Revised Description of *Penicillium ulaiense* and Its Role as a Pathogen of Citrus Fruits

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ABSTRACT

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Penicillium ulaiense causes whisker mold, a recently described postharvest disease of citrus fruits. A new, more comprehensive description of the fungus and related taxonomic information are given. This description is based on examinations of the type isolate and 33 other isolates from citrus fruits in seven citrus-growing areas of the world. *P. ulaiense* was confirmed unique from *P. italicum,* the cause of citrus blue mold, by arbitrarily primed polymerase chain reaction analysis and morphological criteria. *P. ulaiense, P. italicum,* and *P. digitatum* (the cause of citrus green mold) showed similar temperature-growth relationships, but *P. ulaiense* grew more slowly. *P. ulaiense* was pathogenic but only moderately virulent to commercial varieties of citrus. *P. italicum* and *P. digitatum* formed lesions 80 mm in diameter on lemons and oranges three to five times faster than did *P. ulaiense.* The number of conidia required to produce decay in 50% of inoculated lemons was 100–1,000 times greater for *P. ulaiense* than those required for *P. italicum* and *P. digitatum.* Isolates of *P. ulaiense* collected in the United States were relatively resistant to the fungicides imazalil, thiabendazole, and *o*-phenylphenol; mean fungicide concentrations producing 50% inhibition for 17 isolates were 0.7 (±0.2), 45.7 (±14.1), and 12.5 (±5.4) μg/ml, respectively. Growth of all isolates collected in other countries was inhibited completely by 0.2 μg of imazalil or 10 μg of thiabendazole per milliliter. *P. ulaiense* was isolated frequently from California packinghouses but not from citrus groves.

Additional keywords: fungicide resistance, taxonomy.

According to Pitt (20), the genus *Penicillium* includes 150 species. Relatively few species are economically important plant pathogens. Among the most notable species are *P. italicum* Wehmer and *P. digitatum* (Pers.:Fr.) Sacc., which cause blue mold and green mold of citrus fruits, respectively. Postharvest losses of citrus fruit caused by *P. digitatum* and *P. italicum* can account for more than 90% of all postharvest losses in semiarid production areas of the world (4). For this reason, virtually all decay control strategies in California citrus packinghouses are aimed at controlling blue and green molds.

In 1987, mycologists in Taiwan published a description of *P. ulaiense* Hsieh, Su, & Tzean sp. nov. (12). This report was a mycological description only and did not address aspects of pathogenicity. The report has received little attention because of the limited distribution of the journal. Independent of this work, researchers collected isolates of *P. ulaiense* in California in 1987, but the uniqueness of these isolates was initially overlooked because they resembled *P. italicum,* especially in colony color. These isolates attracted our interest in 1992 because they were much more resistant to postharvest fungicides than is *P. italicum.* Subsequently, we isolated *P. ulaiense* frequently from decayed citrus fruits collected in California packinghouses. These isolates matched the description of the Taiwan type. A disease note was published, and the disease common name "whisker mold" was proposed (11) and accepted (15).

This paper provides a revised, comprehensive description of *P. ulaiense* and evidence that it is a distinct species. Several aspects of the physiology and pathogenicity of *P. ulaiense* are compared with those of *P. digitatum* and *P. italicum.*

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MATERIALS AND METHODS

Isolates. Thirty-three isolates of *P. ulaiense* were collected from decaying citrus fruits found in seven citrus-growing areas of the world. Two isolates were collected in California in 1987, and the remainder were collected in 1992 and 1993 (Table 1). The Taiwan type *P. ulaiense* (isolate 1640) was collected from a rotting orange along a roadside in Ulai, Taiwan, and was kindly provided by S.-S. Tzean of the Department of Plant Pathology and Entomology, National Taiwan University, Taipei.

All isolates were taken from stock cultures maintained for long-term storage on silica gel (19) at 3 C and transferred to slants of potato-dextrose agar (PDA) to prepare inoculum for the various tests.

Taxonomy. The description and classification of *P. ulaiense* followed the methodology of Pitt (20) and are based on examinations of 34 isolates listed in Table 1. Petri dishes containing Czapek agar (Cz), Czapek-yeast extract agar (CYA), malt extract agar (MEA), or 25% glycerol nitrate agar (G25N) were stab inoculated with pure cultures of *P. ulaiense* and incubated at 25 C for 7 days. In addition, cultures on CYA were incubated at 5 and 37 C for 7 days. Diameter, texture, and color of the colonies and microscopic morphological characteristics were recorded. The composition of the media has been described in detail (20). The only modification to Pitt's identification scheme was the use of Cz (23) in addition to MEA, CYA, and G25N. On Cz, *P. ulaiense* shows characteristic structures unique to this medium. Capitalized names of colors in the description were taken from the Methuen Handbook of Colour (14). Several isolates of *P. italicum* and *P. digitatum* were examined in the same manner to determine distinguishing characteristics among the three species and as known controls. For scanning electron microscopy, cultures were fixed in the vapors of 2% osmium tetroxide for 2 days, air dried for 3 days, and sputter coated with gold (29).

Arbitrarily primed polymerase chain reaction. The percentage

of genetic similarity between *P. italicum* and *P. ulaiense* was estimated by arbitrarily primed polymerase chain reaction (ap-PCR) analysis. Certain other species, which are not morphologically similar, were included to provide an array of genetic types. A total of 14 isolates assigned to six species were analyzed: *P. digitatum,* isolates 1, 83, and 1020; *P. italicum,* isolates 1233, 1293, and 1418; *P. ulaiense,* isolates 1610, 1616, 1632, and 1640 (type); *P. expansum* Link, isolates 1446 and 1448; *P. citreonigrum* Dierckx, isolate 1433; and *P. variabile* Sopp, isolate 1460. Each isolate was single spored and increased on PDA slants. Spore suspensions were prepared by flooding 7- to 10-day-old slant cultures with 4–5 ml of sterile deionized water containing 0.01% Triton X-100 (Sigma Chemical Co., St. Louis, MO), vortexing for 5–10 s, and filtering the spore suspension through double-layered cheesecloth to remove hyphal fragments.

Flasks containing 100 ml of potato-dextrose broth (PDB) were inoculated with a 0.5-ml spore suspension from slants and incu-

TABLE 1. Origin of *Penicillium* isolates

Species Isolate	Source	Origin[a]	Isolation date
P. ulaiense			
1609	Lemon	Camarillo, California	8 July 1987
1610	Lemon	Saticoy, California (A)[b]	14 July 1987
1612	Grapefruit	Yuma, Arizona (A)	29 April 1992
1613	Grapefruit	Yuma, Arizona (A)	29 April 1992
1614	Grapefruit	Yuma, Arizona (A)	29 April 1992
1615	Orange	Yuma, Arizona (A)	29 April 1992
1616	Orange	Yuma, Arizona (A)	29 April 1992
1617	Orange	Yuma, Arizona (A)	29 April 1992
1618	Lemon	Riverside, California (A)	12 May 1992
1619	Lemon	Oxnard, California (A)	14 May 1992
1620	Lemon	Oxnard, California (A)	14 May 1992
1621	Lemon	Oxnard, California (A)	14 May 1992
1622	Air	Oxnard, California (A)	14 May 1992
1623	Lemon	Oxnard, California (B)	14 May 1992
1624	Lemon	Oxnard, California (B)	14 May 1992
1625	Lemon	Oxnard, California (B)	14 May 1992
1626	Air	Oxnard, California (B)	14 May 1992
1627	Lemon	Saticoy, California (A)	14 May 1992
1628	Lemon	Saticoy, California (A)	14 May 1992
1629	Lemon	Saticoy, California (A)	14 May 1992
1630	Air	Saticoy, California (A)	14 May 1992
1631	Air	Saticoy, California (A)	14 May 1992
1632	Grapefruit	Riverside, California (B)	11 June 1992
1634	Orange	Riverside, California (B)	11 June 1992
1636	Orange	Riverside, California (C)	11 June 1992
1637	Orange	Riverside, California (C)	11 June 1992
1640	Orange	Ulai, Taiwan	4 March 1987
1641	Grapefruit	Corona, California (A)	23 June 1992
1642	Grapefruit	Corona, California (A)	23 June 1992
1648	Lemon	Oxnard, California (A)	24 April 1992
1653	Mandarin	Jujuy, Argentina	August 1993
1659	Lemon	New Zealand	22 July 1993
1662	Grapefruit	Florida	28 May 1993
1664	Grapefruit	Mission, Texas	28 April 1993
P. digitatum			
1	Lemon	California	5 April 1965
83	Orange	Grove, Riverside, California	17 December 1981
151	Lemon	Santa Paula, California	1 December 1986
1020	Orange	Grove, Riverside, California	8 March 1989
P. italicum			
1233	Orange	Riverside, California	1 June 1979
1293	Orange	Grove, Ivanhoe, California	21 October 1987
1418	Lemon	Oxnard, California	18 July 1990
P. expansum			
1446	Apple	Hudson Valley, New York	Before 1991
1448	Apple	Hudson Valley, New York	Before 1991
P. citreonigrum			
1433	Water[c]	Hudson Valley, New York	Before 1991
P. variabile			
1460	Lemon	Riverside, California	12 May 1992

[a] All isolates were collected from packinghouses except where indicated as having been collected from a grove; Taiwan type was collected from a rotting, roadside-litter orange.

[b] Letters in parentheses designate different packinghouses within the same city.

[c] Water from apple packinghouse fruit dump tank

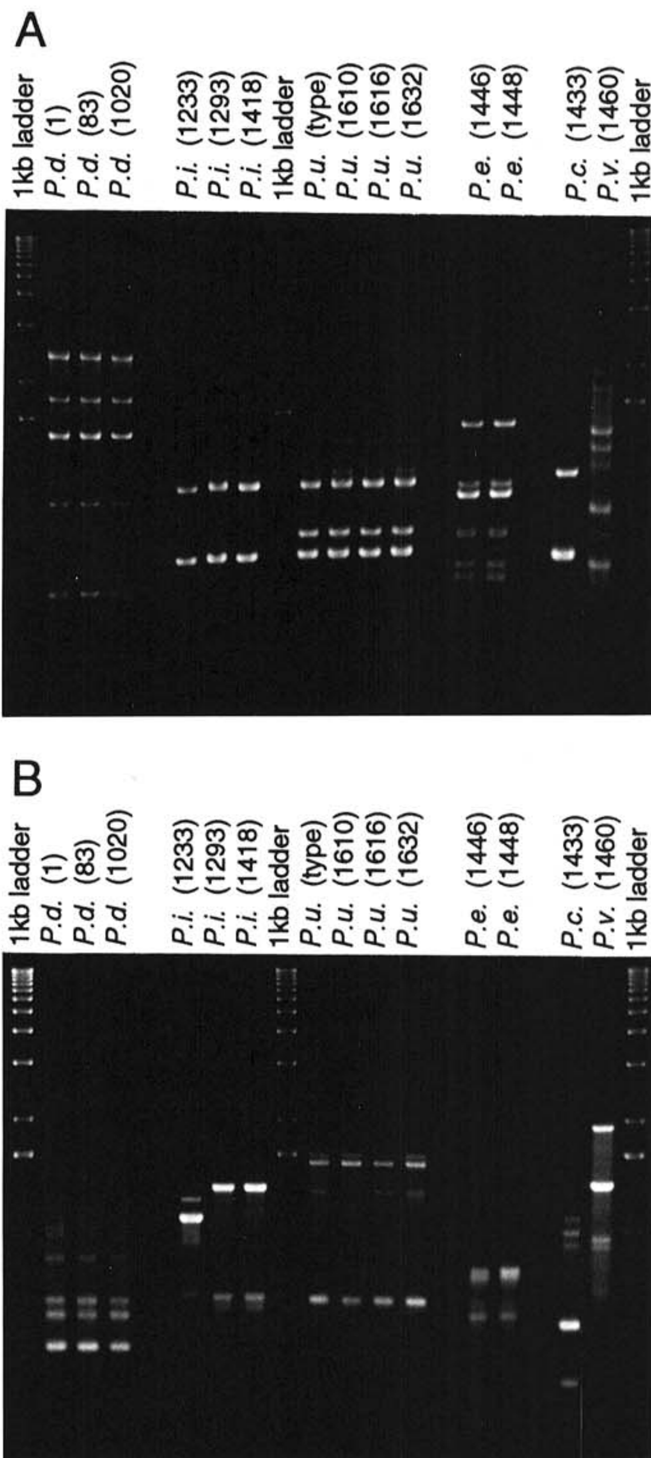

Fig. 1. Arbitrarily primed polymerase chain reaction (ap-PCR) analysis of total DNA extracted from mycelia of *Penicillium* spp. grown in potato-dextrose broth. PCR products were electrophoresed for 2 h at 80 V in 1.5% (w/v) agarose gel. Amplified DNA was produced with **A,** primer $(CAG)_5$ and **B,** primer $(GACAC)_3$. *P.d.* = *P. digitatum; P.i.* = *P. italicum; P.u.* = *P. ulaiense; P.e.* = *P. expansum; P.c.* = *P. citreonigrum;* and *P.v.* = *P. variabile.* Numbers in parentheses refer to isolates listed in Table 1.

bated on a rotary shaker at 25 C for 4 days. Mycelial mats were collected by filtration, washed with 200–300 ml of sterile water, and freeze-dried. The mycelium was powdered, and total genomic DNA was extracted (24). Arbitrary portions of the genome of each isolate were amplified with six oligonucleotide primers derived from eukaryotic, minisatellite DNA sequences: 5′-dT-CCTCCTCCTCCTCC-3′ (8), 5′-dGACAGACAGACAGACA-3′ (31), 5′-dCAGCAGCAGCAGCAG-3′, 5′-dGTGGTGGTGGT-GGTG-3′, 5′-dTGTCTGTCTGTCTGTC-3′, and 5′-dGACA-CGACACGACAC-3′. These primers have been designated in the text as $(TCC)_5$, $(GACA)_4$, $(CAG)_5$, $(GTG)_5$, $(TGTC)_4$, and $(GACAC)_3$, respectively. Primers were synthesized by the Biotechnology Instrumentation Facility, University of California, Riverside, and purified according to Sambrook et al (26). Amplification was performed in 20-μl reaction volumes containing 10–100 ng of total DNA, 2 μl of 10× *Taq* polymerase buffer, 1 μl of 30 mM $MgCl_2$, 2 μl of 2 mM dNTP, 1 μl of 20 μM primer, 1 unit of *Taq*1 DNA polymerase (Promega, Madison, WI), and 12.8 μl of sterile deionized water. The ap-PCR parameters included an initial denaturing at 95 C for 5 min followed by 30 cycles of denaturing at 95 C for 30 s, annealing at 58 C for primers $(CAG)_5$ and $(GTG)_5$ or at 42 C for all other primers for 30 s, and extension at 72 C for 90 s. After amplification, 3 μl of loading dye was added to each reaction mixture, and 4 μl of reaction mixture, plus dye, was loaded onto 1.5% (w/v) agarose gels and electrophoresed 2 h at 80 V. Amplified DNA was stained with ethidium bromide and visualized with UV light, and the position of the bands was compared to a standard 1-kb ladder.

Banding patterns of ap-PCR products (Fig. 1) for each primer were analyzed with a binomial system based on the presence or absence of a given ap-PCR product: 1 if a given product was present for an isolate and 0 if it was absent. Data matrices from all six primers were used in cluster analyses with the microcomputer program NTSYS-pc 1.60 (25) to determine isolate relationships. Dendrograms (Fig. 2) were produced by the unweighted paired group method with arithmetic means and Dice's similarity coefficient (3).

Coremium production. Five experiments were performed to determine factors responsible for variability in coremium production. *P. ulaiense* is often observed in mixed infections, leading to speculation that the presence of other pathogens might stimulate coremium production. Nontreated, surface-sterilized lemons (*Citrus limon* (L.) N. L. Burm. 'Eureka') were wound inoculated with *P. ulaiense* alone or with *P. ulaiense* and another citrus pathogen. *Phytophthora citrophthora* (R. E. Sm. & E. H. Sm.) Leonian or *Geotrichum candidum* Link were inoculated 1 wk prior to inoculation with *P. ulaiense* or *P. italicum*, whereas *P. digitatum* and *P. italicum* were mixed with *P. ulaiense* inoculum. A steel puncture-wounding tool (1 mm in diameter and 2 mm long) was dipped into a dense spore suspension (10^7 spores per milliliter) of the pathogen(s) and forced into the rind near the equator of each fruit. Fruit were stored at 23 C. Decaying fruit were examined for variation in coremium production for 2 wk after inoculation with *P. ulaiense*.

The same pathogen combinations as above were stabbed at three equidistant locations on petri dishes containing Harding's medium (H-25; PDA, 2 g of neopeptone, and 2 g of yeast extract per liter) (9). *P. ulaiense* inoculum was placed at one site; and *P. citrophthora, G. candidum, P. digitatum,* or *P. italicum* were inoculated at the two remaining sites on the same petri dish. The plates were incubated at 25 C and examined every 2 days for variation in coremium production.

The effect of postharvest fungicide treatments on coremium production was evaluated with surface-sterilized lemons that were dipped for 1 min in 3 g of thiabendazole (2-[4-thiazolyl]benzimidazole; Freshguard 598, 98.5% a.i., FMC, Riverside, CA) per liter in water. Three thiabendazole-treated and three nontreated lemons were placed in plastic shoe boxes. The fruit in each box were wound inoculated as described above with one of seven isolates of *P. ulaiense* or one isolate of *P. italicum* (for comparison). The boxes were covered with perforated cellophane and stored at 15 C. Fruit were examined 13 days after inoculation for variation in coremium production.

Dicloran (2,6-dichloro-4-nitroaniline; technical 90% a.i., Nor-Am Chemical Co., Wilmington, DE) was reported to enhance fascicle production in *Penicillium* spp. (16). Petri plates containing dicloran-amended H-25 (20 μg/ml) and H-25 agar alone were stab inoculated with *P. ulaiense* as described above, incubated at 25 C, and examined for coremium production.

The effect of commercial fruit treatments on coremium production was evaluated. Grapefruit (*C.* ×*paradisi* Macfady 'Ruby') and oranges (*C. sinensis* (L.) Osbeck 'Valencia') were collected from two southern California packinghouses. The grapefruit were treated commercially with 2 g of imazalil (Freshguard 700, 44.6% a.i., FMC) plus 3 g of thiabendazole per liter in wax, and the oranges were treated with 5 g of thiabendazole per liter in wax. A set of three grapefruit and three oranges (two treated and one nontreated control) was placed in each of eight plastic shoe boxes. Each fruit set was wound inoculated with one of seven isolates of *P. ulaiense* and one isolate of *P. italicum* as described above. Fruit were evaluated periodically over 21 days at 15 C for variation in coremium production.

Habitat. Citrus packinghouses and groves in southern California were assayed to determine the distribution and frequency of occurrence of *P. ulaiense*. Petri plates containing H-25 agar were exposed to the atmosphere in three packinghouses (1–5 min, depending on location) and incubated for 4 days at 25 C, and colonies of *P. ulaiense, P. italicum,* and *P. digitatum* were counted.

In citrus groves, three methods of detecting *P. ulaiense* were used. 1) Blue, sporulating, coremiform Penicillia were isolated from decaying fruit on grove floors and identified in the laboratory. 2) Since we had previously determined that California isolates successfully rotted fungicide-treated fruit, we attempted to trap *P. ulaiense* with fungicide-treated fruit. Ten sets of five fruits each were placed at random locations throughout an orange grove at the University of California, Riverside. Each fruit set consisted of three lemons and two oranges. Lemons were not treated or were treated with 0.25 g or 1 g of imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole; technical, 97.5% a.i., Janssen Pharmaceutica, Beerse, Belgium) per liter (1-min dip). Oranges were not treated or were treated with 1 g of imazalil per liter (1-min dip). Fruit were surface sterilized in 70% ethanol, treated with fungicide, and puncture wounded eight or nine times with a sterile, 19-pin flower holder (1-mm-diameter pins arranged equidistantly on a 12-mm-diameter metal plate). Fruits were then placed on the orange grove floor without disturbing the natural surroundings. The orange grove had been recently irrigated, and there were many rotting fruits on the ground. After 4 days exposure to the grove floor environment,

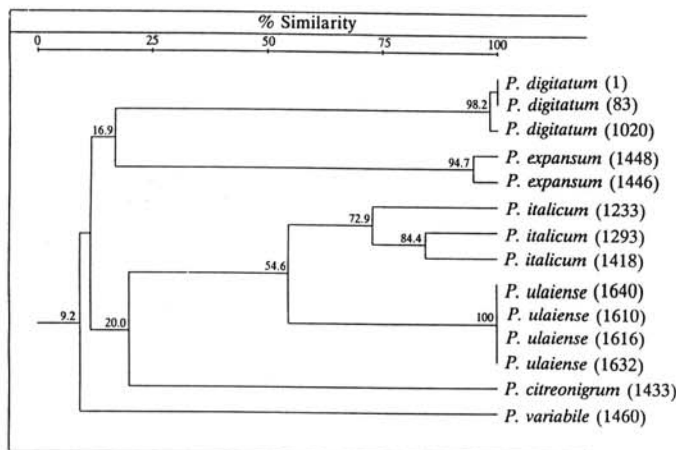

Fig. 2. Dendrogram constructed from arbitrarily primed polymerase chain reaction data indicating the percentage of similarity of comigrating bands within and between various *Penicillium* spp. The dendrogram was produced with the NTSYS microcomputer program (25).

fruits were placed in plastic bags and held at room temperature for decay development. At 8 and 11 days after placement in the grove, fruit were examined for the presence of molds resembling *P. ulaiense*. Molds suspected to be *P. ulaiense* were isolated in pure culture and identified. 3) Nine University of California farm advisers were given descriptions of *P. ulaiense* and photographs of whisker mold. They were requested to collect (from citrus groves only) molds resembling *P. ulaiense* and return them for positive identification.

World distribution. The same descriptions of *P. ulaiense* and photographs of whisker mold were sent to researchers familiar with *Penicillium* molds of citrus outside California. They were requested to collect any molds resembling *P. ulaiense* in their vicinities and return them for positive identification.

Temperature-growth relation. Petri plates containing 15 ml of MEA were stab inoculated (one inoculation per plate) with a needle that had been dipped into a dense spore suspension of *P. ulaiense, P. italicum,* or *P. digitatum.* The plates were incubated at 5, 12, 15, 18, 20, 25, 27, 30, 33, and 36 C, and colony diameters were measured at 8 days. To determine significant differences in temperature-growth response between species, linear regression analysis was performed on mean colony diameters for each species up to the point of highest growth rate (i.e., 25 C for *P. ulaiense* and 27 C for *P. italicum* and *P. digitatum*), and analysis of variance (ANOVA) was performed on the slopes of those regression lines. Isolates tested included six of *P. ulaiense* (1610, 1616, 1632, 1640, 1642, and 1648), three of *P. digitatum* (1, 151, and 1020), and three of *P. italicum* (1233, 1293, and 1418). Each treatment was replicated three times, and the experiment was repeated three times.

Virulence. Two measures of virulence were evaluated: aggressiveness and parasitic fitness. Aggressiveness of *P. ulaiense, P. digitatum,* and *P. italicum* was assessed as the rate of lesion expansion on oranges and lemons. Oranges and lemons were hand-picked from groves at the University of California, Riverside. Fruit were washed in tap water and surface sterilized in 70% ethanol. The same 12 isolates used in the temperature-growth tests were used in this test. Wound inoculation was performed as described in the coremium production experiments. A set of fruit consisting of six oranges and six lemons was inoculated with each isolate. Fruit were placed in plastic shoe boxes (six fruit per box) and incubated at 15 C. Lesion diameter, which included the sporulating area, the white mycelial margin, and the surrounding soft, water-soaked area, was measured at 8, 13, and 18 days. The number of days required for development of an 80-mm-diameter lesion was determined by direct observation or by interpolation from a linear regression line.

Parasitic fitness of *P. ulaiense, P. digitatum,* and *P. italicum* was evaluated by determining the inoculum efficiency of several isolates of each species on lemons. Lemons were obtained from a local packinghouse prior to any postharvest treatment. Fruit were surface sterilized with 70% ethanol and separated randomly into groups of 25. Inocula were prepared as described for the ap-PCR experiments. Each fruit in a group was inoculated with one of seven isolates at four equidistant locations near the fruit equator with a repeating syringe (Hamilton Company, Reno, NV) calibrated to deliver 5 μl of spore suspension. The syringe made a wound 1 mm wide and 2 mm deep. The concentration of spores was determined with a hemacytometer. The number of spores per inoculation site was calculated from the concentration of spores and the volume delivered. Isolates tested included three of *P. ulaiense* (1610, 1640, and 1642), two of *P. italicum* (1233 and 1418), and two of *P. digitatum* (1 and 1020). The incidence of decay was evaluated at 6 days at 22 C and 100% relative humidity. The experiment was repeated three times.

Fungicide sensitivity. Twenty isolates of *P. ulaiense* were evaluated for sensitivity to imazalil, thiabendazole, and *o*-phenylphenol (Dow Chemical, Midland, MI). Spore suspensions were prepared as described for the ap-PCR experiments and adjusted to 10^6 spores per milliliter on the basis of the optical density of the suspension (18). The suspension (1 ml) was added to 15 ml of molten plug agar (H-25 agar supplemented with 6 g of agar per liter to increase agar firmness) at 50 C, dispensed into petri dishes, and incubated for 24 h at 25 C. *P. ulaiense* spores began germinating at 12 h and reached 100% germination by 24 h. Plugs (4 mm in diameter) from these plates were placed in the centers of plates containing fungicide-amended H-25 agar. Fungicide concentrations (per milliliter) in agar plates were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 μg of imazalil; 0, 10, 20, 30, and 40 μg of thiabendazole; and 0, 5, 10, 20, and 30 μg of *o*-phenylphenol. Colony diameters were measured at 7 days at 25 C. A linear regression of percent inhibition versus fungicide concentration was calculated, and the EC_{50} (fungicide concentration producing 50% inhibition) was determined by interpolation from the regression line.

Yeast extract, malt extract, agar, neopeptone, PDA, and PDB used alone or in various media were purchased from Difco Laboratories, Detroit, MI.

RESULTS

Taxonomy. A revised description for *P. ulaiense* follows. Colonies on MEA (Fig. 3A) at 7 days at 25 C were 15–17 mm in diameter and plane. They had discrete coremia, which were usually borne in distinct annular or concentric zones. The coremia were claviform and 2–8 mm long and had white, sterile stalks 0.15–0.40 mm wide and apices 0.4–1.5 mm wide and 0.4–2.0 mm long (Fig. 4A). Conidia were borne abundantly from mycelium and coremium apices but not from coremium stalks. The conidia were Greenish grey (26C2) to Greyish green (26C3) in mass. Subsurface mycelial cords often appeared after 7–14 days of incubation. Yellowish orange (4A5-4A7) soluble pigment was often produced. The colony reverse was pale or Yellowish orange (4A5-4A7) at 7 days but usually became Orange (5A7) by 14 days.

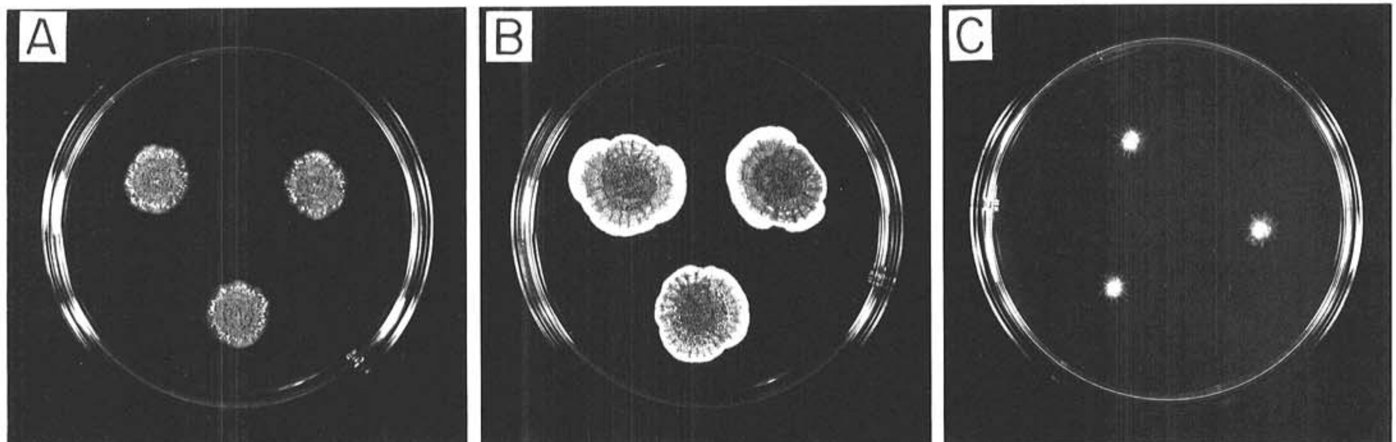

Fig. 3. *Penicillium ulaiense* colonies at 7 days at 25 C on **A,** malt extract agar, **B,** Czapek-yeast extract agar, and **C,** Czapek agar.

A pronounced musty odor was produced.

Colonies on CYA (Fig. 3B) at 7 days at 25 C were 15–20 mm in diameter, plane, and sometimes slightly umbonate and had coremia forming abundantly. The coremia apices were often white from delayed sporulation, especially at colony centers. Coremia were claviform, produced in somewhat annular zones, and borne on well-defined, sterile, white stipes, typically 2–6 mm long (usually shorter than those grown on MEA). Conidia were borne from surface mycelium as well as from coremium apices. The conidia in mass were the same color as those on MEA, and sporulation was delayed compared with that on MEA. Colonies lacked exudate. Colony reverse was radially sulcate, and reverse color was usually pale to white or buff but sometimes Yellowish orange (4A5-4A7), which usually appeared after 7 days and intensified with time. A pronounced, musty odor was produced.

Colonies on Cz (Fig. 3C) at 7 days at 25 C were 1–3 mm in diameter and more green than those on CYA. Subsurface mycelial cords extended 1–2 mm beyond colony margins and sometimes emerged to form pinhead tufts of spores (i.e., nascent coremia). Occasionally, mycelial cords were absent at 7 days but usually appeared later and permeated the entire plate at 2–3 wk. Coremia were shorter than those on CYA or MEA and appeared later (i.e., after 7 days).

Colonies on G25N at 7 days at 25 C were 15–20 mm in diameter, lightly sulcate, moderately deep, dense, and white or buff.

At 7 days at 5 C on CYA, microcolonies or colonies up to 2 mm in diameter were produced; normal growth resumed when plates were placed at 25 C. There was no growth on CYA at 37 C; growth did not resume at 25 C.

Conidiophores (Fig. 4B) were borne from surface or subsurface hyphae and from within coremium stalks; stipes of indeterminate length and 2.4–3.7 μm wide were very sinuous and had smooth walls. Penicilli were irregularly branched, often divergent, mostly terverticillate, and intertwined into a dense hymenial layer; there were two to three rami per penicillus, 6–16 $\times$ 2.5–4.0 μm. Metulae were arranged in verticils of two to four measuring 10–22 $\times$ 2.5–4.0 μm with smooth walls and were frequently apically inflated. Phialides (Fig. 4C) were closely packed, three to five per metula, 10–17 $\times$ 2.5–4.0 μm, and ampulliform to cylindroidal, tapering abruptly to long cylindroidal collula (mostly 2–3 $\times$ 2 μm); collula were sometimes indistinguishable from the first few conidia and sometimes approached the length of the phialide. Conidia (Fig. 4D) were borne as cylinders from the collula and became enlarged, rounded, and elongated with maturation; they had smooth walls and were mostly cylindrical but included a variety of other shapes, were extremely variable, 1.5–5.0 $\times$ 3.0–13 μm, and were borne in long disordered chains.

Distinguishing characteristics. Slow growth, grey green conidia in mass, formation of 1- to 8-mm-long coremia, reverse orange-soluble pigment (especially on MEA), subsurface mycelial cords (especially on Cz), and the ability to rot citrus fruits are characteristics that distinguish *P. ulaiense* from other *Penicillium* spp. *P. ulaiense* is most appropriately placed with *P. italicum* and *P. digitatum* in subgenus *Penicillium,* section *Cylindrosporum,* series *Italica* as defined by Pitt (20).

Variation. All isolates behaved similarly but differed from the type in that growth of the type was slightly faster and coremia were longer with less sporulation than other isolates. Orange reverse-colony coloration on MEA and CYA and mycelial cords on Cz or MEA were present in all other isolates examined except the type. Orange reverse-colony coloration and mycelial cords (in all isolates examined except the type) were most conspicuous and most consistently produced on MEA and Cz, respectively.

Affinities. *P. ulaiense* shows a clear relationship with *P. italicum.* It is similar to *P. italicum* in color of conidia in mass and in morphology of phialides and conidia. It differs from *P. italicum* in that its growth is slower on CYA, Cz, and MEA; it forms distinct coremia rather than inconspicuous tufts of spores. Production of coremia in *P. italicum* is variable: they are not produced often, but when produced, they are smaller (usually <1 mm long) and much less conspicuous than those of *P. ulaiense*, which forms conspicuous coremia consistently, with occasional morphological variation. The concentric pattern of *P. ulaiense* coremia production on MEA is easily distinguished from the deep, fast-growing, tufted coremia characteristic of *P. italicum.* Reverse colors in *P. ulaiense* colonies are not dark brown, as are those of *P. italicum,* but rather are pale or orangish yellow as are those on MEA and CYA.

Deposition of cultures. The Taiwan type (isolate 1640) and a California isolate of *P. ulaiense* (isolate 1642) have been deposited at the following culture collection centers: Northern Regional Research Center, Peoria, IL; American Type Culture Collection (1640, ATCC 90828; 1642, ATCC 90829), Rockville, MD; and Centraalbureau voor Schimmelcultures (1640, CBS 261.94; 1642, CBS 262.94), Baarn, The Netherlands.

ap-PCR. All isolates of the *Penicillium* species analyzed produced banding patterns of amplified DNA fragments that were unique to each species (Fig. 1). *P. ulaiense* had the greatest similarity with *P. italicum* (54.6%, Fig. 2). All other species showed ≤20% banding similarity with other species but ≥94.7% similarity within the species (except *P. italicum* with 72.9% similarity to isolate 1233). All four isolates of *P. ulaiense,* including the Taiwan type, had identical banding patterns with each of six primers.

Coremium production. No pathogen combinations, either in vitro or in fruit, consistently affected coremium production. PDA amended with dicloran (20 μg/ml) reduced radial growth but did not enhance coremium production. In contrast, H-25 agar amended with thiabendazole (10–30 μg/ml) appeared to enhance

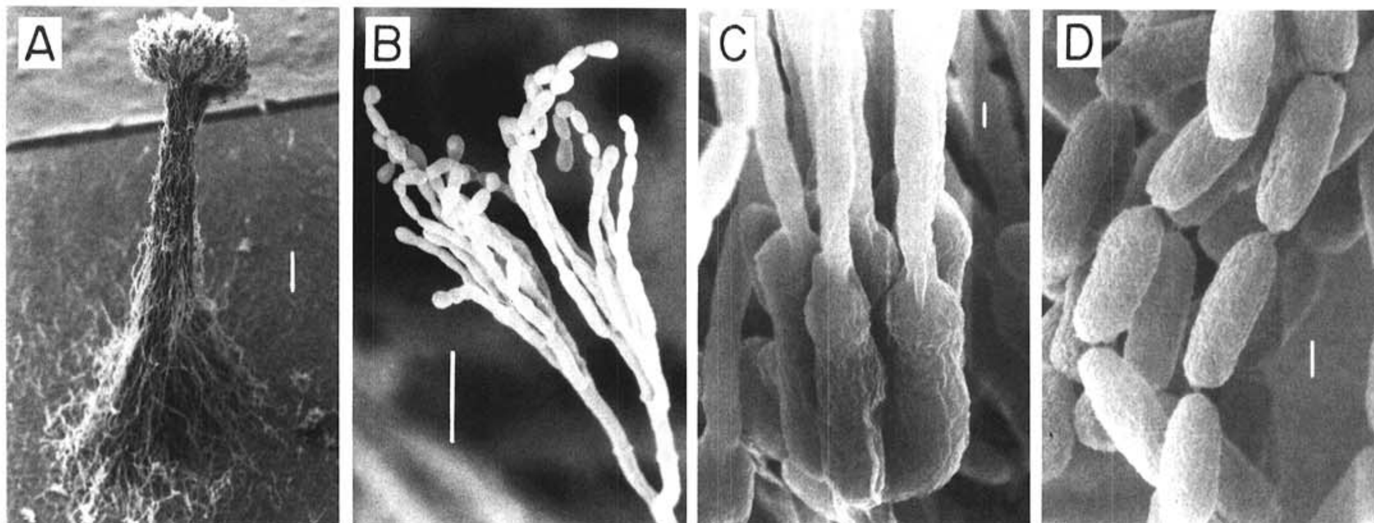

Fig. 4. Scanning electron micrographs of *Penicillium ulaiense:* **A,** coremium (size bar = 100 μm); **B,** penicillus (size bar = 10 μm); **C,** verticil of phialides (size bar = 1.0 μm); and **D,** chains of conidia (size bar = 1.0 μm).

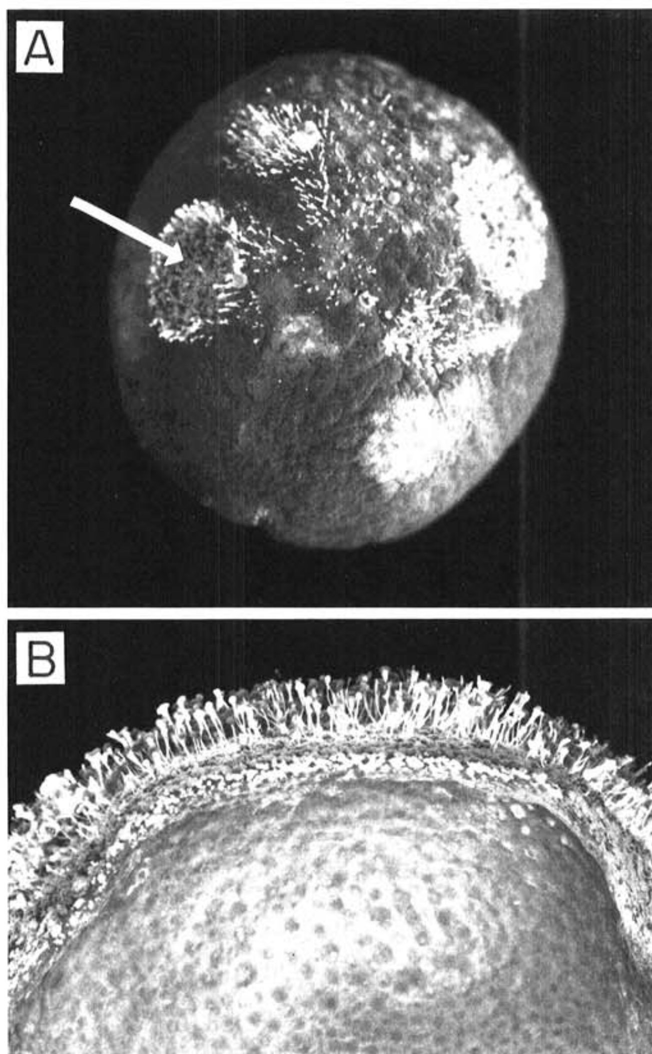

Fig. 5. A, Mixed infection of *Pencillium ulaiense* with *P. digitatum* on Valencia orange as commonly seen in packinghouses (arrow points to *P. ulaiense* lesion); **B,** *P. ulaiense* on Valencia orange 2–3 wk (15 C) after wound inoculation with pure culture.

the production of dense coremia but delayed sporulation by several days.

Disease symptoms. Whisker mold is usually found on stored citrus fruits as a mixed infection with *P. digitatum.* In mixed infections, coremia are strikingly contrasted with the olive green, velvety spore mass produced by *P. digitatum* and may be arranged in concentric circles or circular patches or scattered randomly and quite uniformly over the entire fruit surface (Fig. 5A). Occasionally, whisker mold has been observed as the sole cause of decay (Fig. 5B). On decaying fruit, coremia often vary in length and shape of stalk and head (e.g., flattened and/or fanlike) and often bear sterile tips, so that sporulation is less abundant than with blue or green mold.

Disease symptoms varied considerably in fruit that had been wound inoculated with *P. ulaiense.* Typically, decay proceeded at a slow but steady rate, and there was abundant sporulation. The advancing lesion had a thin, white mycelial margin and a water-soaked border of variable width (2–10 mm). The water-soaked border was often orangish red, especially in lemons. In oranges, this border sometimes darkened and eventually appeared to arrest lesion advance. Decay occasionally consisted of a small (2–5 mm in diameter), black, sunken lesion without sporulation. This lesion sometimes continued to increase in size and to eventually sporulate, or its development became arrested.

Coremia were not always prominent features on lesions. When coremia were not produced, decay appeared identical to that produced by *P. italicum* but proceeded at a slower rate. Often coremia were not present until the later stages of decay (Fig. 5B).

Habitat. *P. ulaiense* was isolated from all 15 packinghouses visited in southern and central California. Usually, *P. ulaiense* was found in mixed infections with *P. digitatum* and other citrus pathogens (Fig. 5A). *P. ulaiense* comprised 1–20% of the *Penicillium* colonies on H-25 agar exposed for 1–5 min to the atmosphere of three packinghouses.

P. ulaiense was not detected in citrus groves, despite numerous attempts. Every blue mold found with inconspicuous coremia was identified as *P. italicum.* The attempt to trap *P. ulaiense* in fungicide-treated fruit placed in a citrus grove was unsuccessful; only nontreated fruit rotted with *P. italicum* and *P. digitatum.* No mold samples were received for identification from California farm advisers.

World distribution. To date, *P. ulaiense* has been positively identified from Argentina, Arizona, Australia, California, Florida, Italy, New Zealand, South Africa, Taiwan, and Texas (Table

TABLE 2. Reports of *Penicillium ulaiense* in citrus-growing areas of the world

Location	Researcher	Findings		
		Positive identification	Detected without positive identification	Inconclusive
Argentina, Jujuy	L. Carrillo	X	...	...
Argentina, Tucumán	B. Stein	...	...	X
Arizona	G. J. Holmes	X	...	...
Australia, New South Wales	B. L. Wild	X	...	...
Brazil	W. R. W. Ribeiro	...	...	X
California[a]	G. J. Holmes	X	...	...
Florida	E. Brown	X	...	...
Greece	A. Manouilidis-Chitzanidis	...	...	X
Israel, Bet Dagan	E. Cohen	...	X	...
Italy, Sicily	G. Lanza	X	...	...
Italy, Sardinia	M. Schirra	...	...	X
New Zealand	R. R. Stange	X	...	...
South Africa	L. Schutte	X	...	...
Spain, Valencia	P. Bradbury	...	...	X
Spain, Valencia	J. J. Tuset	...	...	X
Taiwan, Ulai	S.-S. Tzean	X	...	...
Texas	M. Skaria	X	...	...
Turkey, Adana	M. Biçici	...	...	X
Uruguay	L. A. Diaz	...	...	X

[a] Collected from packinghouses in all citrus-growing districts (i.e., coastal, desert, inland valleys, and central valley).

TABLE 3. Colony diameters (mm) of *Penicillium ulaiense, P. digitatum*, and *P. italicum* at 8 days incubation on malt extract agar at nine temperatures

Species Isolate	Temperature (C)								
	5	12	15	18	20	25	27	30	33
P. ulaiense									
1610	2.0[a]	15.0	17.0	21.0	22.0	24.0	18.0	7.0	0.0
1616	2.0	14.0	16.0	17.0	18.0	21.0	16.0	7.0	0.0
1632	3.0	15.0	16.0	18.0	18.0	23.0	17.0	0.0	0.0
1640	2.0	12.0	19.0	23.0	25.0	23.0	17.0	4.0	0.0
1642	3.0	11.0	16.0	20.0	23.0	28.0	19.0	0.0	0.0
1648	2.0	12.0	16.0	18.0	20.0	25.0	17.0	0.0	0.0
Mean	2.3	13.2	16.7	19.5	21.0	24.0	17.3	3.0	0.0
SE[b]	0.2	0.8	0.5	1.0	1.3	1.1	0.5	1.6	0.0
P. italicum									
1233	4.0	27.0	39.0	49.0	50.0	58.0	57.0	31.0	0.0
1293	4.0	25.0	33.0	41.0	42.0	49.0	53.0	30.0	0.0
1418	5.0	22.0	26.0	32.0	34.0	42.0	47.0	22.0	0.0
Mean	4.3	24.7	32.7	40.7	42.0	49.7	52.3	27.7	0.0
SE	0.4	1.8	1.6	6.0	5.7	5.7	3.6	3.5	0.0
P. digitatum									
1	0.0	5.0	12.0	13.0	13.0	23.0	33.0	11.0	0.0
151	0.0	8.0	13.0	19.0	21.0	32.0	31.0	22.0	0.0
1020	0.0	17.0	31.0	38.0	39.0	40.0	37.0	15.0	0.0
Mean	0.0	10.0	18.7	23.3	24.3	31.7	33.7	16.0	0.0
SE	0.0	4.4	7.6	9.2	9.4	6.0	2.2	3.9	0.0

[a] Values represent the average colony diameter (mm) for three replicates.
[b] Standard error of the mean.

TABLE 4. Days[a] required at 15 C for development of an 80-mm-diameter lesion after wound inoculation of oranges and lemons with *Penicillium ulaiense, P. italicum*, or *P. digitatum*

	P. ulaiense[b]	*P. italicum*[c]	*P. digitatum*[c]
Lemons	30	11 (2.7)	8 (3.8)
Oranges	49	15 (3.3)	9 (5.4)

[a] Days estimated from regression line or from direct observation.
[b] Values are the means of six replicates each of six isolates ($n = 36$).
[c] Values are the means of six replicates each of three isolates ($n = 18$). Values in parentheses are virulence factors relative to *P. ulaiense*, calculated as days *P. ulaiense*/days *P. digitatum* or *P. italicum*.

2). Researchers in other areas have neither confirmed nor denied the presence of *P. ulaiense*.

Temperature-growth relation. *P. ulaiense* showed a temperature-growth relation similar to that of *P. digitatum* and *P. italicum* but grew more slowly at most temperatures (Table 3). Slopes derived from temperature-growth regression lines of each species were significantly different as determined by ANOVA and Fisher's LSD ($P < 0.05$). Maximum growth of *P. ulaiense* on MEA occurred at 25 C. At 5 C, microcolonies 1–2 mm in diameter occasionally formed; at 33 C and above, spores were killed (i.e., they did not resume growth at room temperature). Orange reverse-colony coloration was produced consistently at incubation temperatures of 15, 18, and 20 C in all isolates tested except the Taiwan type.

Virulence. *P. ulaiense* was less aggressive as a pathogen of citrus fruits than were *P. digitatum* and *P. italicum*, which decayed lemons and oranges approximately three to five times faster than did *P. ulaiense* (Table 4). Inoculum of *P. ulaiense* was less efficient in causing infection than were inocula of *P. digitatum* and *P. italicum*. Approximately 100–1,000 times more *P. ulaiense* spores per inoculation site were required to produce 50% infection in wound-inoculated lemons (Fig. 6) than were required for *P. italicum* and *P. digitatum*.

Fungicide sensitivity. *P. ulaiense* isolates from Taiwan, New Zealand, and Argentina were sensitive (i.e., complete inhibition) to imazalil at 0.2 μg/ml and to thiabendazole at 10 μg/ml but formed colonies on all concentrations of *o*-phenylphenol tested ($EC_{50} \approx 10$ μg/ml). However, all California isolates were resistant to both imazalil and thiabendazole. Sensitivity of California isolates to *o*-phenylphenol was similar to that of the Taiwan type. Mean EC_{50} values for 17 California isolates were 0.7 (±0.2) for

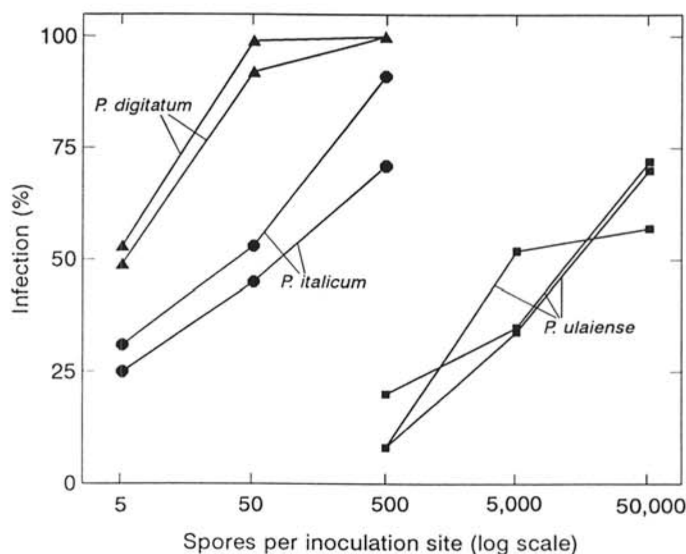

Fig. 6. Effect of inoculum level of three *Penicillium* spp. on infection of lemon fruit. Each line represents a single isolate. Inoculum volume = 5 μl.

imazalil, 45.7 (±14.1) for thiabendazole, and 12.5 (±5.4) μg/ml for *o*-phenylphenol (Table 5).

DISCUSSION

We believe that *P. ulaiense* is a distinct species, evidenced by its morphology and growth on standard media and by ap-PCR analysis. The most distinctive feature of *P. ulaiense* is the formation of 2- to 8-mm-long coremia, which were produced characteristically on MEA but also on CYA, Cz, and citrus fruits. Additional distinguishing features include colony-reverse orange coloration and mycelial cords on Cz and MEA. The type isolate failed to produce orange reverse coloration or mycelial cords on any medium at any temperature tested and may be showing signs of deterioration. However, we have not yet observed this type of deterioration in any other isolate.

The ap-PCR analysis was a useful tool in distinguishing *Penicillium* spp. Within-species similarity of ap-PCR products among isolates of *P. digitatum, P. ulaiense*, and *P. expansum* was sur-

prisingly high (>94%), considering the temporal and geographic distribution of the isolates (Fig. 2). Work with ap-PCR on nematodes (30) showed that ≤20% banding similarity is normally observed between species and >90% similarity is normally observed within species. Similarity of bands between *P. ulaiense* and *P. italicum* at 54.6% is intermediate. However, since no variation was detected in the four isolates of *P. ulaiense,* we feel that this level of similarity, coupled with the morphological and ecological differences discussed below, is sufficient to justify *P. ulaiense* as a separate species. Further ap-PCR work on *P. italicum* and *P. ulaiense* with more isolates would be a logical next step.

In addition to classical *Penicillium* taxonomy, isozyme analysis (2) and secondary metabolite profiles (7) have been used to identify species. ap-PCR analysis could provide yet another tool for identifying and classifying the genus *Penicillium.*

Mislivec (16) reported enhanced fascicle production in many *Penicillium* spp. grown on media amended with dicloran. This effect was not observed in *P. ulaiense.* Coremium production in *P. ulaiense* was variable on thiabendazole-treated fruit observed in packinghouses. *P. ulaiense* grown on H-25 agar amended with sublethal levels of thiabendazole (10–30 μg/ml for California isolates) produced colonies with dense coremia and delayed sporulation (7–14 days, depending on concentration). This influence of thiabendazole on coremium production could not be reproduced on thiabendazole-treated fruit inoculated with *P. ulaiense.* Coremium production in *P. ulaiense* is photosensitive (G. J. Holmes, *unpublished*), which agrees with other studies on coremiform Penicillia (1), but this does not seem to account for all the variation observed in coremium production. Certain isolates quite consistently produce longer, more discrete coremia (e.g., type). Coremia and their length appear to play an important role in air dispersal of spores. The frequency of contamination of cultures in our laboratory is much higher for *P. ulaiense* than for *P. digitatum* or *P. italicum.*

Although the Taiwan type *P. ulaiense* was isolated from a solitary rotting orange, the species was not investigated as a pathogen (12). We found that *P. ulaiense* was pathogenic to all commercial varieties of citrus fruits and moderately virulent (aggressive or parasitically fit) on lemons and oranges. The growth of *P. ulaiense* was slower than that of *P. italicum* and *P. digitatum* on fruit and on MEA medium. It should be noted that *P. digitatum* grows more quickly than does *P. italicum* on PDA or H-25 (6; G. J. Holmes, *unpublished*) but not on MEA. In addition, a higher spore concentration was required to produce active lesions on lemons and oranges compared with the other two species.

All California isolates of *P. ulaiense* grew on levels of imazalil and thiabendazole that completely inhibited the type strain of *P. ulaiense* and fungicide-sensitive *P. digitatum* and *P. italicum.* Previous work with fungicide-resistant isolates of *P. digitatum* showed that this species is less resistant to imazalil and thiabendazole than is *P. ulaiense* (10). In vitro resistance of both *P. ulaiense* and *P. digitatum* correlates to loss of decay control in laboratory and field tests (10; G. J. Holmes, *unpublished*). Sensitivity of *P. ulaiense* isolates from Taiwan, New Zealand, and Argentina to imazalil and thiabendazole and resistance of California isolates to these fungicides suggests that California isolates were selected by fungicides used intensively in packinghouses. Analogous circumstances have been reported for pome fruits, where the thiabendazole-resistant, weak pathogens *P. solitum* Westling and *P. crustosum* Thom became more parasitically fit on benzimidazole-treated fruit (21,22). Currently, whisker mold appears to be of minor economic importance, although decay losses caused by this pathogen have not been evaluated. While *P. ulaiense* is only moderately virulent on citrus and is rarely found alone on diseased fruit, its abundance in packinghouses and its resistance to fungicides make it a pathogen of increasing concern.

The initial isolation of *P. ulaiense* in Taiwan is not strong evidence of the species' origin. Only one isolate has been collected in Taiwan, and little is known of its distribution or frequency of occurrence there (S.-S. Tzean, *personal communication*). *P. ulaiense* was first collected in California in 1987 but was probably present in packinghouses and groves at a very low frequency for many years. *P. ulaiense* appears to be distributed worldwide on the basis of our survey of citrus-growing areas of the world, and other researchers are beginning to investigate the disease (27). Given the current high frequency of *P. ulaiense* in packinghouses and the vast amount of research on postharvest diseases of citrus and their causal agents (4,5,13,17,28), it is difficult to explain how this species could have gone undetected. One possible explanation is that *P. ulaiense* was mistaken for *P. italicum;* the colony color is virtually identical, and both species form coremia. In our world survey, many citrus pathologists recalled seeing *Penicillium* molds with the characteristics of *P. ulaiense* but thought they were variants of *P. italicum.* In earlier times, however, it is difficult to believe that the meticulous pioneer citrus pathologists H. S. Fawcett and L. J. Klotz (5,13) would have missed *P. ulaiense* if it were as abundant in their time (1920s–1960s) as it is today. A more plausible explanation is that *P. ulaiense* has always been a rare species because of its low parasitic fitness on citrus fruit compared with *P. digitatum* and *P. italicum.* In 1982, the intensive commercial use of imazalil began in California, and the selection of imazalil-resistant biotypes of *Penicillium* spp. followed. Under imazalil selection pressure, imazalil-resistant populations of *P. ulaiense* may have acquired a higher parasitic fitness in packinghouses than they possessed in groves. In California groves today, wild-type *P. ulaiense* is probably present at low frequencies as it was in earlier times (5,13). Nonetheless, we have received grove-collected, fungicide-sensitive isolates from Argentina, Australia and New Zealand.

TABLE 5. Sensitivity of 20 isolates of *Penicillium ulaiense* to imazalil, thiabendazole, and *o*-phenylphenol

	EC_{50}[a] (μg/ml)		
Isolate	Imazalil	Thiabendazole	*o*-Phenylphenol
1640	<0.2[b]	<10	...[c]
1653	<0.2	<10	...
1659	<0.2	<10	...
1609	0.7 (92.7)[d]	47.0 (1.2)	8.8 (3.3)
1610	0.6 (146.4)	44.6 (1.4)	10.6 (4.2)
1614	0.4 (272.8)	51.0 (2.0)	14.0 (4.4)
1616	0.5 (137.1)	63.0 (0.8)	11.7 (4.0)
1617	0.7 (88.3)	46.0 (1.6)	11.8 (3.9)
1618	0.4 (211.6)	34.6 (1.3)	...
1620	1.0 (59.7)	57.7 (0.8)	13.5 (4.1)
1622	1.0 (62.0)	27.8 (1.9)	27.0 (1.5)
1623	0.8 (78.6)	51.5 (0.9)	18.4 (2.7)
1625	0.6 (143.1)	48.8 (1.2)	13.5 (3.1)
1626	0.5 (130.5)	31.2 (1.8)	8.3 (3.0)
1629	0.7 (120.0)	53.2 (0.9)	11.4 (3.2)
1630	0.7 (85.5)	35.5 (1.2)	10.6 (3.8)
1632	0.7 (109.8)	45.5 (1.3)	9.7 (3.6)
1634	0.8 (67.9)	45.5 (1.4)	9.8 (3.4)
1636	0.5 (145.4)	42.2 (1.4)	8.8 (3.3)
1637	0.8 (107.9)	52.3 (1.2)	11.4 (3.5)
Mean[e]	0.7	45.7	12.5
SD[f]	0.2	14.1	5.4

[a] Concentration that causes 50% inhibition of radial growth.
[b] < = Complete inhibition of growth at this concentration.
[c] Insufficient data.
[d] Number in parentheses is the slope of the regression line.
[e] EC_{50} values for sensitive isolates were not included in calculation of mean.
[f] Standard deviation.

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