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Cymbidium black rot caused by an aberrant strain of *Phytophthora multivesiculata* in Taiwan

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ABSTRACT

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In 1996 and 1997, a severe outbreak of a disease on Cymbidium orchid (*Cymbidium hybridium*) occurred in a garden in I-Lan, Taiwan, with numerous plants showing black rot symptoms on leaves, pseudostems and roots and dying. A *Phytophthora*-like microorganism was constantly isolated from the diseased plants and two of the eleven isolates collected from this garden were used in this study. Inoculation tests were carried out using zoospore suspensions to fulfill Koch's postulates. Results showed that both isolates (P96013 and P96016) were pathogenic on *C. hybridium* with the same black rot symptoms as those developed on the naturally infected plants. The pathogenicity of these two *Phytophthora*-like isolates was confirmed as the organism reisolated from the inoculated plants was morphologically identical to the culture of the original inoculum. In addition to *C. hybridium*, the two *Phytophthora*-like isolates were also pathogenic on other Asian *Cymbidium* species, including *C. ensifolium, C. sinense* and *C. rubrigemmum*, causing black rot symptoms and death of plants. Both *Phytophthora*-like isolates were similar to *Phytophthora multivesiculata* in most of the morphological characteristics. However, they were different from *P. multivesiculata* in some features such as dimensions of sporangia, oogonia and oospores as well as maximum growth temperature. The ITS sequences of these two *Phytophthora*-like isolates were identical to that of *P. multivesiculata*. Based on the morphological characteristics and the molecular data, both *Phytophthora*-like isolates from *C. hybridium* were identified as an aberrant strain of *Phytophthora multivesiculata*. This is the first report of the aberrant strain of *Phytophthora multivesiculata* in Taiwan. Diseased orchid plants in the garden of Yuan-Shan, I-Lan county, were destroyed in 1998 and this aberrant strain of *P. multivesiculata* was not found in the survey of 1998 and 1999 in I-Lan county or any other orchid production areas of Taiwan.

Keywords: Aberrant strain, *Phytophthora multivesiculata*, Cymbidium orchid, *Cymbidium hybridium*, New disease, Identification.

INTRODUCTION

Orchids are important ornamental crops in Taiwan. The island has the ideal climate for breeding and growing many economically important species of orchids. However,

the climate is also conducive to outbreaks and dissemination of numerous pests on orchids. There are over 20 diseases of orchids recorded in Taiwan [2, 9], and diseases caused by *Phytophthora* spp. are among the important ones. During the survey of 1996 and 1997, a serious disease of the Cymbidium orchid (*Cymbidium hybridium* Hort.) was found in a commercial garden for production of orchid flowers in Yuan-Shan, I-Lan county, Taiwan, with numerous plants showing symptoms of black rot on leaves, pseudostems and flowers and dying. Isolation from diseased plants collected from this garden resulted in a *Phytophthora*-like microorganism which is previously unknown in Taiwan [1, 5]. The objective of this study was to determine identity of this new *Phytophthora*-like pathogen of *C. hybridium* in Taiwan, using both morphological characteristics and molecular data, including the soluble protein pattern [3] and ITS sequence [7].

MATERIALS AND METHODS

Isolation and cultures

Diseased plants of Cymbidium orchids (*C. hybridium*) showing symptoms of water-soaked black-rot lesions on leaves and pseudostems as well as root rots were collected in 1996 and 1997 from an orchid garden located at Yuan-Shan, I-Lan County, Taiwan. To isolate the microorganism from the diseased plants, small pieces (ab. 7 × 7 × 5 mm) of plant tissues were removed from the margin of lesions on leaves, surface sterilized in 0.5 % sodium hypochloride for 30 min and placed on a clarified V-8 juice agar (CVA) medium (5% clarified V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) supplemented with 100 ppm of ampicillin, 50 ppm of mycostatin and 10 ppm of pentachloronitrobenzene [11]. After incubation at room temperature for 1-2 days, agar blocks containing mycelial mats were removed from the edge of the fungal colony derived from each tissue and individually transferred onto a fresh 5% V-8 juice agar (5% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) plate. Single-zoospore isolates from these VA cultures were established and two of the isolates, P96013 and P96016, were used for subsequent tests. To prepare stock cultures, agar blocks containing mycelial mats were removed from 5-7-day-old VA cultures, submerged in sterile distilled water in test tubes and stored at 24°C [4].

The *Phytophthora* species used for comparison with *Phytophthora*-like cultures from diseased plants of *C. hybridium* were *P. erthroseptica* Pethybridge, isolate p64156; *P. megasperma* Dechersler, isolate p58817 (courtesy of Dr. H. H. Ho, USA), and *P. sojae* Kaufmann and Gerdemann, race 1 and race 3 (courtesy of Dr. W. H. Ko).

Morphological studies

For studying morphological characteristics of isolates P96013 and P96016, large amounts of zoosporangia were produced by the method described by Hwang *et al.* [10]. Zoosporangia were incubated at 15°C for 30 min to stimulate release of zoospores, and the resulting zoospore suspension was stored in an ice box and used for inoculation of orchid plants within half an hour.

For studying formation of hyphal swellings, five to ten pieces of agar discs (5×5×3 mm) containing mycelial mats were removed from the periphery of the *Phytophthora*-like colonies grown on CVA for 5-7 days and placed in sterile distilled water in a Petri dish (60 mm diam.). After incubation at room temperature for 24 h, the agar discs were examined under a compound microscope for formation of hyphal swellings. Mycelia growing on the agar blocks were also examined for the formation of hyphal swellings and chlamydospores.

For studying sexual reproduction, each single zoospore isolate of *Phytophthora*-like organism was grown on 10% VA (10% V-8 juice, 0.02% $CaCO_3$ and 2% Bacto agar) at 24°C in darkness for 14 days and then incubated at 24°C under light for another 14 days for the maturation of oospores. Fifty mature oospores were randomly selected from each isolate and measured for size under a compound microscope. The size of oogonia and antheridia was also measured.

Temperature on growth of *Phytophthora* spp.

To determine the effect of temperature on the growth

of the *Phytophthora*-like microorganism, agar discs (5 mm diam.) containing mycelial mats were removed from the periphery of 5-7 days old colonies on 5% CVA using a cork borer (6 mm diam.) and were each transferred to the edge of a 5% CVA plate. The inoculated dishes were incubated at 10, 15, 18, 21, 24, 27, 29, 30, 33, 35 or 36^{o}C in darkness. The colony diameter in each dish was measured daily, either until the mycelium growth reached the opposite edge of the Petri dish or for a maximum of 10 days after inoculation. There were four dishes for each temperature and the experiment was repeated twice.

Pathogenicity tests

Plants of *C. hybridum*, cultivar Lady Spring 'Nancy', were grown in pots for 6-12 months and used for the inoculation tests. Inoculation was done by spraying 50 ml of the zoospore suspension ($10^{4\sim5}$ zoospores/ml) on each plant until runoff, and the excess solution was poured on the pseudostems. For the treatment of control, plants were sprayed with sterile distill water only. Each inoculated or control plant was covered with a transparent plastic bag for 72 h to maintain high moisture, placed at 20-24^{o}C in a walk-in growth chamber (S-Tai Co., Taiwan) and examined daily for symptoms of the disease on the plants for a period of 14 days. There were five plants for each treatment and the experiment was repeated once. To reisolate the pathogen, lesions were excised from the inoculated plants, surface sterilized and placed on the selective medium by the method described previously. The procedures of Koch's postulates were fulfilled to confirm pathogenicity of each *Phytophthora*-like isolate.

To determine the pathogenicity of the *Phytophthora* -like isolates from *C. hybridum* on other Asian Cymbidium species, including *C. sinense*, *C. ensifolium* and *C. rubrigemmum*, were used for inoculation, using the same procedure described in the inoculation of *C. hybridum*..

Soluble Proteins Pattern Analysis

To compare the electrophoretic patterns of the soluble protein in mycelia, cultures of *Phytophthora*-like isolates P96013 and P96016 from *C. hybridum* and other species of *Phytophthora*, including *P. erthroseptica*, isolate p64156; *P. megasperma*, isolate p58817 and *P. sojae* race 1 and race 3 were grown in 5% V8 broth (5% V-8 juice and 0.02% $CaCO_3$) in 250 ml flasks, 50 ml/flask, for 7 days at 24^{o}C in darkness. Mycelial mats in each flask were collected, extracted, and analyzed for soluble proteins, using the procedure of electrophoresis described previously [6]. Standard protein markers (Sigma company, USA) of 250, 150, 100, 75, 50, 37, and 25 kD each of 10 microgram were loaded in a separate well.

Sequence Analysis

DNA preparation. Mycelia of each *Phytophthora*- like isolate of P96013 and P96016 from *C. hybridum* were inoculated on cellophane membrane placed on 5% VA in a Petri dish. After incubation at 24^{o}C for 7-10 days, mycelia on the cellophane membrane were harvested and lyophilized. About 20 mg of the lyophilized mycelia was ground in liquid nitrogen and the DNA was extracted using a Genomic DNA Purification Kit (GeneMark Technology Co., Taichung, Taiwan) according to the manufacturer's protocol.

PCR amplification. The ribosomal internal transcribed spacer (ITS) regions of isolates P96013 and P96016 from *C. hybridum*, including ITS1-5.8S rDNA-ITS2, plus partial 18S rRNA and 28S rRNA were amplified using primers ITS4 and ITS5 [13]. The 25 μl of PCR reaction mixture consisted of 0.2 μg of template DNA, 0.2 μM of each primer, 200 μM of each dNTP (GeneMark, Taiwan), 1X polymerase chain reaction (PCR) buffer and 1.0 unit of ZyM Taq DNA polymerase (Zymeset Biology, Taiwan). The PCR was performed with a denaturing step at 94^{o}C for 3 min, followed by 35 cycles of 94^{o}C for 45 sec, 50^{o}C for 45 sec, 72^{o}C for 45 sec, and a final elongation at 72^{o}C for 7 min. The amplification was carried out using a GeneAmp 9700 system (Applied Biosystems, USA), and the PCR products were examined by electrophoresis on a 1.5% agarose gel using a Mupid-2plus Electrophoresis System (Advance, Japan).

DNA sequencing and sequence alignment. Sequencing of the PCR products was performed by the Seeing Bioscience Company (Taipei, Taiwan), with primers

ITS4, ITS5, 5.8S2-1 (5′TCGCACAT CGATGAAGAACG) and 5.8S2-2 (5′TACGGACA CTGATACAGGCAT). The ITS sequences obtained were compiled and edited using the Vector NTI software v. 10.0 (InforMax Inc., USA), uploaded directly to NCBI (National Center for Biotechnology Center) (http://www.ncbi.nlm.nih.gov) and compared with sequences in GenBank databases using BLAST software [14] to search for the closest *Phytophthora* species. Meanwhile, the sequence of ITS1-5.8S rDNA-ITS2 of the isolate P96013 from cymbidium was submitted to NCBI and the accession no. is FJ147195.

RESULTS

Symptoms of disease and isolatation of pathogen

The unusual *Phytophthora* species associated with black rot of *C. hybridium* was found in both years of 1996 and 1997 in an isolated garden located at Yuan-Shan, I-Lan, a northeastern county of Taiwan. The initial symptoms of the disease on infected plants were formation of bleached and water-soaked spots on leaves and psedostems, which turned into black in 3-5 days (Fig. 1-A, B). Some of the infected leaves drooped prematurely. All of the infected plants died eventually, due to severe black rots of pseudostems and leaves. Because of the wet weather in the spring of 1996 and 1997, the disease spread rapidly in this garden with more than 90% of cultivated Cymbidium plants infected and killed. Eleven single zoospore isolates of *Phytophthora*-like organisms showing similar cultural characteristics were isolated from diseased plants of *C. hybridium* collected in this orchid garden, including 5 isolates in 1996 and 6 isolates in 1997. Two of the isolates, P96013 and P96016, were used in the studies of morphological characteristics, pathogenicity and molecular features.

Pathogenicity tests

Results of artificial inoculation tests showed that both isolates P96013 and P96016 of *Phytophthora*-like organisms were pathogenic on *C. hybridium*, causing symptoms of black rot on leaves and pseudostems within 3-7 days after inoculation. The pathogen did not require wounding treatment for successful infection of the host plants. In addition to *C. hybridium*, isolates P96013 and P96016 were also pathogenic on several other Asian *Cymbidium* species, including *C. sinense*, *C. ensifolium*, and *C. rubrigemmum*. Most of the inoculated plants died one month after the appearance of black rot symptoms. The *Phytophthora*-like pathogen re-isolated from the inoculated orchid plants were identical to the original culture in morphological characteristics.

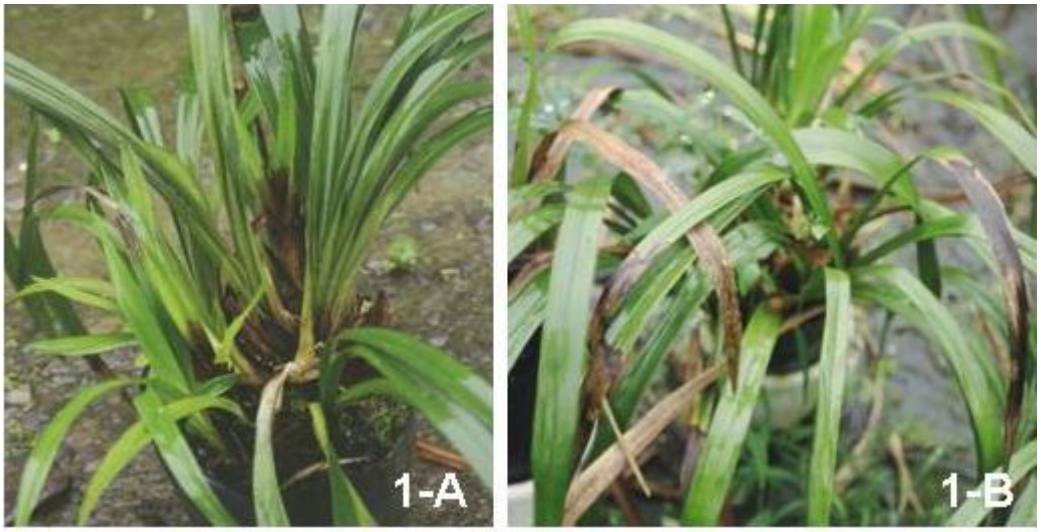

Fig.1. Plants of *Cymbidium hybridium* naturally infected by an aberrant strain of *Phytophthora multivesiculata* showing symptoms of black rot on pseudostems (A) and leaves (B).

Morphological and culture characteristics

Both isolates P96013 and P96016 of *Phytophthora*-like organisms from *C. hybridium* grew on 5% CVA and PDA in Petri dishes and the colonies produced fluffy aerial mycelia without formation of distinctive colony patterns (Fig. 2). A few sporangia were formed on VA culture. However, when agar blocks of VA culture were transferred into sterile distilled water and incubated under light for overnight or 24 h, sporangiophores were formed abundantly from the newly produced mycelia. Usually one sporangium was formed on a long, slender sporangiophore. Sporangia were non-papillate, non-deciduous and ovoid (Fig. 3-C), 42-(53.8)-64 × 32-(40.8)-54 μm in size with length: breadth (L/B) ratio of 1.1-(1.3)-1.6. External proliferation sporangia were formed after releasing zoospores (Fig. 3-D). Chlamydospores were abscent. Hyphal swellings with distinctive catenulation were produced on cultures of VA or in water (Fig. 3-A, B). Both isolates P96013 and P96016 were homothallic and each isolate was capable of producing oospores when cultured alone on 10% VA under dark conditions. Oogonia were spherical with a slightly wavy-wall pattern (Fig. 3-E, F, and G). Oospores were mostly aplerotic. Oogonia and oospores were 33-(50.1)-56 μm and 28-(43.7)-50 μm in diameter, respectively. Antheridia were amphigynous, mostly spherical, and 7-(13.4)-18 × 9-(14.0)-16.4 μm in diameter. No significant differences in the dimensions of the reproductive structures were observed between P96013 and P96016 isolates. Based on the morphological characteristics of sporangia and sexual structures, the *Phytophthora*-like isolates P96013 and P96016 from *C. hybrida* are categorized into Waterhouse's group VI [(15)].

The temperature range for growth of isolates P96013 and P96016 was 10 to 29^{o}C and there was no growth at 30^{o}C (Fig. 4). The optimum temperature for growth of both isolates was 24^{o}C with an average linear growth rate of 3.8 mm/day.

Electrophoresis

The electrophoretic patterns of the soluble mycelial proteins were identical for both P96013 and P96016 isolates of *Phytophthora*-like organisms from *C. hybridium* (Fig. 5). However, the soluble protein patterns of these two isolates were different from the patterns of other *Phytophthora* species, including *P. sojae* race 1 and race 3, *P. erythroseptica* p64156 and *P. megasperma* p58817.

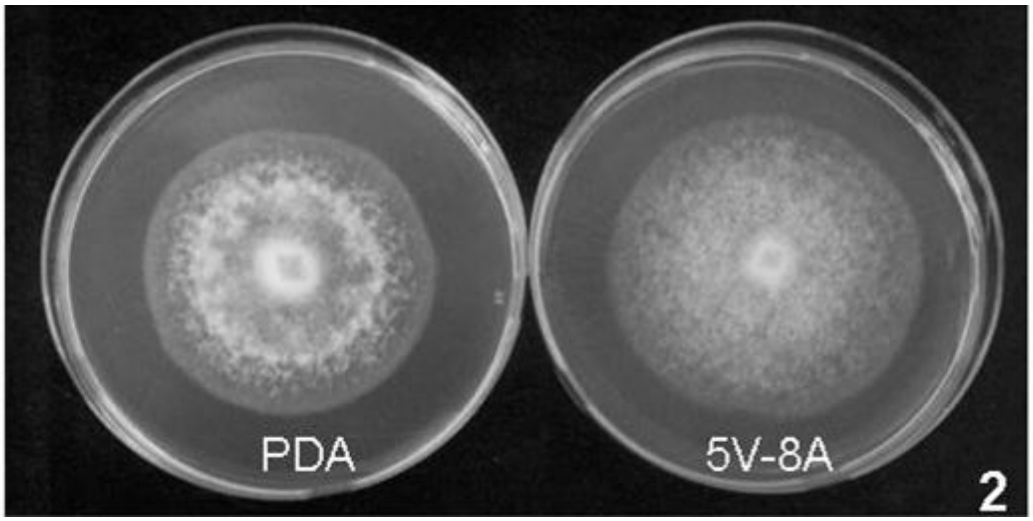

Fig. 2. Colony of an aberrant *Phytophthora multivesiculata*, isolate P96013, grown on 5% CVA (right) and PDA (Left) for 10 days at 24^{o}C.

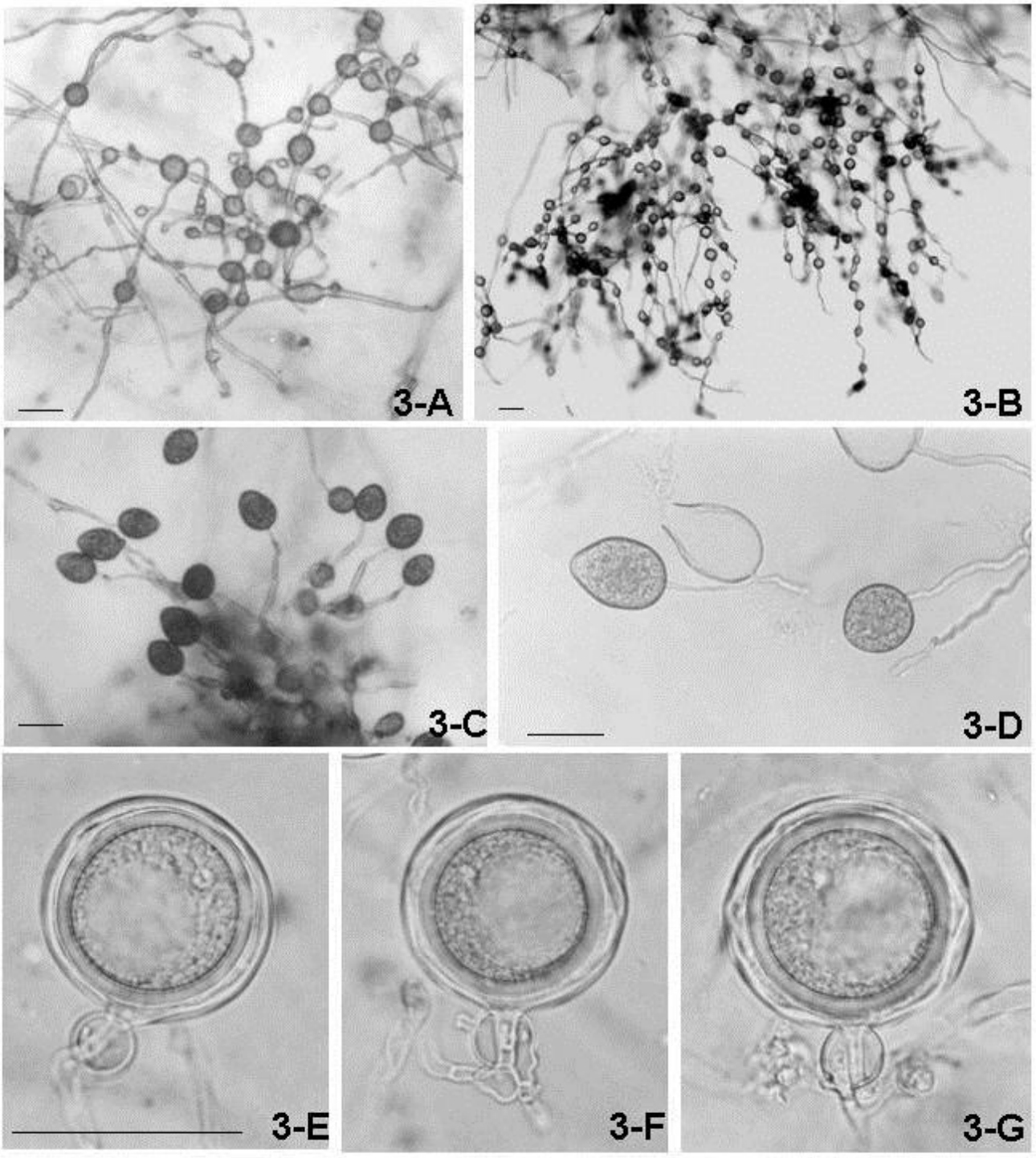

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Fig. 3. Morphological characteristics of an aberrant *Phytophthora multivesiculata*, isolate P96013, from a diseased plant of *Cymbidium hybridium* in Yuan-Shan, I-Lan, Taiwan. Note isolate P96013 produced chain-like hyphal swellings (A) on 5% CVA cultures and chain-like hyphal swellings (B), primary sporangia (C) and external proliferate sporangia (D) in distilled water. Note also oospores of isolate P96013 (E, F) and isolate P96016 (G) formed after growing on 10% VA for 30 days. (Bar = 50 mm).

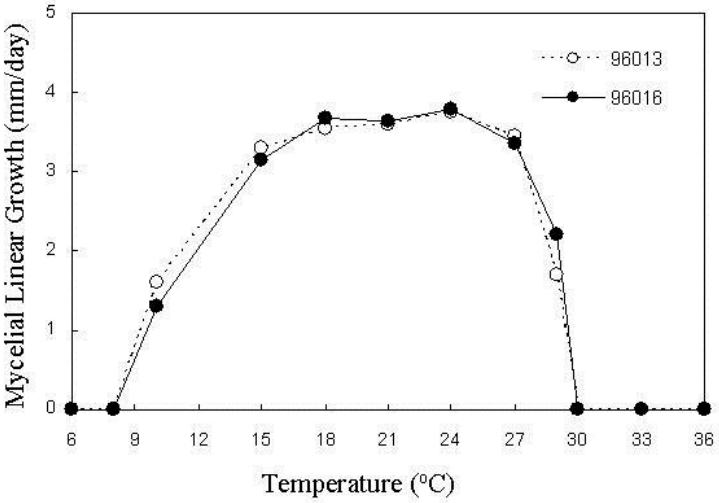

Fig. 4. Daily growth rates of the aberrant *Phytophthora multivesiculata*, isolates P96013 and P96016, on 5% CVA cultures at different temperatures.

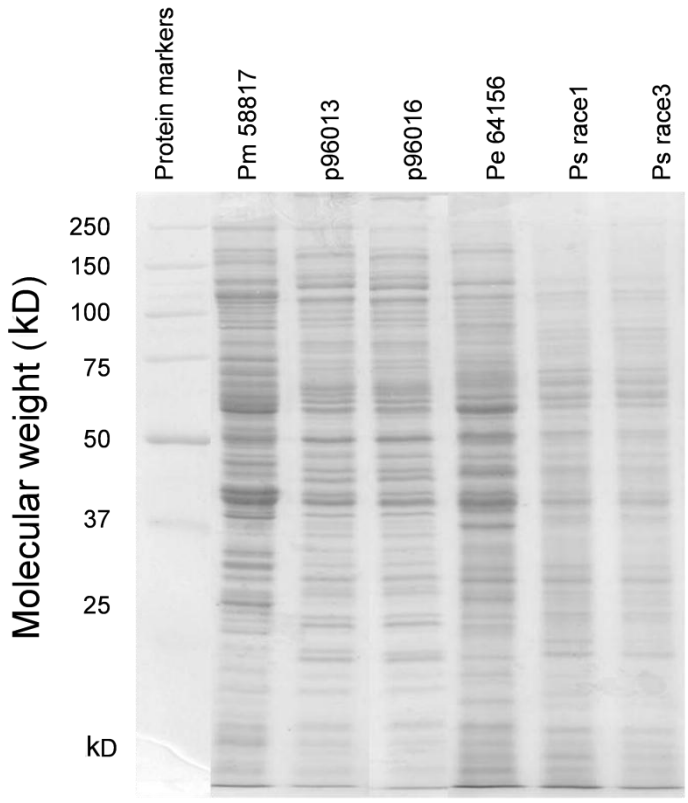

Fig. 5. Water-soluble mycelial protein patterns of different *Phytophthora* species generated by SDS-PAGE electrophoresis. Pm = *P. megasperma*; Pe = P. *erthroseptica*; Ps = *P. sojae.* Isolates P96013 and P96016 = aberrant *Phytophthora multivesiculata* from diseased plants of *Cymbidium hybridium* in Yuan-Shan, I-Lan, Taiwan

ITS sequence

The sequence of ITS region, including ITS1-5.8S-ITS2, of the isolates P96013 (accession no. 147195) and P96016 of *Phytophthora* -like organisms from C. *hybridium* were 803 bp. BLAST analysis of isolates P96013 and P96016 revealed a 100% similarity (803/803) to that of *P. multivesiculata* CDL05.1 (accession no. DQ835678) from Vietnam, and CBS 545.96 (AF266790) from the Netherlands.

DISCUSSION

The morphological and pathological features of the two homothallic isolates, P96013 and P96016, of *Phytophthora*-like organisms from *C. hybridium* in Yuan-Shan, I-Lan, Taiwan were similar to the species *Phytophthora multivesiculata* reported by Ilieva *et al.* [12]. Both isolates P96013 and P96016 from *C. hybridium* in Taiwan produced chain-like hyphal swellings with distinctive catenulate structures. This feature of chain-like hyphal swellings was identical to that of *P. multivesiculata*. However, the two Taiwanese isolates were different from *P. multivesiculata* in having larger sporangia [42-(53.8)-64 × 32-(40.8)-54 μm for the Taiwanese isolates but 45 × 33 μm for the Netherland isolates], larger oogonia [33-(50.1)-56 μm in diameter for the Taiwanese isolates but 28-(41)-50 μm for the Netherland isolates] and larger oospores [28-(43.7)-50 μm for, the Taiwanese isolates but 24-(33)-42 μm for the Netherland isolates]. Also, oogonia of the two Taiwanese isolates from *C. hybridium* had echinulated wall but oogonia of the Netherland isolates of *P. multivesiculata* had smooth walls. In additions, the maximum temperature for growth of the Taiwanese isolates was 29℃, which was 6℃ lower than that of the Netherland isolates of *P. multivesiculata* (with 35℃ as the maximum growth temperature).

ITS sequencing of genomic rDNA has been proven useful for studying phylogenetic relationships of *Phytophthora* species [7]. The ITS sequence of the isolates P96013 and P96016 from *C. hybridium* in Yuan-Shan, Taiwan were identical to that of *P. multivesiculata* but they were different from other species of *Phytophthora* in Waterhouse's group 6 (including *P. erythroseptica*, *P. cinnamomi* Rands, *P. cryptogea* Pethybridge & Lafferty, *P. drechsleri* Tucker and *P. melonis* Katsura), available in GenBank, as they shared ITS1-5.8S-ITS2 sequence identity for only 90% or lower. The soluble protein pattern of the isolates P96013 and P96016 from *C. hybridium* in Taiwan was also different from other closed related species of *Phytophthora* (*P. megasperma*, *P. erythroseptica* and *P. sojae*). Based on all the data presented in this study, it was concluded that the black rot disease of *C. hybridium* in the garden in I-Lan county, Taiwan, is caused by an aberrant strain of *P. multivesiculata*.

Previous records [1,9] showed that only *Phytophthora nicotianae* Breda de Haan (= *P. parasitica* Dastur) and *Phytophthroa palmivora* (Butler) Butler were reported as causal agents of black rot of cymbidium in Taiwan. This is the first report of an aberrant strain of *P. multivesiculata* as a black rot of pathogen of *C. hybridium* in Taiwan. The origin of this pathogen in the garden of Yuan-Shan, I-Lan, remains unknown. Diseased orchid plants in the garden of Yuan-Shan, I-Lan county, were destroyed in 1998 and the black rot disease of orchids caused by this aberrant strain of *P. multivesiculata* was not found in the survey of 1998 and 1999 in I-Lan county or any other orchid production areas of Taiwan. Although *P. multivesiculata* was reported as a pathogen of *Cymbidium* orchids in the Netherlands in 1998 [12] and in New Zealand in 2004 [8], none of the causal agents were described as the aberrant strain of *P. multivesiculata* reported in our study.

This study indicates that both isolates (P96013 and P96016) of the aberrant strain of *P. multivesiculata* are highly pathogenic on *C. hybridium* as well as other Asian species of *Cymbidium*, including *C. ensifolium*, *C. sinense* and *C. rubrigemmum*. Therefore, this aberrant strain of *P. multivesiculata* may pose a potential threat to the orchid industry in Taiwan. However, the aberrant *P. multivesiculata* isolates from diseased plants of *C. hybridium* in Taiwan are incapable of growing at a temperature higher than 29^{o}C and therefore, they are difficult to oversummer in the low altitude areas of southern Taiwan, where the temperature is often above 30^{o}C in the summer period. This high summer temperature may account for the failure of this aberrant stain of *P multivesiculata* to cause black rot disease on Cymbidium orchids in Central and Southern Taiwan (data not shown).

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摘　要

陳麗鈴[1]、安寶貞[2,3]、王姻婷[2]. 2011. 一種非典型 *Phytophthora multivesiculata* 引起的虎頭蘭黑腐病. 植病會刊 20: 1-10.
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1996 年與 1997 年宜蘭員山一處虎頭蘭園出現嚴重的黑腐病，許多植株之偽球莖、葉片及根部均出現黑褐色水浸狀病斑，並嚴重腐敗。從罹病組織分離到一種未曾在台灣發表過的疫病菌，共獲得 11 支菌株。將純粹培養的游走子接種在健康的虎頭蘭植株上，可以造成相同的病徵，且相同的病菌可從發病組織上分離得到。此外，該疫病菌可以引起四季蘭、觀音素心蘭及報歲蘭產生水浸狀斑及黑腐病徵。該菌的特性為：在 5% V-8 瓊脂上菌絲可生長溫度為 10-29℃，最適生長溫度為 24℃。在水中，可以產生大量胞囊，胞囊無乳凸、橢圓形、頂生，游走子釋放後，多數形成再生外生胞囊 (external proliferate sporangia)。不形成厚膜孢子；但無論固態培養或在水中均可產生大量成直角分歧、成長串唸珠狀的菌絲膨脹體 (hyphal swellings)，為該菌的特徵。該菌為同絲型 (homothallic)，在 V-8 瓊脂上單獨培養可形成大量卵孢子，藏卵器表面成波浪狀，不平滑；藏精器單生底著。依據該菌的形態特徵與核醣體內轉錄區間 DNA 序列 (ITS) 的相似度，該菌被鑑定為非典型的 *Phytophthora multivesiculata*。該病害並未在其他蘭花與其他地區出現。

關鍵詞：*Phytophthora multivesiculata*、非典型、虎頭蘭新病害、病害鑑定。