible for terbinafine-mediated growth inhibition in *Py. insidiosum*. This possibility is especially likely in light of the high *in vitro* concentrations needed to see growth inhibition by terbinafine.

We used GC-MS to detect the sterol end products identified in various organisms—i.e., cholesterol (animals), stigmasterol (plants), ergosterol (fungi), and fucosterol (algae and oomycetes) (13)—to find evidence to support the presence of the sterol biosynthetic pathway in *Py. insidiosum*. We were able to detect ergosterol in the fungus *Ca. albicans*. However, no sterol end products were detected in *Py. insidiosum*, even when the starting amount of sterol extract was 7-fold increased versus that used for *Ca. albicans* (Fig. 2). Taken together, the bioinformatic and GC-MS findings present a coherent picture that *Py. insidiosum* lacks a complete set of sterol biosynthetic enzymes, which leads to its failure to produce the sterol end products.

The mevalonate pathway generates dimethylallyldiphosphate, which is a precursor of the sterol biosynthetic pathway. Based on the oomycete genome searches for the homologous enzymes present in the mevalonate and sterol biosynthetic pathways (Fig. 3), we found that the oomycetes in the Saprolegniales subgroup (i.e., *Ap. euteiches* and *Sa. parasitica*) possess a nearly complete set of the enzymes in such pathways, whereas the oomycetes in the subgroups Pythiales, Peronosporales, and Albuginales (i.e., *Pythium* species, *Phytophthora* species, *Hyaloperonospora* species, and *Albugo* species) contain a restricted number of such enzymes (Tables 2 and 3). This finding is consistent with the findings that many oomycetes (including *Py. insidiosum*) of the non-Saprolegniales subgroups are sterol auxotrophs (11, 13), while the oomycetes of the subgroup Saprolegniales can synthesize their own sterols (i.e., cholesterol and fucosterol), as clearly shown for *Ap. euteiches* (12). The ability to synthesize sterols in the oomycetes of the Saprolegniales subgroup and the fungi could indicate their derivation from an ancient common ancestor. However, even if so, during the course of evolution, some critical enzymes involved in sterol synthesis (i.e., HMG1, HMG2, ERG1, ERG7, and ERG9) have been lost in the oomycetes of the non-Saprolegniales subgroups. As a compensatory mechanism, the non-Saprolegniales oomycetes increased their capacity to acquire exogenous sterol by greatly expanding the number of genes encoding sterol-binding and -uptake proteins—the elicitor gene family (36–38). In contrast, this elicitor gene expansion is absent in the Saprolegniales oomycetes (36, 38).

In the sterol biosynthetic pathway, ERG1 and ERG11 are the target enzymes of terbinafine and itraconazole, respectively. The enzyme-encoding sequence of ERG11, but not ERG1, was identified in the genomes of all organisms included in this study (Table 3). The *ERG11* sequences were then used for investigation of the phylogenetic relationship among oomycetes, fungi, algae, and a diatom. The resulting *ERG11*-based tree located most of the oomycetes in group 2 and the remaining oomycetes (*Ap. euteiches* and *Sa. parasitica*) and all other organisms (fungi, algae, and diatom) in group 1 (Fig. 4). Based on the BLAST search using the query sequence from *S. cerevisiae*, the E values among the sterol-producing Saprolegniales oomycetes (E value range, -80 to -68) were much lower than that of the sterol auxotrophic, non-Saprolegniales oomycetes (E value range, -17 to -10) (Table 3). This finding suggests that the rate of gene modification (i.e., mutation, insertion, or deletion) of *ERG11* since divergence of the oomycetes from the common ancestor with the fungi (represented by *S. cerevisiae*) was higher for the non-Saprolegniales oomycetes than that for the Saprolegniales oomycetes. This relationship is also found for other enzymes, i.e., ERG6, ERG24, ERG25, and ERG26 (Table 3), and it suggests that there was less selective pressure on the non-Saprolegniales oomycetes to maintain the sterol biosynthetic pathway.

In conclusion, the *in vitro* susceptibility testing demonstrated that the *Py. insidiosum* isolates were sensitive to the antifungal agents, but only at concentrations that are difficult or impossible to achieve *in vivo*. These results explain the clinical resistance of the drugs in the treatment of most pythiosis patients. The genome analysis of *Py. insidiosum* demonstrated that the organism contains an incomplete set of sterol biosynthetic enzymes. GC-MS analysis did not detect any sterol end products in *Py. insidiosum*. Clinical resistance of antifungal drugs in *Py. insidiosum* results from modifications or losses of some of the genes encoding the drug target sterol biosynthetic enzymes.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, at the Faculty of Medicine, Ramathibodi Hospital, Mahidol University (approval no. MURA2011/412/S3). Informed consent was not obtained from patients (from whom microorganisms were obtained) because the data were analyzed anonymously, and the institutional ethics committee waived the need for written informed consent from the participants.

Microorganisms and growth conditions. Thirty isolates of *Py. insidiosum* from patients with vascular pythiosis ($n = 10$), ocular pythiosis ($n = 7$), cutaneous pythiosis ($n = 3$), and undefined forms of pythiosis ($n = 10$) were recruited in this study. The identity of each *Py. insidiosum* isolate was confirmed by zoospore induction (39, 40) and ribosomal DNA (rDNA) sequence homology analysis (41). All organisms were maintained on SD agar (pH 7.2) at room temperature.

Ten small pieces of SD agar containing a 7-day-old actively growing colony of *Py. insidiosum* (strain Pi-S) were transferred into 100 ml of SD broth (pH 7.2) and incubated, with shaking (150 rpm), at 37°C for 10 days. The organism was killed with 0.02% (wt/vol) thimerosal (Sigma). The hyphal mat was collected by filtration of the cultured broth through a 0.22- μ m-pore-size membrane (Durapore; Merck Millipore). *Candida albicans* strain ATCC 90028 was cultured in yeast extract-peptone-dextrose medium, with shaking (250 rpm), at 30°C for 3 days. The yeast cells were collected by centrifugation at $6,000 \times g$ for 10 min. Harvested organisms were lyophilized using a Genevac EZ-2 Elite machine and stored at -30°C until used for sterol extraction.

***In vitro* antifungal susceptibility assay.** The radial growth of *Py. insidiosum* was determined using the methods described by Brown et al. and Krajaejun et al. (32, 42), with some modifications. Briefly, *Py. insidiosum* was subcultured on SD agar at 37°C for 5 days. A 5-mm-diameter SD agar plug with actively growing mycelium was cut from the edge of a young *Py. insidiosum* colony and placed on SD agar (pH 7.2) containing various concentrations (128, 64, 32, 8, 2, 0.5, and 0 [no-drug control] mg/liter) of terbinafine (purity, 100% [Novartis]) or itraconazole (purity, 100% [Janssen Pharmaceutical]). The agar plate was incubated at 37°C for 2 days. Two colony diameters of each *Py. insidiosum* isolate were measured at day 2. All isolates were tested in duplicate. The average colony diameter was subtracted by 5 mm (the diameter in millimeters of an agar plug) and then divided by 2 to obtain a mean radial growth. The percentage of radial growth of each isolate, after exposure to the antifungal drug, was calculated based on the growth of the same isolate with no drug (control). The percentages of radial growths of *Py. insidiosum* exposed to different drug concentrations were statistically compared using the GraphPad Prism program version 5.00 (GraphPad Software, USA) and one-way analysis of variance (ANOVA) with Dunnett's posttest.

Sterol extraction. Sterol extraction was performed using the methods of Axelsson et al. (43) and Madoui et al. (12), with some modifications. Briefly, the lyophilized samples (~200 mg of either *Ca. albicans* yeasts or *Py. insidiosum* hyphae), 10 μ g of 5 α -cholestane (Sigma [used as an internal control]), and 3 ml of 1% (wt/vol) KOH in methanol were mixed and incubated at 80°C for 90 min. After the reaction mixture was cooled to room temperature, 1 ml of water and 2 ml of hexane were sequentially added to the mixture and incubated on a rocker (BioSan MR-1) overnight at room temperature. The organic phase (upper layer), containing sterols, was transferred to a new glass tube and evaporated using nitrogen gas. The residual material was derivatized at 60°C for 30 min using 20 μ l of pyridine (Sigma) and 50 μ l of N, O-bis(trimethylsilyl) trifluoroacetamide (Thermo Scientific). The derivatized material was dissolved in 500 μ l toluene and subjected to gas chromatography-mass spectrometry (GC-MS).

Four commercially available sterols (including cholesterol [Tokyo Chemical Industry], ergosterol [Acros Organics], stigmasterol [Tokyo Chemical Industry], and fucosterol [Sigma]) were mixed and served as the standard for GC-MS analysis. After addition of 5 α -cholestane, the sterol standard mixture was derivatized (as described above) and dissolved in 500 μ l toluene.

GC-MS. The sterol extracts and the sterol standard mixture were analyzed using GC-MS (model 7890A-5975C [Agilent Technologies]; capillary column [model 190915-433HP-5MS] with 5% phenyl methyl siloxane [size, 30 m by 250 μ m by 0.25 μ m]). The electro-ionization mass spectrometry was set to the scan mode with an m/z range of 50 to 500. The selected-ion monitoring (SIM) mode was used to analyze trace amounts of sterol compounds: 5 α -cholestane masses (m/z of 217, 357, and 372), cholesterol masses (m/z of 129, 329, 368, and 458), ergosterol masses (m/z of 131, 337, 363, and 468), stigmasterol masses (m/z of 83, 255, 394, and 484), and fucosterol masses (m/z of 257, 296, 386, and 484). Temperatures were set as follows: injector, 260°C; oven, 3°C/min increments from 230°C to 239°C, 1°C/min increments to 280°C, and then 280°C for 2 min; MS source, 230°C; and MS quadrupole, 150°C.

Sequence homology analysis. Genomes or transcriptomes of 17 oomycetes (14, 17, 19, 22–28, 44, 45), two fungi (*Candida albicans* strain SC5314 and *Aspergillus fumigatus* strain Af293) (46, 47), and one

each of diatom (48), microalga (45), brown tide alga (49), and brown alga (50) were retrieved from public databases (Table 4). The BLAST 2.2.28+ program (<http://www.ncbi.nlm.nih.gov/>) was locally installed for genome search analysis. The sequences of 22 enzymes (including ERG1 to -13, ERG19, ERG20, ERG24 to -27, HMG1, HMG2, and IDI1) involved in endogenous sterol production of the *Saccharomyces cerevisiae* strain S288c (51) were obtained from the NCBI database (Fig. 3; Tables 1 to 3) and used to BLAST search against all genomes or transcriptomes included in this study. The cutoff E value was 10^{-6} .

Phylogenetic analysis. Phylogenetic analysis of the *ERG11* homologs, coding for the lanosterol 14- α demethylase (which is the drug target of itraconazole) from all microorganisms, was performed online at www.phylogeny.fr (Table 3) (52). Briefly, the sequences were aligned using the MUSCLE program (53). The phylogenetic relationship was analyzed by neighbor joining with 1,000 bootstraps (54) and the Jones-Taylor-Thornton matrix substitution model (55). A phylogenetic tree was generated by TreeDyn (56).

Sequence accession numbers. All sequences of orthologous enzymes in the mevalonate and sterol biosynthetic pathways presented in the genome of *Py. insidiosum* (strain Pi-S) have been submitted to the DNA Data Bank of Japan database, under the accession numbers indicated in Table 1.

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The authors declare they have no conflicts of interest.

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