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SYSTEMATICS

The occurrence and distribution of *Pythium* species on Hainan Island of South China

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ABSTRACT. During the period 2003-2009 a comprehensive study of *Pythium* species in soil and water samples collected from various habitats on Hainan Island was conducted using conductedusing a combination of baiting and selective agar medium technique. A total of 2348 isolates were identified into 29 species: *P. acanthicum*, *P. acanthophoron*, *P. aphanidermatum*, *P. aristosporum*, *P. arrhenomanes*, *P. carolinianum*, *P. catenulatum*, *P. cucurbitacearum*, *P. deliense*, *P. dissotocum*, *P. graminicola*, *P. helicoides*, *P. hydnosporum*, *P. indigoferae*, *P. inflatum, P. intermedium*, *P. irregulare*, *P. marsipium*, *P. middletonii*, *P. monospermum*, *P. myriotylum*, *P. oedochilum*, *P. paroecandrum*, *P. pulchrum*, *P. spinosum*, *P. splendens*, *P. sylvaticum*, *P. ultimum* and *P. vexans*. Except for *P. acanthicum*, *P. aphanidermatum*, *P. catenulatum*, *P. deliense*, *P. dissotocum*, *P. indigoferae*, *P. marsipium*, *P. myriotylum* and *P. splendens*, the remaining 20 species are new records for Hainan. *Pythium cucurbitacearum* is the first record in China. Several species of *Pythium* which are either terrestrial or occasionally found in fresh water were isolated for the first time from fallen leaves submerged in sea water in or near mangrove habitats: *P. vexans*, *P. aphanidermatum*, *P. cucurbitacearum*, *P. helicoides* and *P. middletonii*. It remains to be determined whether they should be treated as halotolerant strains or marine species of *Pythium* but their repeated isolation pointed out their significance in litter decomposition in the marine habitat. By far, *P. vexans* is the most widespread and abundant species followed by *P. deliense*, *P. acanthicum*, *P. aphanidermatum*, *P. helicoides* and *P. splendens*. Of the 23 sampling sites, the greatest diversity of *Pythium* species was found in the cities of Wenchang, Haikao, Wuzhishan, Ledong and their vicinity.

Keywords: Chromista; Ecology; Oomycetes; Pythiaceous Fungi.

INTRODUCTION

The genus *Pythium* Pringsh., with 305 described species (www.mycobank.org), has been classified traditionally with other filamentous, coenocytic, sporangia-producing fungi as "Phycomyetes" (Fitzpatrick, 1930). However, with recent advances in chemical, ultrastructural and molecular studies, *Pythium* spp. are now considered as "fungus-like organisms" or "pseudo-fungi" and are placed in the Kingdom Chromista (Kirk et al., 2008) or Kingdom Straminopila (Webster and Weber, 2007), kingdoms distinct from the Kingdom Fungi (Whittaker, 1969).

Historically, there has also been great confusion regarding the validity of *Pythium* as a distinct genus. The genus was created by Pringsheim (1858) and placed in the family Saprolegniaceae. However, *Pythium* Pringsh. was antedated by both *Pythium* Nees and *Artotrogus* Montagne. Subsequently, the genus *Pythium* Pringsh. was conserved (Plaats-Niterink, 1981). There were attempts to split the genus *Pythium* into two genera to differentiate species with spherical sporangia from those with filamentous sporangia. Schröter (1897) erected the family Pythiaceae in which he described *Pythium* having globose sporangia and *Nematosporangium* with filamentous sporangia. On the other hand, Sparrow (1931) proposed species of *Pythium* with globose sporangia to be placed in a new genus, *Sphaerosporangium*. Others tried to create various infrageneric taxa within *Pythium* but all these proposals have been rejected by Waterhouse (1967), Plaats-Niterink (1981) and Dick (1990). Nevertheless, recent phylogenetic studies based on the molecular data have provided new evidence and rekindled interest to split the genus *Pythium*. Thus Ko et al. (2010) erected a new genus *Aquaperonospora* for species producing rigid, erect and branched sporangiophores forming sporangia synchronously on branchlet tips. Bala et al. (2010) proposed a new genus *Phytopythium* for

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those species with globose to void, often papillate and internally proliferating sporangia. Uzuhashi et al. (2010) restricted the genus *Pythium* to those species with inflated or non-inflated filamentous sporangia while creating four new genera to accommodate species with non filamentous sporangia: *Ovatisporangium* with mainly ovoid to pyriform, sometimes irregular shaped-sporangia, *Elongiosporangium* with clavate to elongate sporangia, *Globisporangium* with globose, sometimes proliferating sporangia and *Pilasporangium* with globose, non-proliferating sporangia. While recognizing the genus concept of *Pythium* is still in a state of flux we prefer to adhere to the classical definition of *Pythium* to include all oomycetous fungi producing non-deciduous sporangia only in water with variable shapes ranging from spherical, subspherical, ovate, obovate, ellipsoidal, pyriform to lobulated and filamentous, with zoospores formed in a membranous vesicle at the tip of an exit tube of the sporangium (Waterhouse, 1974)

In distribution, the species of *Pythium* are cosmopolitan, widely distributed throughout the world ranging from tropical to temperate (Plaats-Niterink, 1981) and even arctic (Hoshino et al., 1999) and antarctic regions (Knox and Paterson, 1973). They exist as saprophytes or parasites in soil, water, on plants, fungi, insects, fish, animals and human beings (Yu, 2001). Economically, they are especially important as pathogens of higher plants, causing serious damage to agricultural crops and turf grasses, leading primarily to soft rot of fruit, rot of roots and stems, and pre- and post-emergence of seeds and seedlings by infecting mainly juvenile or succulent tissues (Hendrix and Campbell, 1973). Whereas *Pythium* diseases are common in tropical to temperate regions, cereal seedlings under snow could also be killed by *Pythium* spp. (Hirane, 1960 ; Lipps, 1980) and an unidentified species of *Pythium* isolated from a colony of diseased leafy liverworts from Signy Island of Antarctic proved to be a potential plant pathogen to local vascular plants, based on artificial inoculation (Bridge et al., 2008). Other species caused diseases in fish (Khulbe, 2009), marine red algae (Takahashi et al., 1977) and mammals including humans (de Cock et al., 1987; Mendoza et al., 1996; Thianprasit et al., 1996). On the other hand, *Pythium* spp. may be of potential benefits to human beings as biological control agents of soil-borne fungal pathogens (Jones, 1995; Wu, 1995) and mosquitoes (Su, 2006; Su et al., 2001), as well as a source of chemicals useful in medicine and food industry (Gandhi and Weete, 1991; Stredansky et al., 2000).

Hainan Island, situated at the southern tip of China, between the 21st and 4th parallels north latitude is separated from the mainland by the Qiongzhou Strait. With an average of 34,000 sq. km., it is only second to Taiwan island in terms of size. The agricultural land is confined primarily to the coastal plain, approximately 1,580 km long. The central portion is occupied by high forested mountains reaching 1,867 m above sea level with mountain streams providing irrigation water for the agricultural land. It is the major tropical agricultural center in China for important crops like rubber, palm, coconut, papaya, avocado, mango, pineapple, pepper, piper, taro, passion fruit, citrus and sisal hemp etc. Its maritime tropical weather favors the survival and spread of zoosporic fungi like *Phytophthora* and *Pythium* in the ecosystem. To date, as a result of a comprehensive survey, 14 species of *Phytophthora* have been found attacking a wide variety of plants in Hainan with their distribution determined (Zeng et al., 2008). However, a similar study was absent for *Pythium* in Hainan. As part of their study of the genus *Pythium* in China, Yu and Ma (1989) isolated 11 species from soil and plant samples in Hainan: *P. acanthicum*, *P. adhaerens*, *P. aphanidermatum*, *P. catenulatum*, *P. deliense*, *P. diclinum*, *P. dissotocum*, *P. indigoferae*, *P. marsipium*, *P. myriotylum* and *P. periilum*, but the locality and frequency of their occurrence on the island were not investigated. During the period 2003-2009 we conducted a comprehensive survey on the occurrence and distribution of *Pythium* species in Hainan.

MATERIALS AND METHODS

Specimen collection

Soil and occasionally water samples were collected from 23 sites throughout Hainan Island from a diversity of habitats, including virgin mountain forests, woods, mangrove swamps, botanical gardens, nurseries, orchards, vegetable fields, rice paddies and areas around ornamental trees/shrubs (Figure 1). Soil samples (about 300-500 g each) were collected from the 5-15 mm of top soil after the surface debris was removed with a clean hand shovel. Each soil sample was placed individually in a clean plastic bag and labeled properly. Three samples were taken at each collection site. Water samples along with submerged fallen leaves were collected with empty mineral water bottles (900 mL). They were brought back to the laboratories as soon as possible for immediate isolation.

Isolation of *Pythium* species

The three soil samples for each collection site were mixed and then placed evenly in 15-cm diam. glass dishes so that the soil in the vessel was 1.5-2 cm deep. Sterile distilled water was added to cover the soil up to about 1.5 cm deep. Water samples were similarly placed in the vessel to a depth of 1.5-2.0 cm. For collected fallen leaves they were first surface cleaned with running tap water and detergent, blotted dried with blotting paper and the lesion spots were cut into smaller pieces (ca. 2×2 mm). Various materials were used for baiting *Pythium* species. Grass leaves and corn grains which have been boiled in distilled water for 15 min were the major baits used. Other baits included boiled hemp seeds (with seed coat removed afterwards) and egg albumen. Freshly collected leaves of *Hevea,* papaya and citrus trees as well as pine needles were cleaned, blotted dry as described previously and cut into small pieces (ca. 2×2 cm). The baits were submerged in the water in each dish. Occasionally, newly germinated cucumber seedlings were also used. The baits were removed

Figure 1. Distribution of *Pythium* species on Hainan Island. 1, Baisha County; 2, Baoting County; 3, Bawangling (Mountain); 4, Changjiang City; 5, Chengmai County; 6, Danzhou City; 7, Diaoluoshan (Mountain); 8, Dongfang City; 9, Dingan County; 10, Haikao City; 11, Jianfengling (Mountain); 12, Ledong City; 13, Limushan (Mountain); 14, Lingao County; 15, Lingshui County; 16, Qionghai City; 17, Qiongzhong County; 18, Shanya City; 19, Tungchang County; 20, Wanning City; 21, Wenchang City; 22, Wuzhishan City; 23, Wuzhishan (Mountain).

after 12-24 hr at room temperature (ca. 25-28°C), surface cleaned with detergent, blotted dry and divided into smaller pieces (ca. 2×2 mm) which were then placed on 9-cm diam. selective agar media plates. There is no selective agar medium specifically for *Pythium* species only. The selective agar medium was formulated to isolate oomycetes: primarily *Pythium* and *Phytophthora*. It was prepared as follows: 3.5 g $CaCO_3$ was mixed thoroughly with 160 ml Campbell V-8 juice and the mixture clarified by filtration through four layers of cheese cloth. Approximately 100 ml of clarified V-8 juice was diluted with distilled water to 1000 ml, boiled to dissolve 20 g Bacto agar and autoclaved at 121°C for 15 min. Prior to pouring the agar medium into sterilized Petri dishes, benomyl (200 µg/ml), pentachloronitrobenzene (100 µg /ml), rifampicin (100 µg /ml), ampicillin (200 µg/ml) and nystatin (50 µg/ml) were added. The inoculated plates were incubated at room temperature. When mycelial colonies appeared in 1-3 days, they were examined under an inverted light microscope to determine their identity as *Pythium* spp., which could be differentiated from *Phytophthora* spp. based on the much faster growth rate and the fine and flexuous hyphae (5-10 µm wide) (Erwin and Ribeiro, 1996). The hyphal tips were transferred to another selective agar plate before the isolate was finally cultured onto regular clarified V-8 juice agar medium. *Pythium* isolates were maintained on V8 agar slants and in small screw-capped glass vials (1.5×10 cm) of sterile distilled water.

Morphological studies

All morphological studies were made by growing the isolates onto 6-cm clarified V-8 juice agar plates. To induce the production of sporangia, small pieces of mycelial agar discs (ca. 2×2 mm) were cut out from the edge of a growing colony and transferred to 6-cm or 9-cm Petri dishes with added sterile distilled water just enough to submerge the mycelial discs. They were left under regular indoor light at room temperature and checked daily with an inverted light microscope for sporangia, which usually appeared in 24-48 h. In most cases, sex organs appeared later in water or on agar plates. When the isolates failed to produce sporangia and/or sex organs in water cultures, small pieces of boiled grass leaves (ca. 2×2 mm) were added to stimulate their production. Alternatively, non-sterile stream/pond water or soil extract was used instead of sterile distilled water to flood the plates. In the case of *P. splendens* which is heterothallic, a pair of plus and minus strains were obtained through the courtesy of Dr. W.H. Ko of the University of Hawaii and individual isolates were paired with one of these mating strains on the same agar plate. They were examined under light microscope for the production of sex organs at the junction of both colonies after 2 wk. For detailed microscopic examinations, small agar discs bearing sporangia or sex organs were mounted directly in a drop of 0.05% cotton blue/lactophenol on a glass slide and minced carefully into smaller pieces with a pair of 1 ml syringe needles. Then a 20×40 mm cover slip was placed on top with slight pressure to spread out the agar and the fungal structures. They were examined and photographed with a Nikon research light microscope (Japan Nikon Corporation, Tokyo, Japan) by interference contrast microscopy. Fifty measurements were made for each reproductive structure at a magnification of ×400. Only normal, fully mature sporangia and sex organs were

recorded. Identification of *Pythium* species was based primarily on the keys and species description provided by Waterhouse (1967, 1968), Plaats-Niterink (1981), Yu and Ma (1989), Dick (1990) and Yu (1998).

Molecular studies

307 isolates of *Pythium* did not produce sporangia and/or sexual structures and could not be identified with certainty based on morphology alone. Selected isolates were studied further by determining the nucleotide sequences of the internal transcribed spacer 1-5.8S ribosomal RNA gene (ITS1). The isolates were grown in 1000 ml 10% V-8 juice solution at 28°C. The mycelium was filtrated, washed with sterile deionized water and dried for DNA extraction. A 1.5-ml Eppendorf microcentrifuge tube was filled one-third up the conical portion with freshly ground mycelium (0.1-0.3 g, wet ground by hand with a mortar and pestle in liquid nitrogen). Four hundred microliters of lysis buffer (50 mM Tris-HCl [pH 7.2], 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol) was added and vortexed so the mixture was homogeneous. After incubation at 65°C for 1 h, 400 µl chloroform : phenol (1 : 1, v : v) was added, vortexed briefly and microcentrifuged at 10,000 ×*g* for 15 min at room temperature. 300-350 µl of the aqueous phase containing the DNA was transferred to a new tube. Ten microliters of 3 M NaOAc (pH 8.0) was added to the aqueous phase followed by 0.54 volumes of isopropanol. After inverting to mix, the liquid was microcentrifuged at 10,000 ×*g* for 10 min at room temperature. The supernatant was poured off and the pellet was rinsed three times with 70% ethanol. The tubes were inverted for 1 min, drained on a paper towel and were placed in a vacuum oven at 50°C for 15 min or until dry. The pellet was resuspended in 200 µl distilled water. For PCR amplification and sequencing of ITS1 region of rDNA, 1 µl of the DNA sample was diluted in distilled water to a final concentration of 10 ng DNA. Primers ITS1 and ITS2 (White et al., 1990) were used for amplification. Twenty-five microliters of reaction mixture containing 10 ng template DNA, 1 µl of each 1 lM primer, 0.5 µl dNTP stock mixture (2 mM each dNTP), 2.5 µl 10 X PCR buffer, 1.5 µl 25 mM $MgCl_2$, 0.5 µl Taq polymerase was subjected to thermal cycling in a Thermal Cycler (heat lip). An initial denaturation step of 3 min at 95°C was followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, extension for 2 min at 72°C and then followed by a final extension step for 7 min at 72°C. The reaction mixture (5 µl) was run on 1.5% agarose gels, stained with ethidium bromide and visualized under UV illumination to determine the number and size of DNA products amplified in the PCR. The PCR products were extracted from gel by Gel Extraction Mini Kit. The ITS I DNA fragments were subcloned into cloning site of pMD18-T vector and transformed into competent cells of *E. coli* strain XL1-Blue. The clones with recombinant plasmid were identified by blue/white colony screening on LB culture plate (containing 200 mg/ml IPTG, and 20 mg/ml X-gal) and colony PCR method. Then the positive clones were sent to Shanghai Sangon Biological Engineering Technology & Service Co. Ltd. for sequencing on both strands.

RESULTS

A total of 2655 isolates of *Pythium* were obtained and 2348 isolates were identified and assigned to 29 species of *Pythium*: *P. acanthicum*, *P. acanthophoron*, *P. aphanidermatum*, *P. aristosporum*, *P. arrhenomanes*, *P. carolinianum*, *P. catenulatum*, *P. cucurbitacearum*, *P. deliense*, *P. dissotocum*, *P. graminicola*, *P. helicoides*, *P. hydnosporum*, *P. indigoferae*, *P. inflatum, P. intermedium*, *P. irregulare*, *P. marsipium*, *P. middletonii*, *P. monospermum*, *P. myriotylum*, *P. oedochilum*, *P. paroecandrum*, *P. pulchrum*, *P. spinosum*, *P. splendens*, *P. sylvaticum*, *P. ultimum* and *P. vexans*. Their occurrence and distribution in Hainan are summarized in Tables 1 and 2 and the sampling sites are represented in Figure 1.

The identification of *Pythium* species was based primarily on the morphological characteristics. However, the identity of 17 isolates that failed to produce sporangia and/or sexual structures was determined or confirmed as a result of a BLAST search with ITS 1 sequences of isolates of *Pythium* species from GenBank (Table 3). These include *P. acanthophoron*, *P. aphanidermatum*, *P. carolinianum*, *P. cucurbitacearum*, *P. helicoides*, *P. myriotylum*, *P. oedochilum*, *P. splendens* and *P. sylvaticum*. Only isolates showing at least 97% identity with known *Pythium* species in GenBank data were accepted. Isolates Py0600a, PyL8-3, Pycatas2, Pyjflwy2 and Pyjflwy1 had low sequence identity (91-94%) with known *Pythium* species from GenBank and were not included in the list of Hainan *Pythium* species. It is quite possible that these isolates might be new species of *Pythium*. However, further morphological studies are needed to characterize these isolates before new taxa could be proposed.

DISCUSSION

Present comprehensive study of the genus *Pythium* in Hainan yielded some interesting results. Considering the warm and wet climate, together with luxurious natural and cultivated plant growth on the island we were surprised that only 29 species of *Pythium* were isolated. The failure in re-isolating *P. diclinum* and *P. adhaerans* recorded by Yu and Ma (1989) could be due to the difference in the isolation techniques and/or change in the ecosystem over time. However, when these two species are added to our list , there are now 31 species recorded in Hainan, just exceeding half of the total of 56 species of *Pythium* found in mainland China (Ho, 2009; Long et al., 2010). Taiwan island, which is slightly larger in size than Hainan but has similar weather and topography, is exceptional in having much richer *Pythium* diversity. A total of 48 *Pythium* species has been recorded in Taiwan and many caused a wide variety of plant diseases on sugarcane, tree seedlings, vegetables, specialty crops and flowering plants (Ho, 2009,

Table 1. Distribution of *Pythium* species on Hainan Island.

Pythium species	Sampling sites of Hainan Island
P. acanthicum (soil)	Baisha County, Baoting County, Bawangling (Mountain), Changjiang County, Haikao City, Jiangfengling (Mountain), Ledong City, Lingshui County, Qionghai County, Shanya City, Tungchang City, Wanning City, Wenchang City, Wuzhishan (Mountain)
P. acanthophoron (soil)	Haikao City, Lingshui County, Wenchang City
P. aphanidermatum (soil, leaves, sea water)	Baisha County, Changjiang County, Dongfang City, Haikao City, Jianfengling (Mountain), Qionghai City, Qiongzhong County, Wuzhishan City, Wuzhishan (Mountain)
P. aristosporum (soil)	Changjiang County
P. arrhenomanes (soil)	Wuzhishan City, Diaoluoshan (Mountain), Bawangling (Mountain), Haikao City, Lingao County
P. carolinianum (soil, leaves, fresh water)	Wuzhishan (Mountain), Haikao City, Wenchang City, Changjiang County, Qiongzhong County, Wanning City
P. catenulatum (soil)	Wuzhishan (Mountain), Wuzhishan City, Changjiang County, Shanya City
P . cucurbitacearum (leaves, sea water)	Wenchang City
P. deliense (soil)	Jiangfengling (Mountain), Haikao City, Wenchang City, Danzhou City, Ledong County, Shanya City, Wuzhishan City, Qionghai City, Qiongzhong County, Baisha County, Lingshui County, Tungchang County, Dongfang City
P. dissotocum (soil)	Bawangling (Mountain)
P. graminicola (soil)	Haikou City, Baisha County
P. helicoides (soil, leaves, sea water)	Wuzhishan (Mountain), Haikou City, Wenchang City, Danzhou City, Changjiang County
P. hydnosporum (soil)	Wanning City
P. indigofereae (soil, leaves, fresh water)	Wuzhishan (Mountain), Jiangfengling (Mountain), Haikao City, Wenchang City, Ledong County, Shanya City
P. inflatum (soil)	Wenchang City, Ledong County, Shanya City, Qiongzhong County
P. intermedium (soil)	Wuzhishan (Mountain), Shanya City, Wuzhishan City
P. irregular (soil)	Limushan (Mountain)
P. marsipium (leaves, fresh water)	Danzhou City, Wuzhishan (Mountain), Jiangfengling (Mountain)
P. middletonii (soil, leaves, sea water)	Wuzhishan (Mountain), Jiangfengling (Mountain), Bawangling (Mountain), Wenchang City
P. monospermum (soil)	Danzhou City, Bawangling (Mountain)
P. myriotylum (soil)	Wuzhishan (Mountain), Wenchang City, Changjiang County, Ledong County, Shanya City, Wuzhishan City, Chengmai County
P. oedochilum (soil)	Haikou City, Wenchang City, Danzhou City, Ledong County, Chengmai County, Tungchang County
P. paroecandrum (soil)	Tungchang County
P. pulchrum (soil)	Wuzhishan City
P. spinosum (soil)	Jiangfengling (Mountain), Bawangling (Mountain), Haikou City, Danzhou City, Ledong County, Shanya City, Baisha City, Tungchang County, Dongfang City
P. splendens (soil)	Wuzhishan (Mountain), Jiangfengling (Mountain), Bawangling(Mountain), Haikou City, Wenchang City, Danzhou City, Ledong County, Wuzhishan City, Qiongzhong County, Qionghai City
P. sylvaticum (soil)	Qiongzhong County
P. ultimum (soil)	Jiangfengling (Mountain)
P. vexans (soil, leaves, fresh water)	Baisha County, Baoting County, Bawangling (Mountain), Changjiang City, Chengmai County, Danzhou City, Diaoluoshan (Mountain), Dongfang City, Haikao City, Jiangfengling (Mountain), Ledong City, Limushan (Mountain), Lingao County, Lingshui County, Qionghai City, Qiongzhong County, Shanya City, Tungchang County, Wanning City, Wenchang City, Wuzhishan City, Wuzhishan (Mountain)

2011). In contrast, there have been only a few reports of plant diseases in Hainan attributable to *Pythium*. *Pythium aphanidermatum* has been reported to cause leaf rot of bromeliad, *Aechmea fasciata* (Zhang et al., 2003) and stem rot of papaya (Liu et al., 2003) whereas *P. vexans* caused patch canker of rubber trees (Zeng et al., 2005). By artificial inoculation, *P. splendens* was proved to be pathogenic to oil palm seedlings (Chen et al., 2008). The paucity of plant diseases caused by *Pythium* spp. in Hainan could be due to the lack of study or the possibility that most of the species of *Pythium* are indigenous and have co-evolved with local flora. The 18 species of *Pythium* isolated from five virgin mountain forests are most likely indigenous to Hainan: *P. acanthicum*, *P. aphanidermatum*, *P. arrhenomanes*, *P. carolinianum*, *P. catenulatum*, *P. dissotocum*, *P. helicoides*, *P. indigoferae*, *P. inflatum*, *P. intermedium*, *P. irregulare*, *P. middletonii*, *P. paroecandrum*, *P. pulchrum*, *P. spinosum*, *P. splendens*, *P. ultimum* and *P. vexans* since the forests have been protected from the public for many years. Conceivably, propagules from these *Pythium* species could be carried by the mountain streams and populated the lowland surrounding the mountains over time. Furthermore, compared with Taiwan, Hainan has much fewer imported plant cultivars which might bring in exotic species of *Pythium*. A parallel example could be found in a similar plant pathogenic pythiaceous genus: *Phytophthora.* Whereas only 14 species of *Phytophthora* were found in Hainan (Zeng et al., 2008), 38 species have been recorded

Table 2. Number of *Pythium* species from sampling sites of Hainan Island.

Sampling sites of Hainan Island	Number of species	*Pythium* species isolated from each sampling site of Hainan Island
Wenchang City	14	*P. acanthium, P. acanthophoron, P. caroliniarum, P. cucurbitacearum, P. deliense, P. helicoides, P. indigoferae, P. inflatum, P. middletonii, P. myriotylum, P. oedochilum, P. ostracodes, P. splendens, P. vexans*
Haikou City	13	*P. acanthicum, P. acanthophoron, P. aphanidermatum, P. arrhenomanes, P. carolinianum, P. deliense, P. graminicola, P. helicoides, P. indigoferae, P. oedochilum, P. spinosum, P. splendens, P. vexans*
Wuzhishan City	13	*P. acanthicum, P. arrhenomanes, P. catenulatum, P. aphanidermatum, P. deliense, P. helicoides, P. intermedium, P. myriotylum, P. pulchrum, P. splendens, P. oedochilum, P. vexans*
Wuzhishan (mountain)	12	*P. acanthicum, P. aphanidermatum, P. carolinianum, P. catenulatum, P. helicoides, P. indigoferae, P. intermedium, P. marsipium, P. middletonii, P. myriotylum, P. splendens, P. vexans*
Ledong County	11	*P. acanthicum, P. catenulatum, P. deliense, P. helicoides, P. indigoferae, P. inflatum, P. myriotylum, P. oedochilum, P. spinosum, P. splendens, P. vexans*
Jianfengling (mountain)	10	*P. acanthicum, P. aphanidermatum, P. deliense, P. indigoferae, P. marsipium, P. middletonii, P. spinosum, P. splendens, P. ultimum, P. vexans*
Shanya City	10	*P. acanthicum, P. catenulatum, P. deliense, P. helicoides, P. indigoferae, P. inflatum, P. intermedium, P. myriotylum, P. spinosum, P. vexans*
Bawangling (mountain)	8	*P. acanthium, P. arrhenomanes, P. dissotocum, P. middletonii, P. monospermum, P. spinosum, P. splendens, P. vexans*
Qiongzhong County	8	*P. aphanidermatum, P. carolinianum, P. deliense, P. helicoides, P. inflatum, P. splendens, P. sylvaticum, P. vexans*
Changjiang County	7	*P. acanthicum, P. aphanidermatum, P. aristosporum*, *P. carolinianum, P. helicoides, P. myriotylum, P. vexans*
Baisha County	7	*P. acanthicum, P. aphanidermatum, P. carolinianum, P. deliense, P. graminicola, P. spinosum, P. vexans*
Qionghai City	6	*P. acanthicum, P. aphanidermatum, P. deliense, P. helicoides, P. splendens, P. vexans*
Tunchang County	6	*P. acanthicum, P. deliense, P. oedochilum, P. paroecandrum, P. spinosum, P. vexans*
Wanning City	5	*P. acanthicum, P. carolinianum, P. spinosum, P. hydnosporum, P. vexans*
Lingshui County	4	*P. acanthium, P. acanthophoron, P. deliense, P. vexans*
Chengmai County	4	*P. graminicola, P. oedochilum, P. myriotylum, P. vexans*
Dongfang City	4	*P. aphanidermatum, P. deliense, P. spinosum, P. vexans*
Lingao County	2	*P. arrhenomanes, P. vexans*
Baoting County	2	*P. acanthicum, P. vexans*
Diaoluoshan (mountain)	2	*P. arrhenomanes, P. vexans*
Limushan (mountain)	1	*P. irregulare*
Dingan County	1	*P. vexans*

Table 3. BLAST closest match of the ITS1 sequence of Hainan *Pythium* isolates with GenBank Database.

Isolate	*Pythium* species	Accession No.	Strain No.	Max. Identity
HPy-1	*P. acanthophoron*	AF216652.1	CBS337.29	97%
		AY598711.1	CBS337.29	97%
HPy-2	*P. aphanidermatum*	AB355599.1	UOP390	100%
		EU162763.1	63A	100%
		AM396563.1	Py-294	100%
Py0600c	*P. carolinianum*	AY987038	ATCC36434	100%
		DQ211524	F-1332	100%
HPy-3	*P. carolinianum*	GU233300.1	F-1551.2A	98%
HPy-4	*P. cucurbitacearum*	FN263243.1	97-1a	99%
		AY598667.1	CBS748.96	99%
		GU258619.1	WPC:7696A281	98%
HPy-5	*P. helicoides*	FJ348741.1	Py-67	100%
		AB217660.1	CBS286.31TA3	100%
		AB217659.1	CBS286.31TA2	100%
HPy-7	*P. helicoides*	AB108059.1	RoPh3C5	99%
		AB108035.1	H5szl C35	99%
		AB108031.1	H5szl C26	99%
Hpy-8	*P. myriotylum*	FJ797577.1	BR1P52426	100%
		FJ79574.1	KRS14	100%
		FJ797576.1	BRIP39907	100%
Hpy-9	*P. oedochilum*	FJ415970.1	PPR18408	97%
		AB259315.1	MAFF712271	97%
		AY598664.1	CBS292.37	97%
Py0410s	*P. splendens*	AY3757242	OPU591	100%
		AY269993	117	100%
		AY269994	461	100%
Hpy-10	*P. sylvaticum*	AB468779.1	UZ307	97%
		GU259304.1	WPC:16365D1276	97%
		GU259107.1	WPC:15580C1703	97%

in Taiwan (Ho et al., 1995). Nevertheless, considering the fact that many species of *Pythium* are potential plant pathogens (Hendrix and Campbell, 1973) more attention should be paid to detect *Pythium*-induced plant diseases.

Of all the *Pythium* species isolated in Hainan, *Pythium vexans* is by far the most common species, encountered both in terms of its occurrence frequency in sampling sites (92%) and the total number of isolates obtained (73.0%). This is followed by *P. deliense, P. acanthicum*, *P. aphanidermatum*, *P. helicoides* and *P. splendens.* On the other hand, *P. ultimum* which is widely spread throughout the world (Plaats-Niterink, 1981) was rarely isolated in Hainan. In other parts of China, Yu and Ma (1989) found that *P. aphanidermatum* was the dominant species and caused serious diseases of vegetables, fruit, tree and corn seedlings, cotton, tobacco etc. (Editorial Council, 1996; Shen and Zhang, 1995) but *P. vexans* was not one of the top 10 commonest species encountered. In fact *P.vexans* was not recovered from Guangxi Province (Liu, 2004; Fu, 2005) or Hanzhou area (Lou, 2005). The most widespread species in both regions is *P. spinosum*. The discrepancies in the findings could be attributable to the different methods of isolation used.

Of 23 sampling sites in Hainan, the largest number of *Pythium* species came from the cities of Wenchang, Wuzhishan and Haikao. However, it may be premature to speculate on the diversity of *Pythium* species in these locations because the greater number of *Pythium* species isolated might be correlated with the higher frequency of sample collections. Twenty species of *Pythium* are new records in Hainan: *P. acanthophoron*, *P. aristosporum*, *P. arrhenomanes*, *P. carolinianum*, *P. cucurbitaceraum*, *P.*

graminocola, *P. helicoides*, *P. hydnosporum*, *P. inflatum*, *P. intermedium*, *P. irregulare*, *P. middletonii*, *P. monospermum*, *P. oedochilum*, *P. paroecandrum*, *P. pulchrum*, *P. spinosum*, *P. sylvaticum*, *P. ultimum* and *P. vexans*. In addition, *P. cucurbitacearum* has never been reported anywhere in China before. In fact, this species has rarely been reported worldwide and its taxonomy is controversial. Since its first discovery in Japan causing seedling damping off and soft rot of cucumber fruits (Takimoto, 1941) it has been reported only once from rotted fruit of *Trichosanthes dioica* in West Bengal (Chaudhuri, 1974). Plaats-Niterink (1981) did not accept it as a valid species of *Pythium* for the lack of latin diagnosis in the original description. Dick (1990) even suggested that it might belong to the genus *Phytophthora*. Unfortunately, our single isolate did not produce sexual structures but based on its high level of similarity in the ITS sequence (99%) with isolates of *P. cucurbiacearum* in the GenBank we decided to tentatively accept this species hoping that further morphological studies and/or isolation might elucidate the taxonomic position of this obscure species. *Pythium splendens* has only been recently reported in mainland China, from Guangxi (Yuan and Lai, 2004) and Zhejiang (Wang et al., 1995) but is rather common in Hainan. Both mating types are present and some fresh isolates were self-fertile.

Our isolation of *P. sylvaticum* is the second record in mainland China after its first discovery in Hanzhou area **(**Lou, 2005). It is heterothallic but unfortunately we did not have the appropriate mating types to induce the production of sex organs. Nevertheless, its identity was confirmed based on molecular studies. Most of the *Pythium* species are terrestrial and occasionally found in fresh water (Plaats-Niterink, 1981). In the present study, the vast majority of *Pythium* isolates were from soil samples with *P. marsipium, P. carolinianum, P. indigoferae* and *P. vexans* isolated from freshwater as well. Of special interest is the species of *Pythium* isolated from fallen leaves submerged in sea water in or near mangrove habitats: 4 isolates of *P. vexans* and 1 isolate each of *P. aphanidermatum*, *P. cucurbitaceanum*, *P. helicoides* and *P. middletonii*. To date there are only 4 marine *Pythium* species: *P. grandisporangium*, *P. marinum*, *P. porphyrae* and *P. salinum* (Plaats-Niterink, 1981). Further studies are needed to determine if these species should be considered as halotolerant strains or added to the short list of marine species of *Pythium*. The fact that they were isolated from the lesions of fallen leaves submerged in seawater suggests that *Pythium* spp. might play an important role in litter decomposition in the saline environment.

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中國海南島腐黴屬種的發生與分佈

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在 2003 年至 2009 年間，我們採用誘餌富集和選擇性培養基分離技術，對從中國海南島各種不同生境中採集的土壤和水體樣品進行了腐黴屬種 (*Pythium*) 的分離和鑒定。共對 2,348 個腐黴屬分離菌株進行了鑒定，從中鑒定出 29 個腐黴種，分別是：棘腐黴（*P. acanthicum*）、刺器腐黴（*P. acanthophoron*）、瓜果腐黴（*P. aphanidermatum*）、芒孢腐黴（*P. aristosporum*）、強雄腐黴（*P. arrhenomanes*）、卡地腐黴（*P. carolinianum*）、鏈狀腐黴（*P. catenulatum*）、葫蘆腐黴（*P. cucurbitacearum*）、德里腐黴（*P. deliense*），寬雄腐黴（*P. dissotocum*），禾生腐黴（*P. graminicola*）、旋柄腐黴（*P. helicoides*）、齒孢腐黴（*P. hydnosporum*）、木蘭腐黴（*P. indigoferae*）、腫囊腐黴（*P. inflatum*）、間型腐黴（*P. intermedium*）、畸雄腐黴（*P. irregulare*）、袋囊腐黴（*P. marsipium*）、奇雄腐黴（*P. middletonii*）、簡囊腐黴（*P. monospermum*）、群結腐黴（*P. myriotylum*）、腫雄腐黴（*P. oedochilum*）、側雄腐黴（*P. paroecandrum*）、絢麗腐黴（*P. pulchrum*）、刺腐黴（*P. spinosum*）、華麗腐黴（*P. splendens*）、林栖草腐黴（*P. sylvaticum*）、終極腐黴（*P. ultimum*）和鐘器腐黴（*P. vexans*）。除了棘腐黴、瓜果腐黴、鏈狀腐黴、德里腐黴、寬雄腐黴、木蘭腐黴、袋囊腐黴、群結腐黴、華麗腐黴等 9 個種外，其餘 20 個種是海南新記錄種，葫蘆腐黴（*P. cucurbitacearum*）是中國首次報導。鐘器腐黴、瓜果腐黴、葫蘆腐黴、旋柄腐黴和奇雄腐黴等幾個腐黴種以往在陸地分離到，也偶你在淡水中分離到，此次是第一次在浸沒於海水中的落葉上分離到。這些腐黴菌株是否應該作為耐鹽菌株或是海洋種來處理，仍然有待進一步研究，但他們在海水生境腐爛的雜物上被重複分離到顯示出其在海水沉積物分解過程中的重要性。到目前為止，鐘器腐黴是分佈最廣、數量最多的腐黴種類，其次依次是德里腐黴、棘腐黴、瓜果腐黴、旋柄腐黴和華麗腐黴。在 23 個樣本採集地點中，分離到的腐黴種類最多的地點是文昌市、海口市、五指山市、樂東縣及其周邊地區。

關鍵詞：假菌界；生態學；卵菌綱；腐黴屬菌物。