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Genotypic variation in *Penicillium chysogenum* from indoor environments

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Abstract: We examined 198 isolates of *P. chysogenum* recovered from 109 houses in Wallaceburg, Ontario, and 25 culture collection isolates including seven ex-type strains. Multilocus genotypes were determined by heteroduplex mobility assay of regions spanning introns in acetyl co-enzyme A synthase, beta-tubulin, thioredoxin reductase and the internal transcribed spacer regions of the nuclear ribosomal subrepeat. Five unique multilocus haplotypes were revealed without evidence of recombination, indicating strictly clonal population structures. Phylogenetic analysis of allele sequences using maximum parsimony resolved three strongly supported lineages. The dominant clade included more than 90% of house isolates in addition to the notable laboratory contaminant isolated by Alexander Fleming in 1929 in Britain. A second clade contained more than 5% of house isolates clustered with the ex-type strains of *P. chysogenum* and *P. notatum*. Follow-up sampling of outdoor air in the locality failed to reveal *P. chysogenum,* confirming the rarity of this fungus in outdoor air.

Key words: DNA sequence analysis, dust biology, fungal population genetics, heteroduplex mobility assay, indoor air quality, indoor molds

INTRODUCTION

Viable fungal spores occur in great numbers in household dust and indoor air. Many of these spores typically arise outdoors in the phylloplane and are carried indoors on air currents and contaminated footwear. Other genera, such as *Aspergillus* Fr. : Fr. and *Penicillium* Link, are poorly represented in outdoor air in contrast to their indoor frequency. Thus, it is widely believed that these molds proliferate indoors, often cryptically, on various substrates including dust itself under dry conditions.

Penicillium chysogenum Thom is perhaps the most common of all Penicillia (Pitt 1980, Raper and Thom 1949), occurring as an agent of food spoilage (Samson et al 1996) as well as a resident of household dust (Davies 1960) and indoor air. *Penicillium chysogenum* is also a well-known contaminant of damp building materials (Chang et al 1995, Gravesen 1999, Hunter and Lea 1995) and indoor finishes (Adan and Samson 1994). This species is one of the few terverticillate Penicillia that typically does not produce mycotoxins of significant mammalian toxicity (Dillon et al 1996, Nielsen and Gravesen 1999, Pitt and Cruikshank 1990). However, *P. chysogenum* has been identified as an important allergen in the indoor environment (Cooley 1999, Cooley et al 1999, Fergusson et al 1984) and as a rare causative agent of opportunistic mycosis in humans (Eschete et al 1981, Hoffman et al 1992).

Charles Thom (1910) described *P. chysogenum* as a contaminant from cheese. The authentic strain of this taxon is deposited as NRRL 807 (Thom's culture No. 26 = ATCC 10107, CBS 306.48 and DAOM 193710). Thom named *P. chysogenum* fundamentally based on the production of yellow guttation droplets on the colony surface, a characteristic he observed consistently during cultivation on a range of growth media. Thom discussed several strains that produced yellow guttation but varied in colony morphology to some degree, and he interpreted *P. chysogenum* in a broad sense to include these variant ''races'' (Thom 1910). Raper and Thom (1949) later expanded this already broad concept to include several taxa described by Dierckx (1901) and Biourge (1923), noting that these authors described taxa based largely on the examination of single strains that represented contrasting forms of *P. chysogenum*. However, they chose to maintain *P. cyaneo-fulvum* Biourge, *P. meleagrinum* Biourge and *P. notatum* Westling to accommodate the variation they observed in examinations

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of hundreds of isolates of the *P. chysogenum* ''series''. Raper and Thom (1949) considered *P. griseoroseum* Dierckx to be a synonym of *P. notatum*. Samson and co-workers (1977) reinforced the broad concept of *P. chysogenum* by further reducing *P. cyaneofulvum, P. meleagrinum* and *P. notatum* to synonymy with *P. chysogenum*.

Pitt (1980) neotypified *P. chysogenum* with a dried culture of IMI 24314 (as Herb. IMI 24314). Although, in his designation, Pitt referred to the specimen as a lectotype, it formally must be considered a neotype because the designated specimen was not examined by the original author (Article 9.2, ICBN) and the use of a living ex-type that has been maintained in culture cannot be assumed to have remained consistent with the original collection despite its pedigree. In his examination of authentic isolates from the *P. chysogenum* group, Pitt (1980) noted that *P. griseoroseum* produced a floccose colony morphology and biverticillate to irregularly terverticillate penicillia. On this basis, Pitt (1980) reclassified *P. griseoroseum* in *Penicillium* subgenus *Furcatum* and neotypified it with a dried culture at least seven transfers removed from the original isolate (Dierckx 3 > Inst. Pasteur 85 > Biourge 29 > Thom 4733.70 > LSHB P39-1930 > CMI 1962 > IMI 92220) (Hennebert 1985). Despite this subgeneric separation, Pitt (1980) considered *P. griseoroseum* to be close to *P. chysogenum*. In part, his decision to retain the former taxon might have been influenced by the historical significance of the strain in question, which is the oldest laboratory-maintained culture of the genus known (Pitt 1980, p. 7). Cruickshank and Pitt (1987) later supported the conspecificity of *P. chysogenum* and *P. griseoroseum* on the basis of isozyme electrophoretic patterns and reduced *P. griseoroseum* into synonymy with the former. Frisvad and Filtenborg (1989) further supported this conclusion by the comparison of mycotoxin profiles. In contrast, the numerical analysis of morphological and physiological data by Bridge et al (1989) excluded Thom's strain of *P. chysogenum* (Cluster 7) from the core of isolates of this species, which included the authentic culture of *P. griseoroseum* (Cluster 14). These authors rationalized the position of the ex-type culture of *P. chysogenum* (IMI 24314) by suggesting that it was an attenuated strain (Bridge et al 1989). However, investigations of *P. chysogenum* ex-type strains by other authors have not demonstrated any significant cultural deterioration (Pitt 1980, Pitt and Samson 1993). Indeed, Raper and Thom (1949) noted that during 40 years of continuous cultivation, the ex-type strain of *P. chysogenum* remained stable.

The modern concept of *P. chysogenum* includes rapidly growing isolates with loosely branched smooth terverticillate conidiophores and smooth ellipsoidal conidia. The production of yellow guttation and yellow diffusible pigment is also characteristic but varies from isolate to isolate. *Penicillium chysogenum* generally is thought to be strictly asexual because no teleomorph has been identified for this species. However, the clonal nature of *P. chysogenum* has not been tested systematically using a modern molecular genetic approach. Several additional taxa recently have been described that appear to be allied with *P. chysogenum*, specifically *P. aethiopicum* Frisvad, *P. dipodomyis* Frisvad, Filtenborg & Wicklow, *P. flavigenum* Frisvad & Sampson. The placement of these taxa relative to *P. chysogenum* similarly has not been investigated using molecular phylogenetic methods.

In the present study, we examined the extent of clonality within a core group of isolates from the *P. chysogenum* group and representative authentic isolates of related taxa and tested the phylogenetic validity of the current species concept of *P. chysogenum* based on the analysis of multilocus haplotype and DNA sequence data.

MATERIALS AND METHODS

Isolation and identification of strains.—More than 700 isolates of *P. chysogenum* were collected from 376 houses in Wallaceburg, Ontario, in 1994. These isolates were grown 14 d at room temperature on modified Leonian's agar (Malloch 1981) and modified Creatine-Sucrose agar medium (CSA) (Frisvad 1993). A duplicate plate of each isolate was incubated 7 d at 37 C. A subset of 198 micromorphologically and physiologically uniform isolates was selected for genetic characterization based on the hypothesis that these isolates represented a single phylogenetic species. Air sampling was conducted at 18 outdoor locations distributed evenly throughout Wallaceburg during late summer 1995, using a Reuter Centrifugal sampler (RCS) (Biotest, Dreieich, Germany) on Rose Bengal agar medium (Malloch 1981) with a sampling volume of 80 L per sample. Growth media were incubated and colonies analyzed as above.

The number assigned to each fungal isolate is unique and consists of an arbitrary ''house number'' coupled with an incremental accession number reflective of the total number of isolates of *P. chysogenum* obtained from that house. Additional isolates included in sequencing studies consisted of authentic strains as well as a geographical range of voucher isolates (TABLE I).

DNA isolation.—Fungal isolates were inoculated centrally on a Petri plate of Weitzman and Silva-Hutner's agar (WSHA) (Weitzman and Silva-Hutner 1967), and grown 7 d at room temperature under 12 h artificial daylight. The plates were flooded with 2 mL of 95% ethanol, and the conidia and mycelium were suspended by gently scraping the surface of the colonies with a sterile bent glass rod. Conidial suspensions were collected in microcentrifuge tubes, centrifuged at 12 000 rpm, and the supernatant was

TABLE I. Strains examined in this study

Species	Strain no.	Status	Substratum and locality
Penicillium aethiopicum Frisvad	CBS 484.84	ex-type	grains of *Hordeum vulgare,* Ethiopia
P. chrysogenum Thom	C8.12		house dust, Wallaceburg, ON, Canada
P. chrysogenum	C8.24		house dust, Wallaceburg, ON, Canada
P. chrysogenum	C200		house dust, Wallaceburg, ON, Canada
P. chrysogenum	C238		house dust, Wallaceburg, ON, Canada
P. chrysogenum	C317.1		house dust, Wallaceburg, ON, Canada
P. chrysogenum	DAOM 155627		paper, Ottawa, ON, Canada
P. chrysogenum	DAOM 155628		paper, Ottawa, ON, Canada
P. chrysogenum	DAOM 155631		paper, Ottawa, ON, Canada
P. chrysogenum	DAOM 167036		*Picea* forest soil, QC, Canada
P. chrysogenum	DAOM 171025		salami, Ottawa, ON, Canada
P. chrysogenum	DAOM 175157		walls of mouldy house, Niagara Falls, ON, Canada
P. chrysogenum	DAOM 175176		*Lycopersicum esculentum* leaves, PEI, Canada
P. chrysogenum	DAOM 175758		office building, OC, Canada
P. chrysogenum	DAOM 178623		*substr. et loc. incert.*
P. chrysogenum	DAOM 190864		grains of *Hordeum,* MB, Canada
P. chrysogenum	DAOM 193710	ex-type	cheese, CT, USA
P. chrysogenum	DAOM 212031		wooden wall studs, AB, Canada
P. chrysogenum	DAOM 215336		wooden wall studs, AB, Canada
P. chrysogenum	DAOM 215337		hemlock lumber, BC, Canada
P. chrysogenum	DAOM 216700		grains of *Hordeum,* Canada
P. chrysogenum	DAOM 216701		*Sesamum indicum,* Korea
P. chrysogenum	DAOM 59494C		*substr. incert.,* Honduras
P. chrysogenum	NRRL 824		laboratory contaminant, London, England
P. dipodomyis (Frisvad et al) Banke et al	NRRL 1485	ex-type	cheek pouch of *Dipodomyis spectabilis,* AZ, USA
P. flavigenum Frisvad & Samson	CBS 419.89	ex-type	flour, Lyngby, Denmark
P. flavigenum	IMI 321909		*substr. et loc. incert.*
P. nalgiovense Laxa	NRRL 911	ex-type	cheese, Nalzovy, Czech Republic
P. notatum Westling	ATCC 10108	ex-type	branches of *Hyssopus* sp., Norway

discarded. The pellets were dried 30 min in a vacuum concentrator centrifuge. This protocol yielded approximately 15 mg of pelleted conidia per vial. Each vial was sufficient for a single DNA isolation.

Approximately 15 mg of sterile, acid-cleaned Dicalite 1400 (diatomaceous earth-based swimming pool filter, Grefco Inc., Torrance, California) was added to each tube of dry, pelleted, ethanol-killed conidia (Scott et al 1999). After the addition of 10 μL of 70% EtOH, the mixture was ground with a sterile glass rod 1 min and suspended in 600 μL of lysis buffer containing 1.4 M NaCl, 2% w/v CTAB, 200 mM Tris·HCl (pH 8.0) and 20 mM EDTA (Weising et al 1995). Tubes were incubated at 65 C for 1 h and were mixed by inversion at 30 min intervals.

After extraction, the tubes were cooled to room temperature and centrifuged at 10 000 rpm for 1 min. The supernatant liquid was extracted twice with chloroform : isoamyl alcohol (24:1), and the DNA was precipitated with 100% isopropanol at −80 C for 10 min. The pellets were rinsed with 70% ethanol, air-dried and resuspended in 200 μL TE (pH 8.0) (Sambrook et al 1989). Ribonuclease A was added to the DNA at a final concentration of 0.2 μg/μL and incubated at 37 C for 30 min. The DNA was extracted with chloroform : isoamyl alcohol and precipitated with 0.3 M sodium acetate and an equal volume of 100% ethanol at −80 C. DNA pellets were rinsed with 70% ethanol, air-dried and resuspended in 100 μL TE (pH 8.0). DNA concentration was adjusted to 60 ng/μL based on spectrophotometry.

DNA preparation and heteroduplex mobility assay (HMA).—Four polymorphic loci consisting of partial regions spanning introns in the genes encoding acetyl co-enzyme A synthase (acuA), beta-tubulin (benA), thioredoxin reductase (trxB) and the region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) of the nuclear ribosomal RNA gene (rDNA) were PCR-amplified using the primers listed in TABLE II. PCR mixtures consisted of 1 unit of *Taq* DNA polymerase (Boehinger Mannheim, Laval, QC), 50 mM KCl, 2.0 mM $MgCl_2$, 250 μM of each dNTP, 0.2 mM of each primer and 60 ng of template DNA in a total reaction volume of 50 μL overlaid with a drop of sterile mineral oil. Reactions were carried out in a PTC-100 thermocycler (MJ Research, Reno, Nevada). Cycling conditions consisted of 30 cycles of 94 C for 30 s, 58 C for 30 s and 72 C for 30 s with a final extension at 72 C for 2 min. Yield was quantified based on ethidium bromide staining and UV visualization after electrophoresis on 1.2% agarose gels.

Heteroduplexing reactions pooled homologous PCR products pairwise in overlapped combinations (pairs comprised numerically adjacent isolates in a sequentially numbered series of PCRs and the first and last isolate of each series) to encompass the entire set. For each locus exam-

TABLE II. Primers sequences employed in this study

Acetyl-CoA synthetase (acuA)	
Source: this study, Genbank L09598, +2102-2452, spanning introns 3 and 4	
acuA-2F (fwd)	5′-ACC GTG TGG GGT GCC CAC AAG CGT TAC ATG-3′
acuA-1R (rvs)	5′-GGT CAG CTC GTC GGC AAT ACC AAC GAC AGC-3′
Beta-tubulin (benA)	
Source: Glass and Donaldson (1995)	
Bt2A (fwd)	5′-GGT AAC CAA ATC GGT GCT GCT TTC-3′
Bt2b (rvs)	5′-ACC CTC AGT GTA GTG ACC CTT GGC-3′
Nuclear ribosomal DNA ITS1, 5.8S and ITS2 region (ITS)	
Source: White et al (1990), fwd; Untereiner et al (1995), rvs	
ITS5 (fwd)	5′-GGA AGT AAA AGT CGT AAC AAG G-3′
WNL1 (rvs)	5′-TAT GCT TAA GTT CAG CGG-3′
Thioredoxin reductase (trxB)	
Source: this study, EMBL X76119, +801-1153, spanning intron 2	
trxB-1F (fwd)	5′-AAC GCG GAG GAG GTC GTT GAG GCT AAC GGT-3′
trxB-1R (rvs)	5′-TTA GAG CAC AGG CTT TGC CTC CTG GTG AGT-3′

ined, PCRs were diluted to 50% of the original concentration with 4 mM EDTA and 50 mM KCl, combined in equimolar proportion in a total volume of 10 μL and overlaid with a drop of sterile mineral oil. Reactions were heated to boiling for 4 min and immediately annealed at 65 C for 6 min. Products of HMA reactions were separated by electrophoresis in gels consisting of 12% acrylamide, 0.2% bisacrylamide and 0.04% ammonium persulfate in 1 × TBE. Immediately before casting, 0.5% agarose and 0.2% TEMED were added to the degassed solution. Gels were cast in a BioRad Protean electrophoresis apparatus (La Jolla, California) at 1 mm thick, allowed to polymerize 4–5 h and run on a vertical electrophoresis apparatus (Protean II, BioRad) at 10 V/cm, 12 C for up to 20 h. Gels were stained for 2 h in ethidium bromide (250 ng/mL) and destained in dH_2O 3–4 h before imaging.

Using HMA, like pairs of isolates were reduced to a single ''proxy'' strain by transitive property of equality (Scott et al 1999, Scott et al 2000). Subsequent rounds of HMA compared proxy isolates ultimately reducing the entire population to a set of genotypically distinct alleles represented by a minimum number of proxy isolates for each locus tested. One or more isolates for each multilocus genotype identified by heteroduplex analysis were sequenced for phylogenetic analysis.

DNA sequencing and analysis.—PCR templates were purified using QIAquick PCR purification kit (Qiagen Inc., Valencia, California) and sequenced using a *Taq* DyeDeoxy cycle sequencing kit (Applied Biosystems Inc., Foster City, California) and the same primers employed for amplification. Extension products were run on an ABI50 fluorescent automated sequencer (Applied Biosystems Inc.).

Alignments of sequences were performed using Clustal X software version 1.64b (Thompson et al 1997) and adjusted by visual inspection. Phylogenetic relationships were inferred from aligned sequences using the maximum parsimony (MP) method found in PAUP* (beta version 4.0b10) (Swofford 2003).

An heuristic search of the benA dataset (29 taxa, 434 bp, gaps treated as missing) was performed employing tree bisection-reconstruction (TBR) branch swapping with MulTrees and steepest descent options activated. We also performed exhaustive searches of individual datasets consisting of sequences of *P. chysogenum* (DAOM 193710 ex-type, NRRL 824 Fleming strain), *P. notatum* (ATCC 10108 extype) and representatives of the Wallaceburg multilocus haplotypes (*P. chysogenum* C8.24, C8.12, C200, C238, C317.1) for each of the four genetic loci (acuA 291 bp, benA 430 bp, ITS 546 bp, trxB 306 bp). In addition, the ITS dataset was expanded to include the sequence of the ex-type strain of *P. griseoroseum* (NRRL 820). Phylogenies of the eight-taxon dataset also were generated from exhaustive searches of a combined three-locus (acuA, benA, trxB) and four-locus datasets. A single multibase indel in the benA dataset was rescored as a single gap, and gaps were treated as a fifth character in analyses of the pruned datasets that included sequences for this locus. Taxa use as outgroups included *P. nalgiovense* NRRL 911 and *P. dipodomyis* NRRL 13485 (29-taxon benA dataset) and *P. chysogenum* NRRL 824 (eight-taxon dataset).

Bootstrap support (Felsenstein 1985) for internal branches was evaluated from 1000 heuristic searches, and groups with a frequency of greater than 50% were retained in the bootstrap consensus trees. Congruence between the three (acuA, benA, trxB) and four loci for eight taxa was measured based on 10 000 heuristic searches (TBR branch swapping with MulTrees and steepest descent options activated) using the partition-homogeneity test (PHT) included in PAUP*.

RESULTS

Penicillium chysogenum was observed in 52% of houses investigated. Isolates of *P. chysogenum* were re-

TABLE III. Haplotype frequencies of indoor *P. chrysogenum* isolates

Proxy isolate	Locus				No. of isolates	Frequency
	acuA	benA	ITS	trxB		
C8.12	A	A	A	A	179	0.904
C317.1	A	C	B	C	5	0.025
C8.24	B	B	B	B	11	0.056
C238	C	B	B	B	2	0.010
C200	A	C	C	C	1	0.005

tained from a subset of houses investigated (109/369 houses), yielding a total of 198 isolates. Multiple isolates were obtained from 30 houses. *Penicillium chysogenum* was not observed in the 18 outdoor air samples taken throughout Wallaceburg during August 1995.

Haplotypes of house dust isolates identified by heteroduplex analysis are given in TABLE III. The multilocus haplotype AAAA (acuA, benA, ITS and trxB, respectively), represented by the isolate C8.12, was the most commonly observed haplotype in the population and represented more than 90% of all isolates. The second most common multilocus haplotype, BBBB, accounted for 5.6% of the isolates studied and is represented by isolate C8.24 (the same house as the representative isolate used for AAAA, above). Thee minor multilocus haplotypes (ACBC, ACCC and CBBB) accommodated the remainder of the isolates. Multiple genotypes were recovered from 27% of houses where multiple isolates were obtained. Allele identities were confirmed by the sequencing of proxy isolates. Genbank accession numbers for these and other sequences used in this study are given in TABLE IV.

Phylogenetic relationships of species in the *P. chysogenum* group were inferred from an heuristic analysis of partial sequences of the beta-tubulin (benA) gene. This dataset included 29 taxa and consisted of a 434 bp region spanning introns 3–5. MP analysis yielded four MPTs 48 steps in length (L) with a con-

TABLE IV. Sequences used or developed in this study

Identification	Strain no.	GenBank accession numbers			
		acuA	benA	ITS	trxB
P. aethiopicum	CBS 484.84	AY371577	AY371605	AY371635	AY371663
P. chrysogenum	C8.12	AY371550	AY371578	AY371608	AY371637
P. chrysogenum	C8.24	AY371552	AY371579	AY371610	AY371640
P. chrysogenum	C200	AY371555	AY371580	AY371614	AY371639
P. chrysogenum	C238	AY371556	AY371581	AY371613	AY371642
P. chrysogenum	C317.1	AY371554	AY371582	AY371612	AY371638
P. chrysogenum	DAOM 155627	AY371561	AY371584	AY371619	AY371647
P. chrysogenum	DAOM 155628	AY371562	AY371585	AY371620	AY371648
P. chrysogenum	DAOM 155631	AY371563	AY371586	AY371629	AY371657
P. chrysogenum	DAOM 167036	AY371564	AY371587	AY371621	AY371649
P. chrysogenum	DAOM 171025	AY371565	AY371588	AY371630	AY371658
P. chrysogenum	DAOM 175157	AY371566	AY371589	AY371622	AY371650
P. chrysogenum	DAOM 175176	AY371567	AY371590	AY371623	AY371651
P. chrysogenum	DAOM 175758	AY371568	AY371591	AY371624	AY371652
P. chrysogenum	DAOM 178623	AY371569	AY371592	AY371631	AY371659
P. chrysogenum	DAOM 190864	AY371570	—	AY371625	AY371653
P. chrysogenum	DAOM 193710	AY371553	AY371594	AY371611	AY371641
P. chrysogenum	DAOM 212031	AY371571	AY371595	AY371626	AY371654
P. chrysogenum	DAOM 215336	AY371572	AY371596	AY371627	AY371655
P. chrysogenum	DAOM 215337	AY371573	AY371597	AY371632	AY371660
P. chrysogenum	DAOM 216700	AY371574	AY371598	AY371628	AY371656
P. chrysogenum	DAOM 216701	AY371575	AY371599	AY371633	AY371661
P. chrysogenum	DAOM 59494C	AY371560	AY371583	AY371618	AY371646
P. chrysogenum	NRRL 824	AY371551	AY371600	AY371609	AY371636
P. dipodomyis	NRRL 13485	AY371557	AY371602	AY371615	AY371644
P. flavigenum	CBS 419.89	—	AY371607	—	—
P. flavigenum	IMI 321909	—	AY371606	—	—
P. griseoroseum	NRRL 820	—	—	AF034857	—
P. nalgiovense	NRRL 911	AY371559	AY371601	AY371617	AY371645
P. notatum	ATCC 10108	AY371576	AY371604	AY371634	AY371662

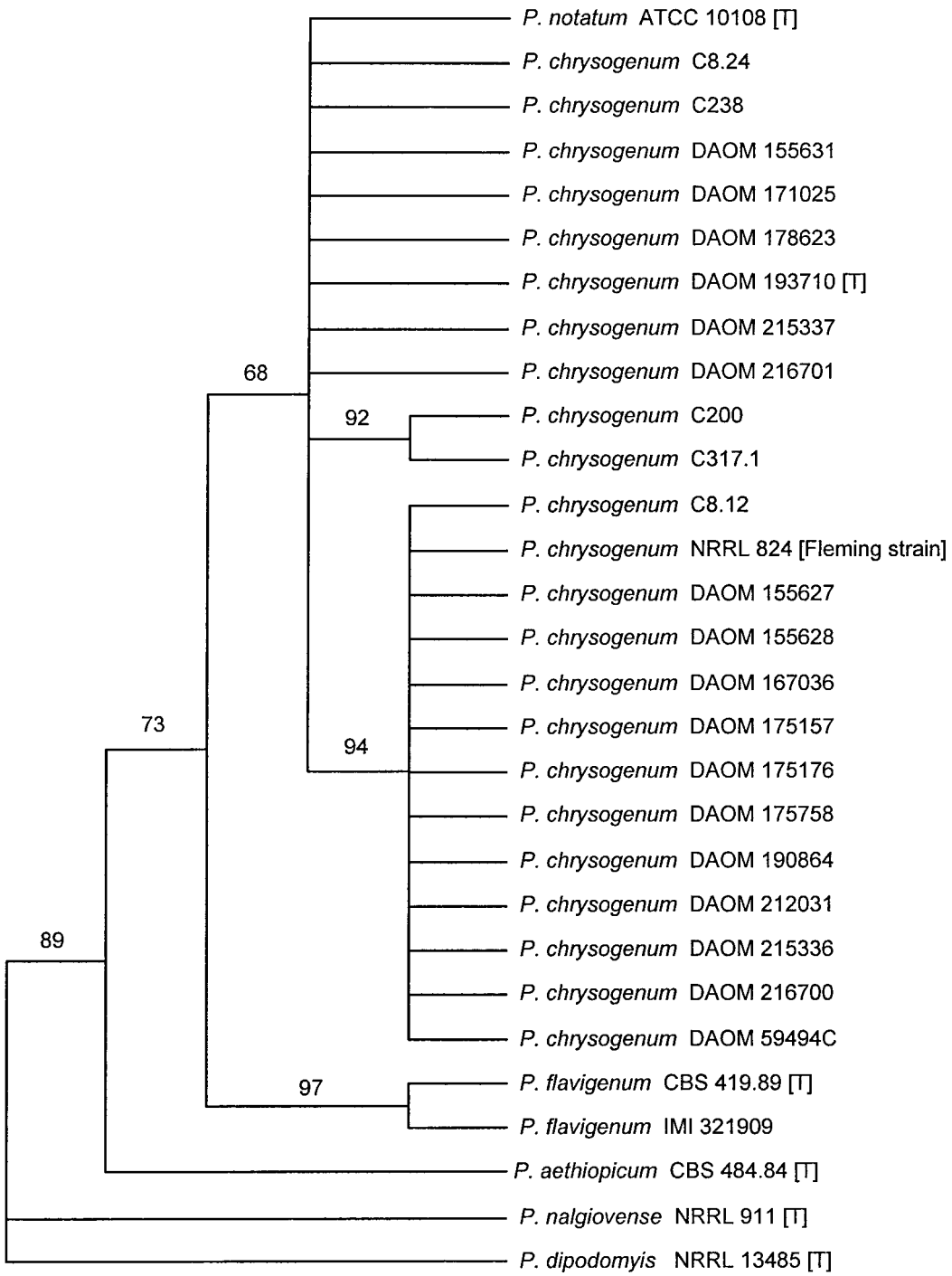

FIG. 1. Phylogenetic relationships of members of the *P. chrysogenum* group inferred from partial beta-tubulin gene sequences. This is a strict consensus of 4 MPTs (L = 48, CI = 0.878, RI = 0.918) generated from a heuristic analysis of 434 bp for 29 taxa. Bootstrap values greater than 50% calculated from 1000 replicates are indicated above the branches. The outgroup taxon are *P. dipodomyis* NRRL 13485 and *P. nalgiovense* NRRL 911.

sistency index (CI) and a retention index (RI) of 0.878 and 0.918, respectively. The strict consensus of these trees (FIG. 1) shows a large well supported lineage (bootstrap support of 73% in 1000 replicates) that corresponds to the *P. chysogenum-P. flavigenum* clade (TreeBase SN1793-5703). In this phylogeny, *P. chysogenum sensu lato* (bootstrap support of 68%) comprised two well supported lineages as well as representative isolates from Canada (British Columbia and Ontario), Norway (ATCC 10108), South Korea

TABLE V. Summary of MPTs produced from each of 4 loci examined

Locus	Number of MPTs	Length (steps)	CI	RI	Total chrs	PI chrs
acuA	1	14	1.000	1.000	291	10
benA	1	12	0.833	0.846	432	10
ITS	1	2	1.000	1.000	546	1
trxB	1	10	1.000	1.000	306	10
4 loci	1	38	0.947	0.962	1575	31
3 loci (w/o ITS)	1	36	0.944	0.961	1029	30

(DAOM 216701) and the United States (DAOM 178623, DAOM 193710). The larger of these clades (bootstrap support of 94%) contained isolates from Canada (including Alberta, Manitoba, Ontario, Prince Edward Island and Quebec), Honduras (DAOM 59494C) and the United Kingdom (NRRL 824). The smaller clade (bootstrap support of 94%) included six isolates obtained from Wallaceburg house dust.

Analysis of individual data from partial acuA, benA and trxB sequences resulted in 10 parsimony-informative characters for each locus (TABLE V). Analysis of ITS data yielded only a single parsimony-informative character (TABLE V). MPTs generated from individual locus datasets showed identical or compatible topologies, supporting the analysis of these datasets in combination (data not shown). Results of 10 000 heuristic searches implementing the PHT ($P = 1.0$) also demonstrated that these topologies are congruent and that sequences from the four different loci may be combined.

Combined analysis of data from partial acuA, benA, ITS and trxB sequences included eight taxa comprising the core of the *P. chysogenum* group. An exhaustive search of the combined dataset (1575 bp, 31 parsimony-informative characters) produced a single MPT (L = 38, CI = 0.947, RI = 962) dividing *P. chysogenum sensu lato* into four well-supported clades (bootstraps 98% or higher) (FIG. 2) (TreeBase SN1793-5704). Clade 1 included the ex-type strains of *P. chysogenum* (DAOM 193710), *P. notatum* (ATCC 10108) and 11 (5.6%) isolates from Wallaceburg house dust (represented in FIG. 2 by C8.24). This lineage was sister of Clade 2, a group that contained two Wallaceburg isolates (1.0% of isolates). Clade 3 consisted of six Wallaceburg isolates (3.0%). The majority of Wallaceburg *P. chysogenum* isolates (179/198, 90.4%) clustered in Clade 4 with the British strain isolated by Alexander Fleming (NRRL 824).

The single dataset MPT based on ITS sequences divided *P. chysogenum sensu lato* into two lineages corresponding to Clades 1/2/3 and Clade 4 from the combined analysis (data not shown) (TreeBase SN1793-5705). The ex-type strain of *P. griseoroseum* (NRRL 820, GenBank AF034857) grouped with the ex-type strains of *P. chysogenum* and *P. notatum,* but this clade was not strongly supported (data not shown).

DISCUSSION

Five unique multilocus haplotypes were revealed without evidence of recombination, indicating strictly clonal population structures in these lineages. Our results support the conclusions of Banke et al (1997) that *P. chysogenum, P. flavigenum, P. nalgiovense* and *P. dipodomyis* are distinct species and that the former two taxa are sister groups. Banke et al (1997) noted a high degree of infraspecific variability in *P. flavigenum* and suggested that this species might consist of several subgroups. Our analysis showed two strains of *P. flavigenum* to be closely related, however these isolates did not share complete sequence homology for the region of beta-tubulin gene examined. Further work is necessary to resolve the phylogenetic structure of this species.

The phylogeny based on partial beta-tubulin sequence showed lineages in *P. chysogenum sensu lato* to be represented across Canada and from a broad geographic range extending to localities in North and Central America, Europe/Scandinavia and Asia (FIG. 1). Our analysis of four gene regions yielded thee topologically compatible trees revealing thee well supported clonal lineages within *P. chysogenum sensu lato*. Applying the phylogenetic species concept advocated by Taylor et al (2000) on the basis of concordance of multiple gene genealogies, these lineages represent distinct phylogenetic species. These lineages collectively may be called the *P. chysogenum* species complex. Our study showed *P. chysogenum sensu stricto* (Clade 1) to be uncommon in the indoor dust mycobiota relative to isolates of Clade 4. Culture collection strains positioned within Clade 1 were isolated as contaminants from raw wood, paper and proteinaceous foods (e.g., sausage, cheese). In contrast, strains clustering with Clade 4 isolates originated from cereals, paper, soil, construction materials and indoor environments, and included the strain isolat-

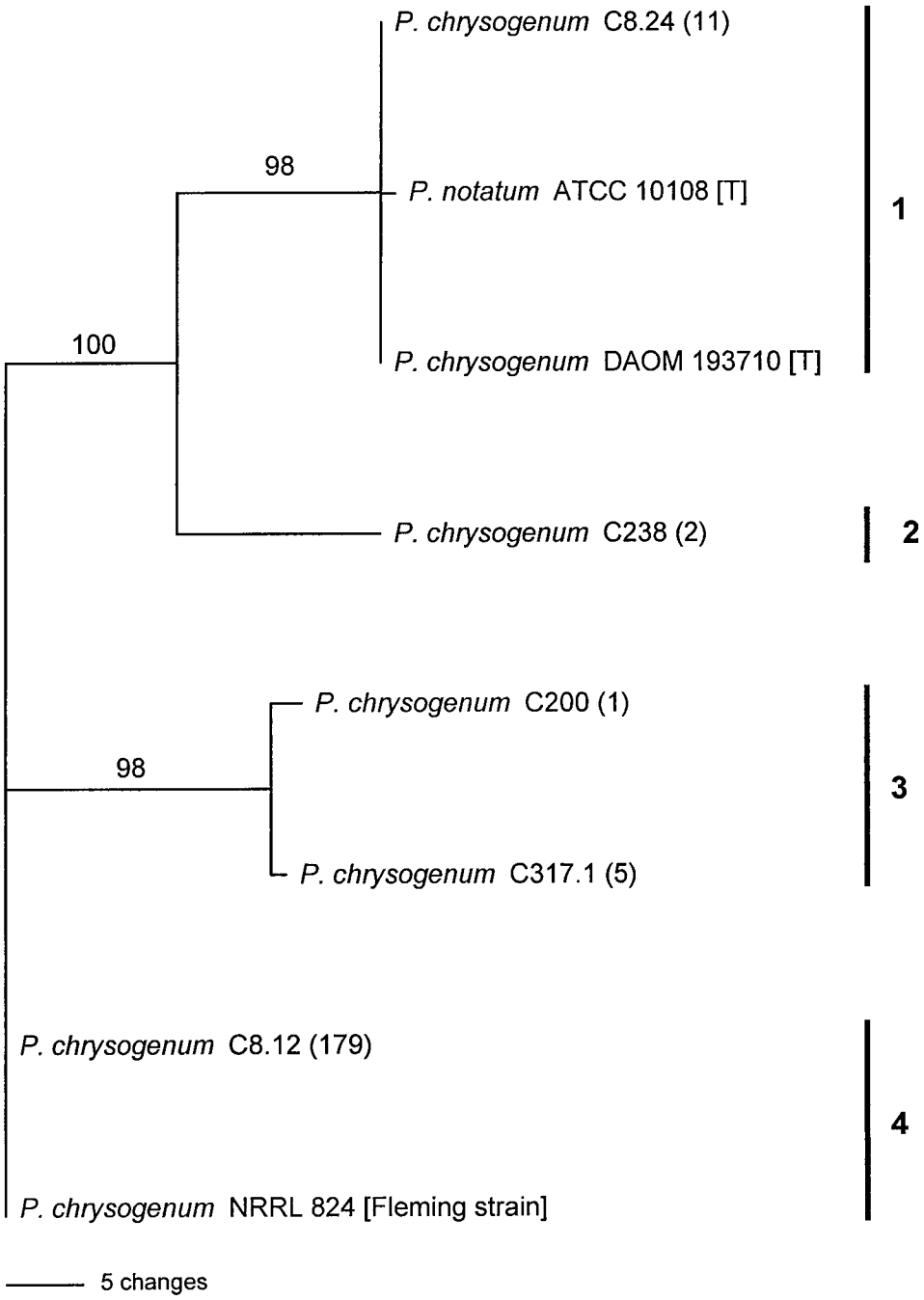

FIG 2. Single MPT inferred from an exhaustive search of the combined acuA, benA, ITS and trxB sequences of authentic or type strains of *P. chrysogenum* strains and Wallaceburg house dust isolates (1575 bp, L = 38, CI = 0.947, RI = 962; 1537 characters are constant, five variable characters are parsimony-uninformative, 31 characters are parsimony-informative). Bootstrap values greater than 50% calculated from 1000 replicates are indicated above the branches. The numbers of Wallaceburg isolates of identical multilocus haplotype are indicated in parentheses.

ed by Alexander Fleming in 1929 as a laboratory plate contaminant (Fleming 1929). The abundance of isolates assigned to Clade 4 suggests that this lineage may have a competitive advantage over other lineages to exploit human-associated indoor niches, although support for this hypothesis would require the investigation of distribution patterns of members of this group in other geographic regions.

The absence of members of the *P. chysogenum* species complex in outdoor air in comparison to the abundance of this group in indoor dust is intriguing. Two competing hypotheses could explain this observation:

(i) Members of the *P. chysogenum* complex form autochthonous populations (i.e., true populations) in household dust and/or on other indoor materials in the presence of superfluous moisture. Consequently, growth and sporulation (amplification) of these fungi indoors over time leads to their quantitative predominance in the dust mycoflora (Bronswijk 1981).

(ii) Viable indoor spores of the *P. chysogenum* complex originate from few airborne members of these taxa in the phyloplane and simply accumulate indoors faster than spores of other taxa as a consequence of their disproportionately longer viability and relatively inefficient removal. It is known that the asexual spores of members of the Trichocomaceae retain viability for a long time under dry conditions (Sussman 1968). Indeed, reculture of dust samples used in this project after a period of 5 yr in dry storage yielded only members of the Trichocomaceae and *Aureobasidium pullulans* (data not shown). In this manner, the number of *P. chysogenum* complex in indoor dust might relate in part to the ''removal'' of other taxa due to attenuated viability. Mechanical factors relating to size distribution also might be important. The spores of members of the *P. chysogenum* complex and related trichocomaceous anamorphs are small in relation to the majority of mitosporic taxa that quantiatively dominate dust in building interiors (e.g., *Cladosporium, Alternaria, Ulocladium*). These small-spored species might be inefficiently removed from the indoor environment by filtration systems in vacuum cleaners and forced-air heating systems. Stetzenbach et al (1999) reported this effect in relation to consumer market vacuum-cleaning devices. Combined, these effects or longer viability and less efficient removal might result in the emergence of an indoor allochthonous population (i.e., pseudo populations) whose constituent elements arise episodically in the phylloplane.

The conspecificity of *P. griseoroseum* and *P. chysogenum* is supported in the present study, in which the ex-type isolate (NRRL 820) clustered with the ex-type cultures of *P. chysogenum* (NRRL 807) and *P. notatum* (NRRL 821) based on the analysis of ITS sequences (data not shown). *Penicillium chysogenum* is thus a later synonym of *P. griseoroseum* based on priority of publication. This conclusion is not surprising because Banke et al (1997) obtained highly similar secondary metabolite profiles for 18 isolates of *P. chysogenum* including the ex-type strain of *P. griseoroseum*. Their proposed conspecificity of *P. chysogenum* with *P. griseoroseum* led Frisvad and co-workers (1990) to recommend conservation of the name due to the industrial importance of this species. They recommended the conservation of *P. chysogenum* as ''the species name for the principal producer of penicillin'' on the basis that the association between the name ''*Penicillium chysogenum*'' and the biochemical characteristic of penicillin biosynthesis was of paramount industrial importance (Frisvad et al 1990a, Lowe and Elander 1983). However, penicillin is known from a range of filamentous fungi (Abraham and Newton 1967, Samson et al 1996) and is speculated to have originated in fungi by horizontal gene transfer from a penicillin-producing actinomycete (Rosewich and Kistler 2000). Kozakiewicz et al (1992) later made a formal proposal to conserve the name *P. chysogenum*. From a nomenclatural standpoint, there do not appear to be existing species or varietal names that can be applied to our clades 3 and 4. Moreover, the erection of new taxa to accommodate these lineages would not conflict with the nomenclatural conservation of *P. chysogenum* and should be pursued. The phylogenetic positions of the major penicillin-producing strains of *P. chysogenum sensu lato* in relation to these new species have not been investigated.

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