Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial β-tubulin sequences

Robert A. Samson[1*], Keith A. Seifert[2], Angelina F.A. Kuijpers[1], Jos A.M.P. Houbraken[1], and Jens C. Frisvad[3]

[1]Centraalbureau voor Schimmelcultures, P.O. Box 85167, 3508 AD Utrecht, the Netherlands, [2]Biodiversity Theme (Mycology & Botany), Environmental Sciences Team, Agriculture and Agri-Food Canada, 960 Carling Ave., Ottawa, K1A 0C6, Canada and [3]Center for Microbial Biotechnology, Biocentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark.

Abstract

Partial β-tubulin sequences were determined for 180 strains representing all accepted species of *Penicillium* subgenus *Penicillium.* The overall phylogenetic structure of the subgenus was determined by a parsimony analysis with each species represented by its type (or other reliably identified) strain. Eight subsequent analyses explored the relationships of three or four strains per species for clades identified from the initial analysis. β-tubulin sequences were excellent species markers, correlating well with phenotypic characters. The phylogeny correlated in general terms with the classification into sections and series proposed in the accompanying monograph. There was good strict consensus support for much of the gene tree, and good bootstrap support for some parts. The phylogenetic analyses suggested that sect. *Viridicata*, the largest section in the subgenus, is divided into three clades. Section *Viridicata* ser. *Viridicata* formed a monophyletic group divided into three subclades supported by strict consensus, with strong bootstrap support for *P. tricolor* (100%), *P. melanoconidium* (99%), *P. polonicum* (87%) and *P. cyclopium* (99%) and moderate support for *P. aurantiogriseum* (79%). The three strains each of *Penicillium freii* and *P. neoechinulatum* had identical sequences. Three strains of *P. viridicatum* had unique sequences, with two strains differing in two or three positions from the type. Within ser. *Camemberti*, *P. palitans* (83%) and *P. crustosum* (99%) were supported by bootstrap. *Penicillium camemberti* and *P. caseifulvum* had identical sequences to each other, and to the type strain of *P. commune*. Section *Viridicata* ser. *Verrucosa* was monophyletic and included two well-supported subclades, one consisting of *P. thymicola* (87%), and the other of *P. verrucosum* (88%), derived within the paraphyletic *P. nordicum*. The phylogeny for sect. *Roqueforti* (100%) was robust, with excellent bootstrap support for all included species, i.e. *P. roqueforti* (100%)*, P. carneum* (94%) and *P. paneum* (100%)*.* In sect. *Penicillium,* Series *Expansa* was paraphyletic, with the monophyletic ser. *Italica* derived within it. The synnematous species in ser. *Claviformia* were a paraphyletic group with the species of ser. *Urticicolae*., including *P. griseofulvum* (99%), derived within. Section *Digitata,* ser. *Digitata* comprised a single well-supported species, *P. digitatum* (100%). The phylogenetic structure of sect. *Chrysogena* had limited strict consensus and bootstrap support. One clade comprises *P. chrysogenum* and *P. flavigenum* (96%)*,* another *P. dipodomyis* and *P. nalgiovense,* and a third *P. mononematosum* and *P. confertum. Penicillium persicinum* and *P. aethiopicum* were weakly placed in this section, both emerging from the backbone of the gene tree. Section *Coronata* (82%) was a well-supported monophyletic group at the base of the phylogram. *Penicillium olsonii* (100%) was basal in the section, with the sibling species *P. bialowiezense* (100%) and *P. brevicompactum* (100%) together forming a well-supported clade (96%).

Keywords: species concepts, terverticillate Penicillia, *Eupenicillium, Ben*A

Introduction

Penicillium species classified in subgenus *Penicillium* are among the most common fungi spoiling food and contaminating indoor environments. Classical monographs and revisions of these moulds often referred to as the asymmetric terverticillate Penicillia, included proposals of many different classifications into sections and series (Thom, 1930; Raper and Thom, 1949; Samson, Stolk and Hadlok, 1976; Pitt, 1979; Frisvad and Filtenborg, 1989; Pitt and Cruickshank, 1990; Stolk *et al.*, 1990). Taxonomic studies that supplemented traditional morphological character sets included many studies of extrolites (Svendsen and Frisvad, 1994; Larsen and Frisvad, 1995; Smedsgaard and Frisvad, 1997), isozymes (Pitt and Cruickshank, 1990), or combinations of phenotypic characters (Bridge *et al.* 1989), usually

analyzed using similarity or clustering methods. Cladistic analyses of gene sequences have been used in the molecular studies of this group, first using ribosomal genes (Skouboe *et al.*, 1996; Peterson, 2000) and later using protein coding genes (Seifert & Louis-Seize, 2000; Peterson 2004). These studies have resolved some questions of species delimitation, but have not provided a statistically robust solution to the the infrageneric classification of the whole subgenus *Penicillium.*

This paper presents the results of the molecular studies that formed part of the polyphasic approach used to arrive at the classification proposed in the companion paper in this volume, which was derived from morphological, physiological, biochemical and molecular data (Table 2; Frisvad and Samson, 2004). Here, we present the results of parsimony analyses of aligned partial β-tubulin sequences for 180 strains, including ex-type or typical cultures, representing the 58 accepted species. Our intent was to find phylogenetic support for the infra-subgeneric classification into sections and series proposed by Frisvad & Samson (2004). In addition, we sought DNA sequence data that could be used to study phylogeny at the species level, facilitate species discovery and identification for other workers, and eventually be used for the development of molecular diagnostics.

Materials and methods

Strains

All strains used in this study are deposited in the Centraalbureau voor Schimmelcultures, and their CBS numbers, along with their GenBank Accession numbers, are included in Table 1. This is a subset of the representative strains of subgenus *Penicillium* used by Frisvad & Samson (2004).

Isolation and maintenance

All strains were isolated and/or grown on malt extract agar (Oxoid CM59) at 24ºC. Cultures used for the molecular study were grown on Malt Peptone (MP) broth using 10% (v/v) of Malt Extract (Brix 10) and 0.1% (w/v) Bacto Peptone (Difco) in 2 mL of medium in 15 mL tubes. The cultures were incubated at 24ºC for 7 d in darkness.

DNA Extraction, PCR amplification, sequencing and analysis

Genomic DNA was isolated using the FastDNA® Kit (Bio101, Carlsbad, USA) following the manufacturer's instructions.

Amplification of the β-tubulin gene was performed using primers Bt2a and Bt2b (Glass & Donaldson, 1995). PCR reactions were performed in 50 µL reaction mixtures containing 1 µL genomic DNA (10 ng/µl), 5 µL PCR buffer, 30 µL ultra pure sterile water, 10 µL dNTP (1 mM), 1 µL of each primer (50 pmol/µL) and 1 µL Taq polymerase (2.5 U/µL DNA) (SpaeroQ, Leiden, The Netherlands). Amplifications were performed in a GeneAmp PCR system 9700 (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands), programmed for 5 cycles of 1 min denaturation at 94°C, followed by primer annealing for 90 s at 68°C, and extension for 2 min at 72°C, with a decrease of annealing temperature of 1°C/cycle, followed by 25 cycles of denaturation at 94°C for 1 min., followed by primer annealing for 90 s at 64°C, extension for 2 min at 72°C, and a final 10 min elongation step at 72°C. After amplification of the β-tubulin template, excess primers and dNTP's were removed from the reaction mixture using a commercial GFX column, PCR DNA Purification kit (Amersham Bioscience, Roosendaal, the Netherlands). Purified PCR fragments were resuspended in 50 µL of TE buffer.

The PCR products were sequenced directly in both directions with primers Bt2a and Bt2b using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Roosendaal, The Netherlands). The cycle sequencing reaction mixture had a total reaction volume of 10 µL, and contained 1 µL of template DNA (10-15 ng/µL), 4 µL Dye terminator RR mix, 4 µL ultra pure sterile water and 1 µL primer (4 pmol/µL). Reactions were run in a GeneAmp PCR system 9700 run in 9600 mode (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands); programmed for 25 cycles of 10 s denaturation at 96°C, followed by primer annealing for 5 s at 50°C and extension for 4 min at 60°C. Sequencing products were purified according to the manufacturer's recommendations with Sephadex G-50 superfine columns (Amersham Bioscience, Roosendaal, The Netherlands) in a multiscreen HV plate (Millipore, Amsterdam, The Netherlands) and with MicroAmp Optical 96-well reaction plate (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). Samples were analyzed on an ABI PRISM 3700 Genetic Analyzer (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). Contigs were assembled using the forward and reverse sequences with the programmes SeqMan and EditSeq from the LaserGene package (DNAStar Inc., Madison, WI).

Table 1. Isolates examined.

Taxon Name	CBS collection number	Other collections numbers	Substratum	Origin	GenBank Number
E. crustaceum	581.67	IBT 24546	Soil	Pakistan, Lahore	AY674446
E. egyptiacum	244.32 **T**	IBT 14684, ATCC 10441, CSIR 707, FRR 2090, IFO 8141, IFO 6094, IFO 8847, IMI 040580, NRRL 2090, QM 1875	Soil	Egypt, Cairo	AY674374
E. egyptiacum	653.82	IBT 14683, CBS 227.81, NRRL 2094, NRRL 22755	Unknown	Unknown	AY496001
E. molle	456.72 **T**	IBT 14682, ATCC 24075, FRR 1542, IFO 31738;, IMI 084589, TRTC 45714, NRRL 13062	Soil	Pakistan	AY674375
E. osmophilum	462.72 **T**	IBT 14679	Soil	Netherlands, Wageningen	AY674376
E. osmophilum	439.73	IBT 14678	Agricultural soil	Netherlands, Wageningen	AY674377
E. osmophilum	184.72	IBT 14677	Soil	Netherlands, Wageningen	AY674379
E. osmophilum	508.73		Agricultural soil	Netherlands, Wageningen	AY674378
E. tularense	431.69	IBT 14789	Soil	USA, California, Tulare Co., Pine Flat	AY674433
P. aethiopicum	484.84 **T**	IBT 21501, FRR 2942, IMI 285524	Grain of *Hordeum vulgare*	Ethiopia, Addis Abeba	AY495983
P. aethiopicum	270.97	IBT 11191	Locust bean gum flour	Denmark	AY495984
P. aethiopicum	287.97	IBT 16873	Soil from tropical room	Canada	AY495985
P. albocoremium	472.84 **T**	IBT 21502, FRR 2931, IMI 285511	Salami	Denmark, Hillerød	AY674326
P. albocoremium	109582	IBT 20068	Cake factory	Denmark, Give	AY674327
P. albocoremium	109614	IBT 19154	Cake	Denmark, Stege	AY674325
P. allii	131.89 **T**	IBT 21503, ATCC 64868, FRR 3184	*Allium sativum*	Egypt, Tell el-Amarna	AY674331
P. allii	109581	IBT 4112, IMI 297905	*Oryza sativa*	Czech Republic, Bohemia	AY674332
P. allii	188.88	IBT 3772	Food	UK	AY674333
P. atramentosum	291.48 **T**	IBT 6616, ATCC 10104, FRR 795, IFO 8137, IMI 039752, IMI 039752ii, LSHB P1, MUCL 29071, MUCL 29126, NRRL 795, QM 7483	French Camembert cheese	USA, Connecticut, Storrs	AY674402
P. aurantiogriseum	792.95	IBT 11325	Apple juice production plant	Denmark	AY674298
P. aurantiogriseum	642.95	IBT 11252	Chicken feed	Denmark	AY674297
P. aurantiogriseum	324.89 **T**	IBT 14016, ATCC 48920, FRR 971, IMI 195050, MUCL 29090, NRRL 971	Unknown	Belgium	AY674296
P. bialowiezense	112882	IBT 13469	Unknown	Denmark	AY674441
P. bialowiezense	227.28 **T**	IBT 23044, IMI 092237, LSHB P71	Soil under conifers	Poland, Bialowiezska Puszcza	AY674439
P. bialowiezense	110104	IBT 20786	Seaweed	Denmark, Belleviebeach	AY674440
P. brevicompactum	110067	IBT 18329	Soil under *Juniperus*	USA, New Mexico	AY674438
P. brevicompactum	480.84	IBT 21507	*Raphanus* sp.	Denmark, Lyngby	AY674434
P. brevicompactum	257.29 **T**	CBS 110071, IBT 23045, ATCC 9056, ATCC 10418, DSM 3825, FRR 862, IMI 040225, LSHB P75, MUCL 30240, MUCL 30241, MUCL 30256, MUCL 30257, MUCL 28647, MUCL 28813, MUCL 28935, NRRL 862, NRRL 863, NRRL 864, NRRL 2011, QM 7496	Unknown	Unknown	AY674437
P. brevicompactum	110068	IBT 13151, WSF 3531	Soil	USA, Wisconsin	AY674436
P. brevicompactum	110069	IBT 18098, FRR 2455	Artificial maple syrup preserved with 650 ppm benzoic acid	Australia, New South Wales, Sydney	AY674435
P. camemberti	299.48 **T**	IBT 21508, ATCC 1105, ATCC 4845, FRR 878, IMI 027831, IMI 092200, LCP 66.584, LSHB P11,MUCL 29790,NCTC 582,NRRL 877,NRRL 878	French Camembert cheese	USA, Connecticut	AY674368
P. camemberti	190.67	IBT 11755	Dutch camembert cheese	Netherlands	AY674369
P. camemberti	112078	IBT 14856	Cheese contaminant, Appenzeller	Switzerland	AY674370
P. carneum	449.78	IBT 21509	Cheddar cheese		AY674384
P. carneum	466.95	IBT 6885, ATCC 46837	Cured meat	Germany	AY674385

P. carneum	112297 **T**	IBT 6884	Mouldy rye bread	Denmark	AY674386
P. caseifulvum	101134 **T**	IBT 21510, IBT 18282	Danablue cheese	Denmark, Kirkeby, Mejeri	AY674372
P. caseifulvum	108956	IBT 19782	Cheese	Denmark	AY674371
P. cavernicola	109557	IBT 5265, FRR 1621, IFO 9341	Butter	Japan	AY674337
P. cavernicola	109556	IBT 3235	Salami	Germany	AY674338
P. cavernicola	109558	IBT 21194	Guacharo Cave	Venezuela	AY674339
P. chrysogenum	776.95	IBT 14462	Lechuguilla cave	USA, New Mexico, Carlsbad	AY495986
P. chrysogenum	775.95	IBT 5304	Air in kitchen	Denmark, Lyngby	AY495987
P. chrysogenum	478.84	IBT 21511	Air, fruit store	Denmark	AY495988
P. chrysogenum	412.69	IBT 23022, FRR 512, IMI 140340, VKM F-1078	Soil	Syria	AY495996
P. chrysogenum	306.48 **T**	IBT 5233, IMI 024314, IMI 092208, ATCC 10106, CCRC 30564, FRR 807, MUCL 29079, MUCL 29145, NRRL 807, NRRL 810, QM 7500	Cheese	USA, Connecticut	AY495981
P. clavigerum	255.94 **T**	CBS 310.48, IBT 21512, NRRL 1003	Man	Canada, Winnipeg, Mannitoba	AY674427
P. clavigerum	113244	IBT 14991, NRRL 1004	Unknown	USA	AY674429
P. clavigerum	112482	IBT 18977, UAMH 2766	Gopher hair	Canada, Cardsan	AY674428
P. commune	279.67	IBT 14135	Roquefort cheese	Netherlands	AY674361
P. commune	311.48 **T**	IBT 6200, ATCC 10428, ATCC 1111, DSM 2211, IFO 5763, IMI 039812ii, IMI 039812iii, NRRL 890, QM 1269	Cheese	USA, Connecticut, Stovis	AY674366
P. commune	115505	CCFC 214793			AY674367
P. concentricum	477.75 **T**	IBT 14571	Colon of deer	Germany, Giessen	AY674413
P. concentricum	191.88	IBT 14606	Soil	USA, South Carolina, Columbia	AY674412
P. confertum	171.87 **T**	IBT 21515, IMI 296930, NRRL A-26904, NRRL 13488	Cheek pouch of *Dipodomys spectabilis*	USA, Arizona	AY674373
P. coprobium	561.90 **T**	IBT 21516, IBT 6932	Pig-feed	Norway	AY674425
P. coprobium	280.97	IBT 15439	Barley	Denmark	AY674423
P. coprobium	562.90	IBT 6900, CCF 2005	Unknown	Czech Republic, Českomoraxská vrchovina hills	AY674424
P. coprophilum	102444	IBT 23268	Woodchip paper behind skirting board	Germany	AY674422
P. coprophilum	186.89	IBT 21517, NRRL 13627	*Andropogon sorghum*	Denmark	AY674420
P. coprophilum	110760 **T**	IBT 5551	Rabbit dung	Netherlands, Baarn	AY674421
P. crustosum	101025	IBT 21518, IBT 14747	Cheese	Portugal, Azores	AY674351
P. crustosum	471.84	IBT 6579, FRR 2929, IMI 285510	*Thymus* sp.	Denmark	AY674352
P. crustosum	115503 **T**	IBT 5528, IBT 6175, IMI 091917, FRR 1669, ATCC 52044, NCTC 4002	Lemon	Scotland, Aberdeen	AY674353
P. cyclopium	101136	IBT 11415,IBT 21519	Harness	Saudi Arabia	AY674308
P. cyclopium	477.84	IBT 5171, FRR 2935, IMI 285516	Grain of *Hordeum vulgare* (barley)	Denmark	AY674309
P. cyclopium	144.45 **T**	IBT 5130, ATCC 8731, ATHUM 2888, CECT 2264, DSM 1250, IMI 089372, LSHB P123, MUCL 15613, NRRL 1888, QM 6839, VKM F-265	Fruit	Norway	AY674310
P. digitatum	101026	IBT 21520, IBT 15179	Chili-mix	Indonesia	AY674403
P. digitatum	136.65 **T**	IBT 23020, DSM 2731	Fruit of *Citrus medica limonium*	Netherlands	AY674404
P. digitatum	115504	DAOM 226630			AY674405
P. dipodomyicola	173.87 **T**	IBT 21521, IMI 296935, NRRL A-27016, NRRL 13487	Cheek pouch of *Dipodomys spectabilis*	USA, Arizona	AY674409
P. dipodomyicola	110421	IBT 16571,RMF A-65	Soil under *Artemisia tridentata* (sage bush)	USA, Wyoming, 16 km north of Rawlins	AY674411
P. dipodomyicola	110422	IBT 19341, FRR 3866	*Oryza sativa* , fumigated with phosphine	Australia, N.S.W. Australia, Sydney, Murrumbidge irrigation area	AY674410
P. dipodomyis	170.87	IBT 21522	Cheek pouch of *Dipodomys spectabilis*	USA, Arizona	AY495989
P. dipodomyis	110413	IBT 17759	Barley	USA, Wyoming, Starr Valley	AY495990
P. dipodomyis	110412 **T**	IBT 5333,IMI 296926,NRRL A-26136,NRRL 13485	*Dipodomys spectabilis* (cheek pouch)	USA, Arizona, 6 km east of Portal	AY495991

P. dipodomyis	112578	IBT 20227	Soil, 14% salt	USA, Utah, Antelope Island, situated in Salt Lake	AY495992
P. discolor	474.84 **T**	IBT 21523	*Raphanus sativus*	Israel	AY674348
P. discolor	278.97	IBT 15145	Dairy cooling device	Denmark	AY674349
P. discolor	271.97	IBT 11512	Acorn	Denmark	AY674350
P. echinulatum	101027	IBT 21524, IBT 12879	Air	Denmark, Hjorring	AY674342
P. echinulatum	317.48 **T**	IBT 6294, ATCC 10434, FRR 1151, IFO 7760, IMI 040028, MUCL 15615, NRRL 1151, QM 7519	Culture contaminant	Canada, Ontario, Ottawa	AY674341
P. echinulatum	337.59	IBT 3232, ATCC 18487, FAT 1019, FRR 637, IFO 6233, IMI 068236, QM 7304	Soil	Japan	AY674340
P. expansum	481.84	IBT 21525	*Brassica oleracea*, 'Brussels sprouts'	Denmark	AY674399
P. expansum	281.97	IBT 15598	Chilled food	Denmark	AY674401
P. expansum	325.48 **T**	IBT 3486, IBT 5101, IBT 5854, IMI 039761ii, ATCC 7861, ATUM 2891, FRR 976, MUCL 29192, NRRL 976, VKM F-275	*Malus sylvestris*	USA	AY674400
P. flavigenum	419.89 **T**	IBT 21526, IBT V1035	Flour	Denmark, Lyngby	AY495993
P. flavigenum	110406	IBT 16616, RMF A58	Soil under *Chrysothmnus nauseosus*	USA, Wyoming, Table rock road/highway 80	AY495994
P. flavigenum	110407	IBT 14060	White beans	USA	AY495995
P. formosanum	211.92 **T**	IBT 21527, IBT 19748	Soil	Taiwan	AY674426
P. freii	794.95	IBT 11273	Chicken feed (cereal)	Denmark	AY674290
P. freii	101486	IBT 11996, CSIR 1876	Barley	South Africa	AY674291
P. freii	112292	IBT 10107, NRRL 5547	Barley	Denmark	AY674292
P. gladioli	332.48 **T**	IBT 14772, ATCC 10448, FRR 939, IMI 034911, IMI 034911ii, LCP 89.202, MUCL 29174, NRRL 939, QM 1955	Corm of *Gladiolus* sp. imported from the Netherlands	USA, District of Columbia, Washington DC	AY674287
P. gladioli	278.47	IBT 14773, ATCC 9437, DSM 2436, IFO 5766, IMI 038567, IMI 038567ii, LSHB P251, LSHB Ad65, NCTC 3994, NRRL 938, QM 6756	Corm of *Gladiolus* sp.	UK, England, Cambridge	AY674288
P. gladioli	815.70	IBT 21528, IBT 14769	Corn of *Gladiolus* sp.	India	AY674289
P. glandicola	333.48	IBT 6592, ATCC 10450, FRR 2036, IMI 040220, MUCL 15621, NRRL 2036, QM 6868	Soil	USA, Illinois, Peoria	AY674416
P. glandicola	498.75 **T**	IBT 21529, IMI 154241	Mouldy wine cork	Portugal	AY674415
P. glandicola	111218	IBT 3291, IMI 297543	Soil	Switzerland, Zürich	AY674414
P. griseofulvum	485.84	IBT 21530	Grain of *Hordeum vulgare*	Denmark, Kalundborg	AY674430
P. griseofulvum	110420	IBT 14319, IMI 351308	*Zea mays* seed	Bulgaria, Vratsa region	AY674431
P. griseofulvum	185.27 **T**	IBT 6740, ATCC 11885, IMI 075832, ATHUM 2893, CECT 2605, DSM 896, IFO 7640, IFO 7641, LCP 79.3245, MUCL 28643, NRRL 2152, NRRL 2300, QM 6902, VKM F-286	Unknown	Belgium	AY674432
P. hirsutum	135.41 **T**	IBT 21531, ATCC 10429, FRR 2032, IFO 6092, IMI 040213, MUCL 15622, NRRL 2032	Aphid, green fly	Netherlands, Baarn	AY674328
P. hirsutum	349.75	IBT 12398, PD 73/1274	Bulb of *Tulipa* sp.	Netherlands	AY674329
P. hirsutum	110100	IBT 10624,NRRL 999	Root of horse raddish	USA, Illinois	AY674330
P. hordei	704.68	IBT 23023	Grain of Hordeum vulgare	Netherlands, Baarn	AY674346
P. hordei	788.70	IBT 23024, IMI 197487	Cereal	United Kingdom	AY674345
P. hordei	701.68 **T**	IBT 17804, IBT 6980, IMI 151748, ATCC 22053, CECT 2290, FRR 815, MUCL 39559	*Hordeum vulgare*	Denmark	AY674347
P. italicum	489.84	IBT 21533	*Raphanus sativus*	Israel, imported to Denmark	AY674396
P. italicum	339.48 **T**	IBT 23029, ATCC 10454, DSM 2754, FRR 983, IMI 039760, MUCL 15608, NRRL 983, QM 7572	Fruit of *Citrus* sp.	USA, California, Riverside, Citrus Experiment Station	AY674398
P. italicum	278.58	IBT 23030, DSM 2428	Fruit of *Citrus sinensis*	Netherlands, Baarn	AY674397
P. marinum	109547	IBT 16713	Sandy soil, mutant of 109549	Tunisia	AY674390
P. marinum	109549	IBT 16712	Sandy soil	Tunisia	AY674391

P. marinum	109550 **T**	IBT 14360	Sandy soil	Japan	AY674392
P. melanoconidium	640.95	IBT 10031	*Panicum miliaceum* imported to Denmark	Unknown	AY674303
P. melanoconidium	218.90	IBT 3443	*Hordeum vulgare*	Denmark, Naestved	AY674302
P. melanoconidium	115506 **T**	IBT 3444, IMI 321503	Wheat	Denmark	AY674304
P. mononematosum	172.87 **T**	IBT 21535, IMI 296925, NRRL 13482	Heavily moulded seed of *Amaranthus* sp.	USA, Arizona, 6 km E of Portal	AY495997
P. mononematosum	112104	IBT 3073, IBT 6071, IBT 5521, IBT 5522, NRRL A26910	Kangaroo rat	USA, Arizona, 8 km east of Portal	AY495998
P. nalgiovense	352.48 **T**	IBT 21536, ATCC 10472, CCF 1728, CCRC 31671, DSM 897, FRR 911, IFO 8112, IMI 039804, MUCL 31194, NRRL 911, QM 7600	Ellischauer cheese	Czechoslovakia	AY495999
P. nalgiovense	318.92	IBT 12383	Sausage, imported from Italy	Denmark	AY496000
P. neoechinulatum	169.87 **T**	CBS 101135, IBT 21537, IBT 3493, NRRL A-26897	Cheek pouch of *Dipodomys spectabilis*	USA, Arizona, 8 km east of Portal	AY674301
P. neoechinulatum	101135	CBS 169.87, IBT 21537, IBT 3493, NRRL A-26897	Cheek pouch of *Dipodomys spectabilis*	USA, Arizona, 8 km east of Portal	AY674299
P. neoechinulatum	110343	IBT 5595, NRRL A-26842	Seed cache, *Dipodomys spectabilis*	USA, Arizona, 6 km east of Portal	AY674300
P. nordicum	112573	IBT 5105,IBT 4736,NRRL 5574	Salami	Italy	AY674317
P. nordicum	110770	IBT 4734,IBT 6728	Sausage	Germany	AY674318
P. nordicum	606.68	IBT 14172	Chicken meat	Germany, former FRG	AY674319
P. nordicum	109541	IBT 6573	Lumpsucker (*Cycloptenus lumpus* eggs)	Denmark	AY674316
P. nordicum	109538	IBT 22528	Fish feed	Denmark	AY674314
P. nordicum	109537	IBT 22532	Jam	Japan	AY674315
P. olsonii	833.88	IBT 21538	Cactus pot soil	Denmark, Lyngby	AY674442
P. olsonii	381.75	IBT 4523, PD 750211/1	*Fragaria* sp.	Netherlands	AY674444
P. olsonii	349.61	IBT 23032	Rubber life-raft	Netherlands	AY674443
P. olsonii	232.60 **T**	IBT 23473, IMI 192502, FRR 432	Root of *Picea* sp.	Austria	AY674445
P. palitans	491.84	IBT 6355, FRR 2948, IMI 285531	Mouldy liver paste	Denmark, Holbeck	AY674363
P. palitans	101031	IBT 21540, IBT 14740	Cocoa	Japan	AY674362
P. palitans	115507	IBT 14741	Unknown	Japan	AY674365
P. palitans	112204	IBT 13421, VKM F-478	Unknown	Russia	AY674364
P. paneum	101032	IBT 21541, IBT 12407	Mouldy rye bread	Denmark	AY674387
P. paneum	464.95	IBT 11839	Rye bread (non preserved)	Denmark, Odense	AY674389
P. paneum	465.95 **T**	IBT 13929	Mouldy baker's yeast	Denmark, Vangede	AY674388
P. persicinum	111235 **T**	IBT 24565, AS3.5891	Soil	China, Qinghai Province	AY495982
P. polonicum	101479	IBT 14320, IMI 351304	Foods	Bulgaria, Vratza region	AY674306
P. polonicum	690.77	IBT 6285, IJFM 3752, IMI 291200	Air	Spain, Canary Islands	AY674307
P. polonicum	222.28 **T**	IBT 12821, IMI 291194, MUCL 29204, NRRL 995	Soil	Poland	AY674305
P. radicicola	112430 **T**	IBT 10696	*Armoracia rusticana* root	Denmark	AY674357
P. radicicola	109551	IBT 22536	Soil, near waterfall by grass	Iceland, Pingveillir	AY674359
P. radicicola	109554	IBT 22526	Onion	Denmark	AY674360
P. radicicola	112425	IBT 3489	Carrot	Denmark	AY674358
P. roqueforti	479.84	IBT 21543	Mouldy baker's yeast	Denmark	AY674382
P. roqueforti	135.67	IBT 19475, MUCL 8491	Blue cheese	Germany, former FRG	AY674380
P. roqueforti	234.38	IBT 19781, IMI 291202	Blue Cheshire cheese	Unknown	AY674381
P. roqueforti	221.30 **T**	IBT 6754, IMI 024313, ATCC 10110, ATCC 1129, CECT 2905, IFO 5459, NCTC 588, NRRL 849, QM 1937	French roquefort cheese	USA	AY674383
P. sclerotigenum	101033 **T**	CBS 343.59, IBT 14346, IBT 21544, ATCC 18488, IFO 6167, IMI 68616, NRRL 3461	Rotting tuber of *Dioscorea batatas*	Japan, Myogo Pref.,Tamba Prov., Sasayama	AY674393
P. sclerotigenum	307.97	IBT 15061, IMI 361520	Blue yams	Philippines	AY674394
P. sclerotigenum	306.97	IBT 13938, IMI 267703	*Dioscori cayenensis*	Jamaica	AY674395
P. solitum	147.86	IBT 21545	Fruit of *Malus sylvestris*	Denmark	AY674355

P. solitum	424.89 **T**	CBS 288.36, IBT 3948, ATCC 9923, FRR 937, IFO 7765, IMI 039810, IMI 092225, LSHB P52, MUCL 28668, MUCL 29173, NRRL 937	Unknown	Germany	AY674354
P. solitum	146.86	IBT 23035	Fruit of *Malus sylvestris*	Denmark	AY674356
P. thymicola	111226	IBT 21560	Air of archive	Czech Republic, Moravia	AY674320
P. thymicola	111227	IBT 5254	Sorghum	Sudan	AY674322
P. thymicola	111225 **T**	IBT 5891	Thyme	Europe, South Europe	AY674321
P. tricolor	637.93	IBT 21547, IMI 357306	*Triticum aestivum*	Canada, Saskatchewan, Prudhomme	AY674312
P. tricolor	636.93	IBT 12471, DAOM 216241	*Triticum aestivum*	Canada, Saskatchewan, Hanley UGG Elevator	AY674311
P. tricolor	635.93 **T**	IBT 12493, DAOM 216240	*Triticum aestivum*	Canada, Saskatchewan, Prince Albert UGG Elevator	AY674313
P. tulipae	109555 **T**	IBT 3458	Tulip bulb	Denmark	AY674344
P. tulipae	111217	IBT 10676	Leaf of Tulip	Denmark	AY674343
P. ulaiense	262.94	IBT 21548	Grapefruit cv. Marsh	USA, California, packinghouse in Corona	AY674406
P. ulaiense	210.92 **T**	IBT 18387	Skin of decaying orange	Taiwan, Ulai	AY674408
P. ulaiense	314.97	IBT 13258	Apricot	Unknown	AY674407
P. venetum	405.92	IBT 21549	*Iris* sp.	South Korea, Suweon	AY674334
P. venetum	201.57	IBT 23039, ATCC 16025, CECT 2812, IMI 019759, MUCL 19012, QM 840	Bulb of *Hyacinthus* sp.	UK, England	AY674335
P. venetum	253.96	IBT 23040	Asparagus	Netherlands	AY674336
P. verrucosum	603.74 **T**	IBT 4733, IBT 12809, ATCC 48957, ATHUM 2897, CECT 2906, FRR 965, IMI 200310, MUCL 28674, MUCL 29186, NRRL 965	Unknown	Belgium, Leuven	AY674323
P. verrucosum	115508	CCFC 213195			AY674324
P. viridicatum	101034	IBT 21551, IBT 15053	Beans	Bulgaria, Hubavene	AY674293
P. viridicatum	390.48 **T**	IBT 23041, ATCC 10515, IFO 7736, IMI 039758, NRRL 963, QM 7683	Air	USA, District of Columbia, Washington D.C.	AY674295
P. viridicatum	109826	IBT 14246, IMI 351305	*Zea mays*	Bulgaria, Viatsa	AY674294
P. vulpinum	101133	IBT 11932, IBT 21552	Melon		AY674417
P. vulpinum	305.63	IBT 3228	Insect	Netherlands	AY674418
P. vulpinum	110772	IBT 19370, IMI 300363	Soil	India, Meghalaya	AY674419

Alignments of the partial β-tubulin gene sequences data were calculated using the software package BioNumerics (Applied Maths BVBA, Sint Martens-Latem, Belgium) and manual adjustments were made by eye to maximize homology.

The phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000). Alignment gaps were treated as a fifth character state, missing data were identified by '?', uninformative characters were excluded and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with simple taxon additions using the first taxon in the alignment as reference. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Other statistics including tree length, consistency index, retention index, rescaled consistency index, and homoplasy index (CI, RI, RC, HI) were also calculated. Sequences were deposited at GenBank (http://www.ncbi.nlm.nih.gov) and accession numbers are given in Table 1. In addition, all sequences are available in a searchable format as part of an identification application at the CBS website, at the following URL http://www.cbs.knaw.nl/penicillium.htm

Nine separate parsimony analyses were made. The first included the type strains of all accepted species, and gave an overall impression of the phylogenetic structure of the β-tubulin gene tree. Subsequent analyses included all strains of particular sections or series of subgenus *Penicillium*, in groupings derived from the first analysis.

Constraint analyses were run to test conflicts between the proposed classification of Frisvad & Samson (2004) and the cladification suggested by the β-tubulin gene trees, using the Kishino-Hasegawa test in PAUP 4.0b10. The constraints implemented are discussed in the Results section below.

Results

The results of the maximum parsimony analyses are presented as phylograms in Figs. 1-9. Each figure represents one of the most parsimonious trees (MPTs) from each of the nine analyses, with lines in bold designating branches present in the strict consensus tree (i.e. 100%) of the MPTs. Because of the large numbers of identical or very similar sequences in some data sets, there were often many topologically equivalent trees among the MPTs, differing in the arrangement of terminal or zero-length branches. Therefore, we generally restricted heuristic searches to 5000 MPTs, to avoid saturating the computer's memory with redundant trees.

Prior to proceeding with the analyses presented here, a preliminary analysis was undertaken using an alignment including all the 180 sequences present in the complete data set. This analysis gave a similar overall species topology to Fig. 1 (results not shown). All species remained monophyletic (except as noted below for other analyses) and the relationships among them were consistent with those shown in Fig. 1. The difficulty of effectively presenting such an analysis in print form, combined with problems inherent in assessing the reliability of a computerized alignment of this size, led us to the series of analyses presented in this paper.

Several preliminary parsimony analyses were run on subsets of the data to assess the reliability of alignments. Separate analyses were run for the exons (which clearly had no alignment ambiguities) and for the introns (which tended to include areas that were more difficult to align satisfactorily). The results of these analyses (not shown here) suggested that in the data set covering the whole subgenus, many of the characters in the introns were responsible for the fine structure of the phylogram, in particular the resolution in the terminal branches. For all analyses reported here, both exons and introns were included.

A first analysis was made for the entire subgenus *Penicillium*; with each species represented by its type (or other reliably identified) strain (Fig. 1). Eight separate analyses were subsequently undertaken to examine the relationships of three or four strains per species for clades identified from the larger data set. These clades did not necessarily receive strong bootstrap support in Fig. 1, but all received 100% consensus support. For each of these analyses, the alignments were individually optimized manually to maximize homology. In most cases, the alignments were unambiguous in the smaller data sets, and separate analyses of exons and introns were unnecessary.

Table 2. Classification of species of subgenus *Penicillium* based on morphology, growth pattern, ecology, extrolites, and partial β–tubulin sequences, based on Frisvad & Samson (2004).

Section *Coronata*	
Ser. *Olsonii*	*P. bialowiezense*
	P. brevicompactum
	P. olsonii
Section *Roqueforti*	
Ser. *Roqueforti*	*P. carneum*
	P. paneum
	P. roqueforti
Section *Chrysogena*	
Ser. *Chrysogena*	*P. chrysogenum*
	P. dipodomyis
	P. flavigenum
	P. nalgiovense
Ser. *Mononematosa*	*P. confertum*
	P. mononematosum
Ser. *Persicina*	*P. persicinum*
Ser. *Aethiopica*	*P. aethiopicum*
Section *Penicillium*	
Ser. *Expansa*	*P. expansum*
	P. marinum
	P. sclerotigenum
Ser. *Claviformia*	*P. clavigerum*
	P. concentricum
	P. coprobium
	P. coprophilum
	P. formosanum
	P. glandicola
	P. vulpinum
Ser. *Urticicolae*	*P. dipodomyicola*
	P. griseofulvum
Ser. *Italica*	*P. italicum*
	P. ulaiense
Ser. *Gladioli*	*P. gladioli*
Section *Digitata*	
Ser. *Digitata*	*P. digitatum*
Section *Viridicata*	
Ser. *Viridicata*	*P. aurantiogriseum*
	P. cyclopium
	P. freii
	P. melanoconidium
	P. neoechinulatum
	P. polonicum
	P. tricolor
	P. viridicatum
Ser. *Corymbifera*	*P. albocoremium*
	P. allii
	P. hirsutum
	P. hordei
	P. radicicola
	P. tulipae
	P. venetum
Ser. *Verrucosa*	*P. nordicum*
	P. thymicola
	P. verrucosum
Ser. *Camemberti*	*P. atramentosum*
	P. camemberti
	P. caseifulvum
	P. commune
	P. crustosum
	P. palitans
Ser. *Solita*	*P. cavernicola*
	P. discolor
	P. echinulatum
	P. solitum

Section *Viridicata*

The phylogenetic analyses for sect. *Viridicata*, the most speciose section in the subgenus, were divided into three sets of species, and the results are shown in Figs. 2-4. *Penicillium gladioli* (sect. *Penicillium,* ser. *Gladioli*) was used as outgroup for these analyses based on its position in Fig. 1. The species formed a monophyletic group, and was apparently related to sect. *Viridicata* based on consensus but not bootstrap support.

Section *Viridicata* ser. *Viridicata* formed a monophyletic group in Fig.1 (86% bootstrap support), and was divided into three subclades supported by strict consensus in Fig. 2, which shows one of eight MPTs. The largest clade included *P. tricolor, P. freii, P. neoechinulatum, P. viridicatum* and *P. aurantio-griseum. Penicillium melanoconidium* formed its own clade, and the sister group relationship with the *P. polonicum/P. cyclopium* clade suggested in Fig. 2 has no bootstrap or consensus support. All but two species in the series had 100% consensus support and there was strong bootstrap support for *P. tricolor* (100%), *P. melanoconidium* (99%), *P. polonicum* (87%) and *P. cyclopium* (99%). The sequences of the three strains of *P. aurantiogriseum* (79%) were identical. This clade was basal to a poorly supported clade (65 %) including strains of three species, *P. freii*, *P. neoechinulatum* and *P. viridicatum*. All strains of *P. freii* and *P. neoechinulatum* had identical sequences. The three strains of *P. viridicatum* each had unique sequences, with the two extra strains differing from the type in two and three positions. These three strains did not form a monophyletic clade.

Fig. 3 represents one of 5000 saved MPTs. The topology of the presented phylogram suggests that ser. *Camemberti, Solita* and *Corymbifera* may be polyphyletic as currently delimited, but the back bone of the tree had weak bootstrap and consensus support. All species had 100% consensus support, except as noted below. Within ser. *Camemberti*, *P. palitans* (83%) was reasonably well-supported by bootstrap. All strains of *Penicillium camemberti* and *P. caseifulvum* had identical sequences to each other, and to the type strain of *P. commune*. Two strains of *P. commune* differed from the type by one bp substitution and thus formed a clade separate from the type. A constraint analysis enforcing the monophyly of strains identified as *P. commune* was accepted (3 steps longer than the MPTs, P=0.0832). *Penicillium crustosum*, a strongly supported species (99%) was considered a member of ser. *Camemberti* by Frisvad & Samson (2004), but based on consensus support was a part of ser. *Solita* in this analysis. The strongly supported species *P. cavernicola* (95%), considered part of sect. *Solita* by Frisvad & Samson (2004), appeared phylogenetically related to ser. *Camemberti* here, but without bootstrap or consensus support. Within ser. *Solita*, *P. echinulatum* (98%) was also a strongly supported, monophyletic species. *Penicillium discolor* and *P. solitum* both formed monophyletic groups in all MPTs, but received weak bootstrap support. These three species formed a distinct clade in the strict consensus analysis, which was weakly supported by bootstrap. Their separation of this clade from ser. *Camemberti* was apparent with consensus support.

Species assigned to ser. *Corymbifera* occurred in four different monophyletic clades, all emerging from the backbone of the strict consensus tree. All the species of ser. *Corymbifera* in this phylogram were monophyletic based on strong bootstrap and strict consensus support. *Penicillium albocoremium* and the well-supported *P. allii* (99%) together comprised a fairly well-supported monophyletic pair of sister species, on a relatively long branch. Similarly, *P. tulipae* and *P. radicicola* were a well-supported clade (93%). *Penicillium radicicola* CBS 112425 occurred basal to the main *P. radicicola* clade that included the ex-type, differing by 3 bp. The β-tubulin sequence of this strain was more similar to *P. tulipae* than to the ex-type of *P. radicicola. Penicillium hordei* forms a well-supported monopheletic clade and is the only species included in ser. *Corymbifera* by Frisvad & Samson (2004) that does not attack plant bulbs.

Constraint analyses were run to enforce the monophyly of ser. *Camemberti, Corymbifera* and *Solita* as defined by Frisvad & Samson (2004). The constraint for ser. *Camemberti* resulted in trees 3 steps longer than the unconstrained MPTs, and was accepted (P=0.0832). The *Corymbifera* constraint was identical to the consensus of the MPTs. A constraint enforcing the monophyly of ser. *Solita* was also accepted (trees 2 steps longer than the MPTs, P=0.1587).

The phylogenetic analysis of sect. *Viridicata* ser. *Verrucosa* is presented in Fig. 4, which is one of 6 MPTs. This series was monophyletic in Fig. 1 (96%) and comprised two well-supported subclades in Fig. 4. One of these was *P. thymicola* (87%). The other consisted of *P. nordicum*, paraphyletic because of a well-supported, nested monophyletic clade consisting of two strains of *P. verrucosum* (87%). Some of the strains of *P. nordicum* (CBS 109541, 109538) differed from the type of *P. nordicum* (CBS 112573) by one bp, and had identical sequences to another strain isolated from jam (CBS 109538).

Section *Roqueforti*

Section *Roqueforti* was supported with 100% bootstrap support in Figs. 1 and 5. Each of the three species in Fig. 5, the single MPT from this analysis, was well-supported by bootstrap, as was the structure of the species phylogeny. The three strains of *P. roqueforti* isolated from blue cheese (CBS 135.67, 234.38, 221.30) were clearly conspecific with a strain from mouldy bakers yeast (CBS 479.84).

The relationship between sect. *Roqueforti* and *Eupenicillium osmophilum* was supported by 72% bootstrap in Fig. 1. *Eupenicillium egypticum* was included as an outgroup in Fig. 5 based on its position in Fig. 1, but the species is not particularly close phylogenetically to sect. *Roqueforti*.

Section *Penicillium*

The phylogenetic relationships among the five sections of sect. *Penicillium* are shown in Figs. 6 and 7. Fig. 6 represents the single MPT from the analysis. Ser. *Expansa* was paraphyletic as circumscribed by Frisvad & Samson (2004), with the monophyletic ser. *Italica* (89%) derived from within it. A constraint analysis enforcing the monophyly of ser. *Expansa* resulted in an acceptable tree 3 steps longer (P=0.2630). The species of ser. *Expansa* were generally strongly (*P. marinum*, *P. sclerotigenum*, both 100%) or moderately well-supported (*P. expansum* 70%) by bootstrap. Of the two species in series *Italica, P. ulaiense* (98%) received strong bootstrap support.

The phylogeny of ser. *Urticicolae* and *Claviformia* are shown in Fig. 7, which represents one of four MPTs. In this analysis, ser. *Urticicolae* was a strongly supported monophyletic group (89%), but was derived from within the apparently paraphyletic ser. *Claviformia.* The two species of ser. *Urticicolae* were on long branches, but only *P. griseofulvum* (99%) was well-supported. There was strong strict consensus support but no bootstrap support for the backbone of this tree. A constraint analysis enforcing the monophyly of ser. *Claviformia* resulted in trees with the same number of steps as the MPTs. All of the synnematous species comprised well-supported, monophyletic groups, generally on fairly long branches. *Penicillium formosanum*, known from a single isolate, also produces synnemata and was included in ser. *Claviformia* by Frisvad & Samson (2004). Its relationship to ser. *Claviformia* was equivocal in this analysis, and the closest species in Fig. 1, *P. vulpinum*, was still rather distant.

Penicillium atramentosum was placed in ser. *Camemberti* by Frisvad and Samson (2004), based on its ability to grow on creatine as sole nitrogen source and its occurrence on cheese. However, it has a unique combination of characters (including the ability to grow at very high pH values and its inability to produce acids) that set it apart from most other terverticillate Penicillia. In Fig. 1, it was a sister species to the remaining terverticillate Penicillia (except sect. *Coronata*) with 95% bootstrap support. The species was included only in Fig. 7, where it was basal to the rest of the tree. The seven strains of *P. atramentosum* analyzed all had identical sequences (data not shown).

Section *Digitata*

Fig. 6 shows that Sect. *Digitata,* ser. *Digitata* comprised a single well-supported species, *P. digitatum*. The relationships of this species to the other sections and series of subgenus *Penicillium* were difficult to evaluate. This species is entirely unique with a series of autapomorphic phenotypic characters and few features in common with other members of subgenus *Penicillium*. All DNA sequence analyses so far place it in subgenus *Penicillium* (Peterson, 2000, this paper).

Section *Chrysogena*

Section *Chrysogena* contained two monophyletic groups according to Fig. 1, but with weak bootstrap support. One clade comprised *P. chrysogenum*, *P. flavigenum* and ser. *Mononematosa*, and had strict consensus support. The other clade included *P. dipodomyis* and *P. nalgiovense* with *P. persicinum,* and lacked consensus support. *Penicillium aethiopicum* and *P. atramentosum* were basal to most of the remaining terverticillate Penicillia. A more detailed phylogeny is shown in Fig. 8, which is one of 39 MPTs. Of the four series, three were monophyletic, but two of these included only one species. Series *Chrysogena* was apparently polyphyletic, with both ser. *Mononematosa* and ser. *Persicina* derived within it. However, a constraint analysis enforcing monophyly of ser. *Chrysogena* resulted in trees 4 steps longer than the MPTs, and was accepted (P=0.3256). Of the species included in ser. *Chrysogena*, *P. flavigenum* (96%) received high bootstrap support. A sibling species relationship between *P. dipodomyis* and *P. nalgiovense* was supported by strict consensus and bootstrap (89%). Series *Mononematosa* received 74% bootstrap support, and included two closely related species with identical sequences, *Pencillium mononematosum* and *P. confertum*. *Penicillium aethiopicum* (100%), the single species of ser. *Aethiopica*, was on a fairly long branch near the base of the tree (100% bootstrap), and also appeared to be remote from sect. *Chrysogena* in Fig. 1.

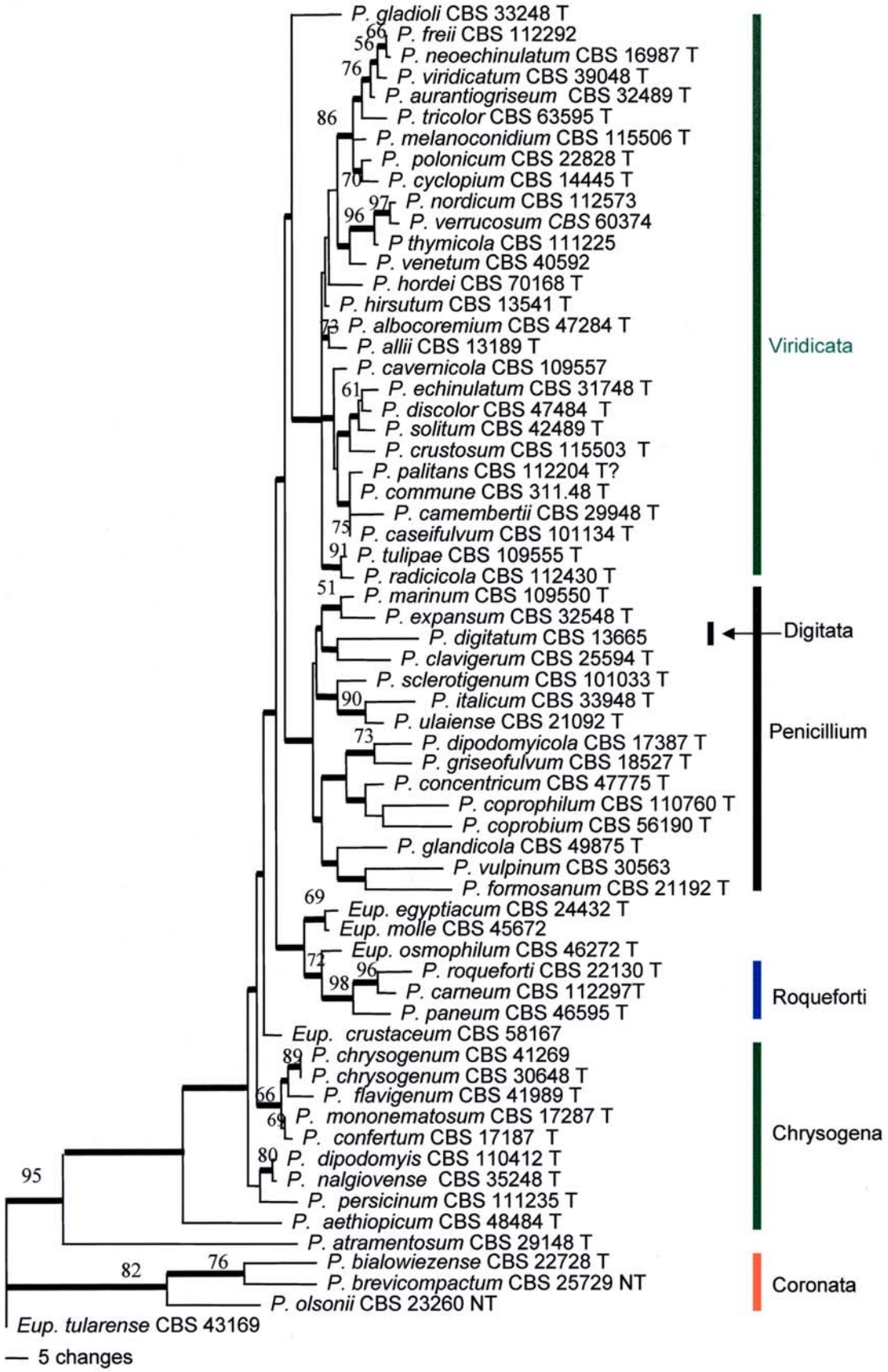

Fig. 1. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, reduced data set comprising mostly type strains. One of 5000 equally most parsimonious trees of 681steps based on a heuristic search with *Eup. tularense* as outgroup. The branches in bold occur in 100% of the equally most parsimonious trees. The numbers represent bootstrap percentages > 50 %. (CI= 0.452 RI= 0.587 RC= 0.266, HI= 0.548). Ex-type cultures are indicated with T.

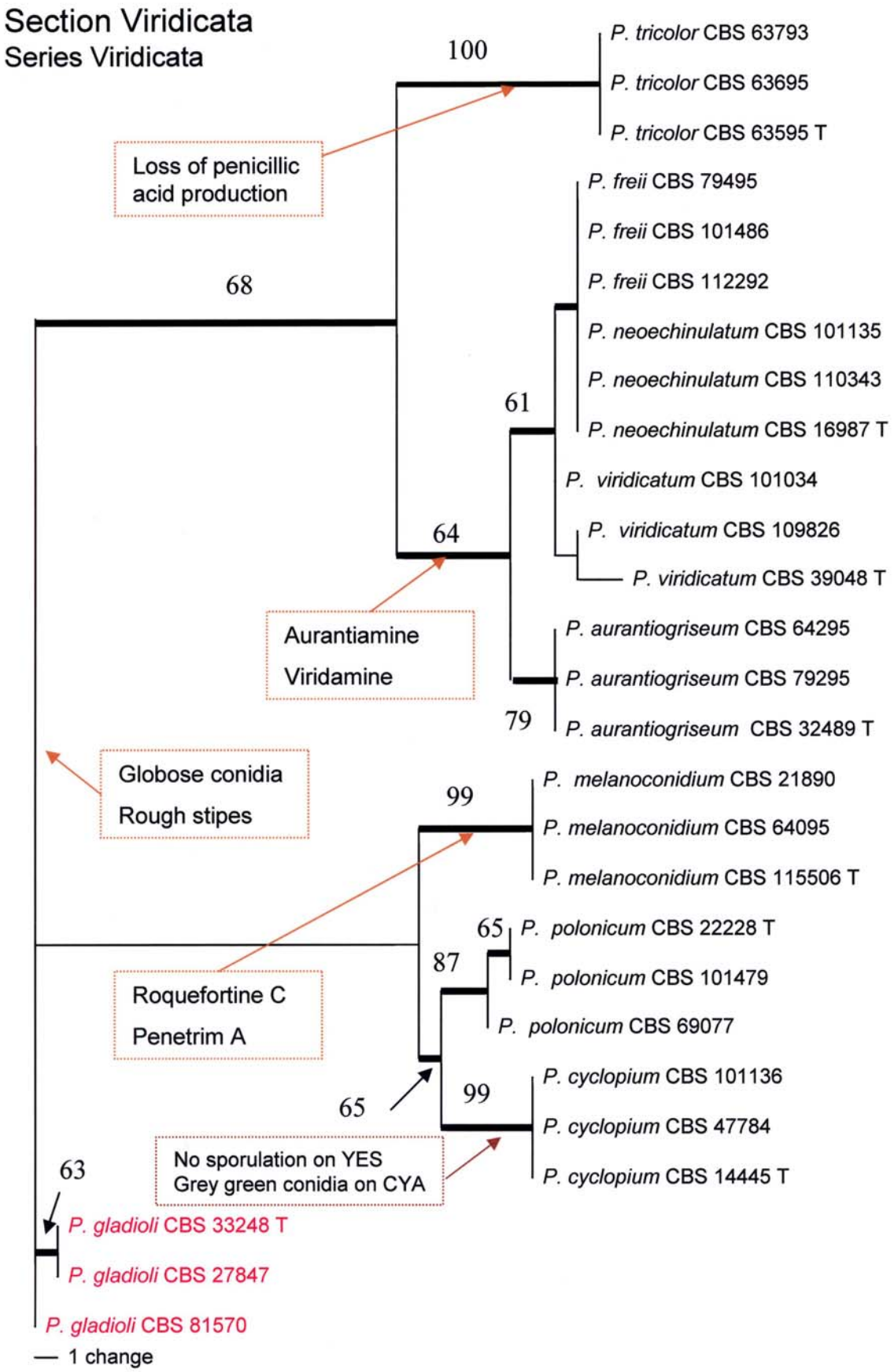

Fig. 2. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Viridicata,* ser. *Viridicata*, including all sequenced strains. One of the eight equally most parsimonious trees of 50 steps based on a heuristic search with *P. gladioli* as outgroup. The branches in bold occur in 100% of the equally most parsimonious trees. The numbers represent bootstrap percentages > 50 %. (CI= 0.900 RI= 0.967 RC= 0.870, HI= 0.100). Ex-type cultures are indicated with T.

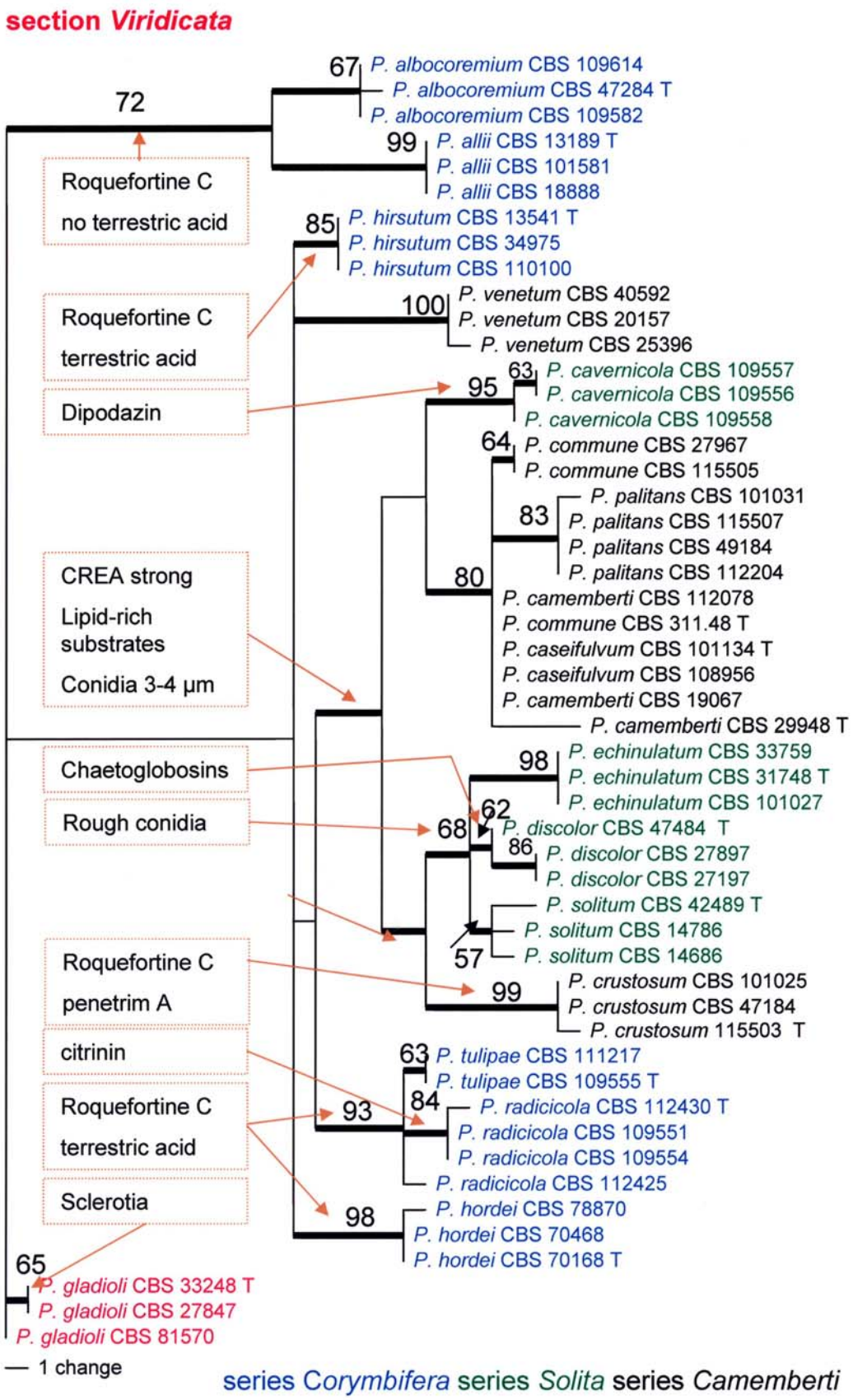

Fig. 3. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Viridicata,* ser. *Corymbifera, Camemberti,* and *Solita*, including all sequenced strains. One of 5000 equally most parsimonious trees of 95 steps based on a heuristic search with *P. gladioli* as outgroup. The branches in bold occur in 100% of the equally most parsimonious trees. The numbers represent bootstrap percentages > 50 %. (CI= 0.811 RI= 0.940 RC= 0.762, HI= 0.189). Ex-type cultures are indicated with T.

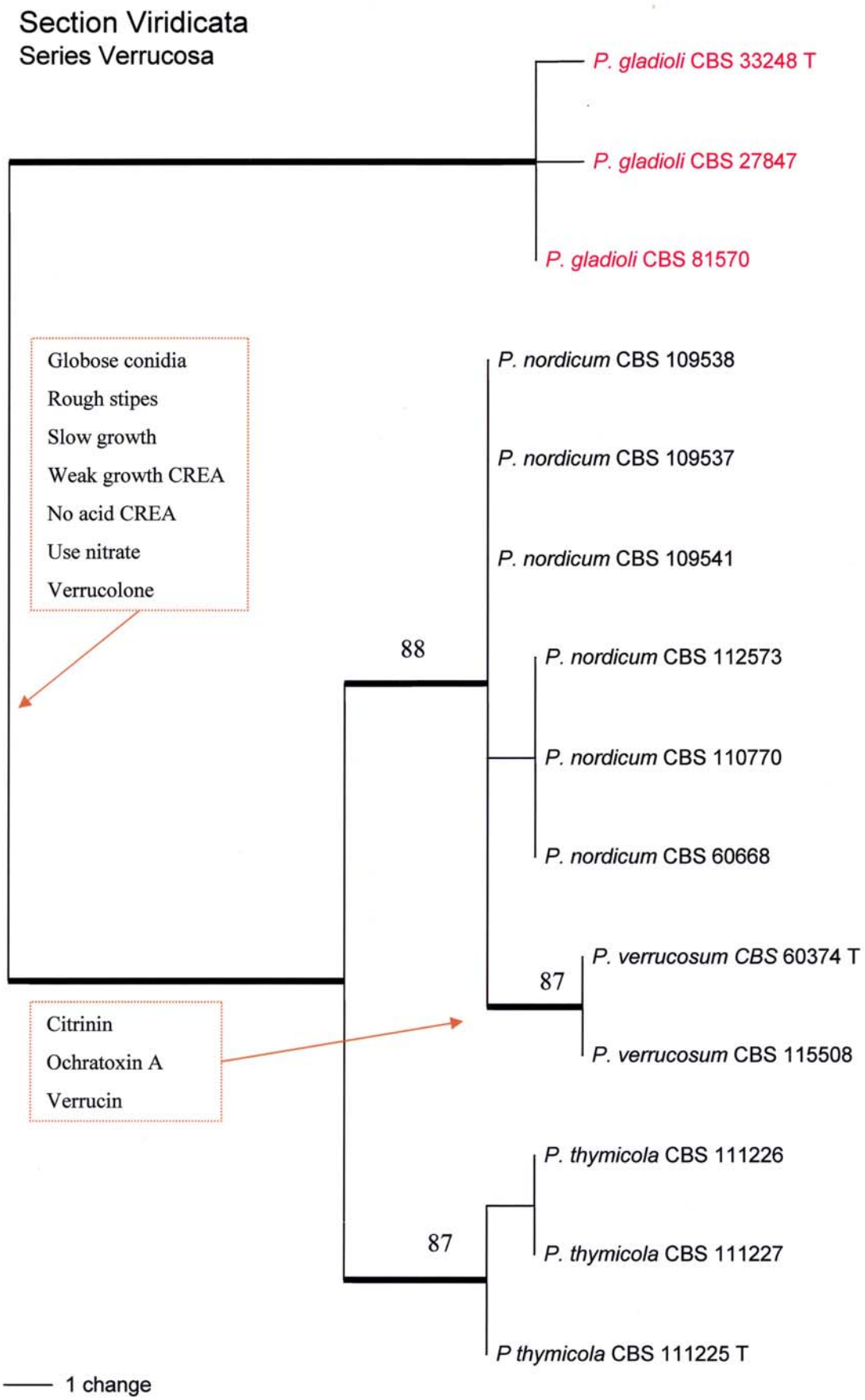

Fig. 4. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Viridicata,* ser. *Verrucosa*, including all sequenced strains. One of the six equally most parsimonious trees of 29 steps based on a heuristic search with *P. gladioli* as the outgroup. The branches in bold occur in 100% of the equally most parsimonious trees. The numbers represent bootstrap percentages > 50 %. (CI= 0.931 RI= 0.969 RC= 0.902, HI= 0.069). Ex-type cultures are indicated with T.

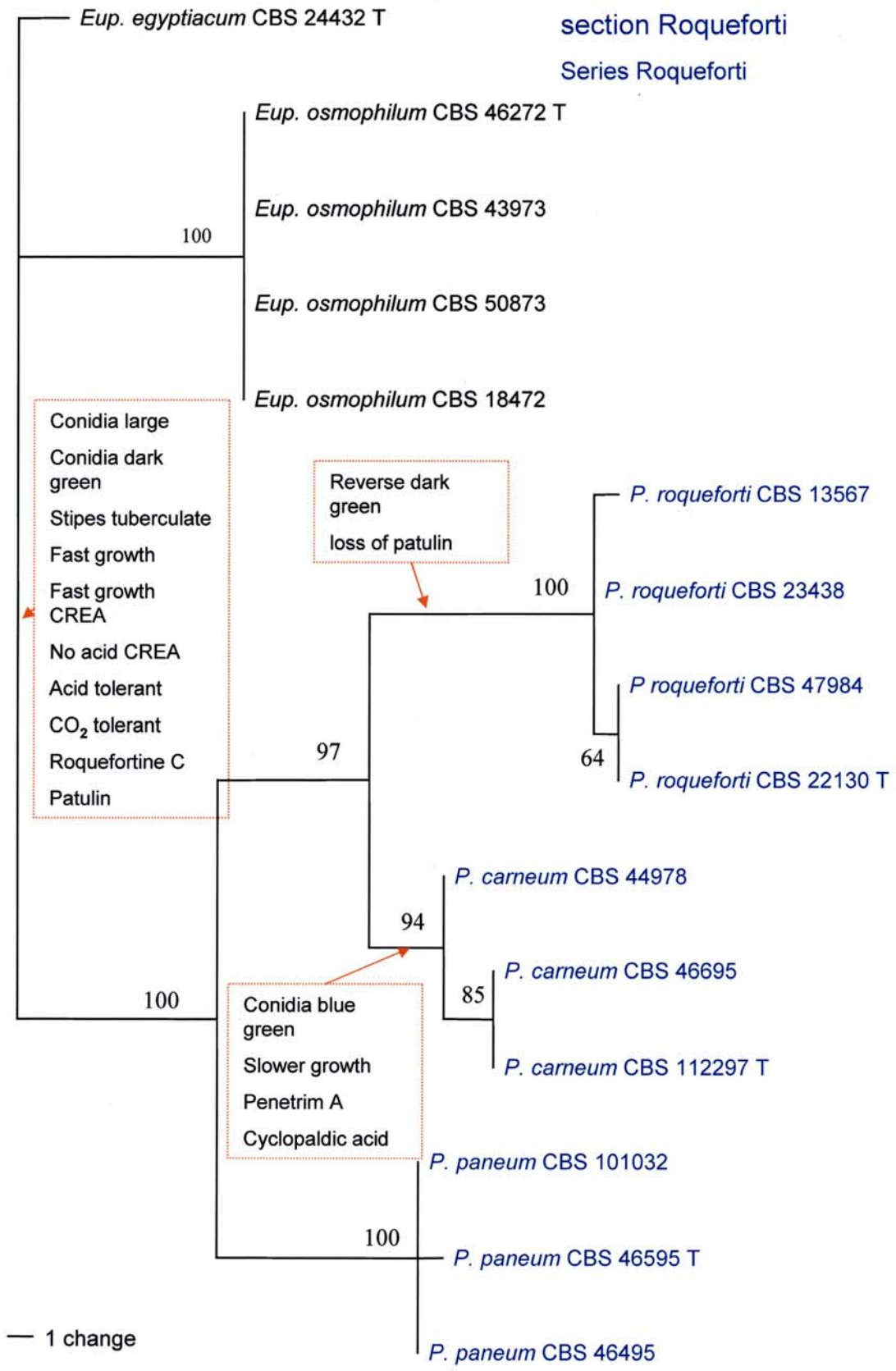

Fig. 5. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Roqueforti,* ser. *Roqueforti*, including all sequenced strains. Single most parsimonious tree of 50 steps based on a heuristic search with *Eup. egyptiacum* as the outgroup. The numbers represent bootstrap percentages > 50 %. (CI= 0.960 RI= 0.986 RC= 0.947, HI= 0.040). Ex-type cultures are indicated with T.

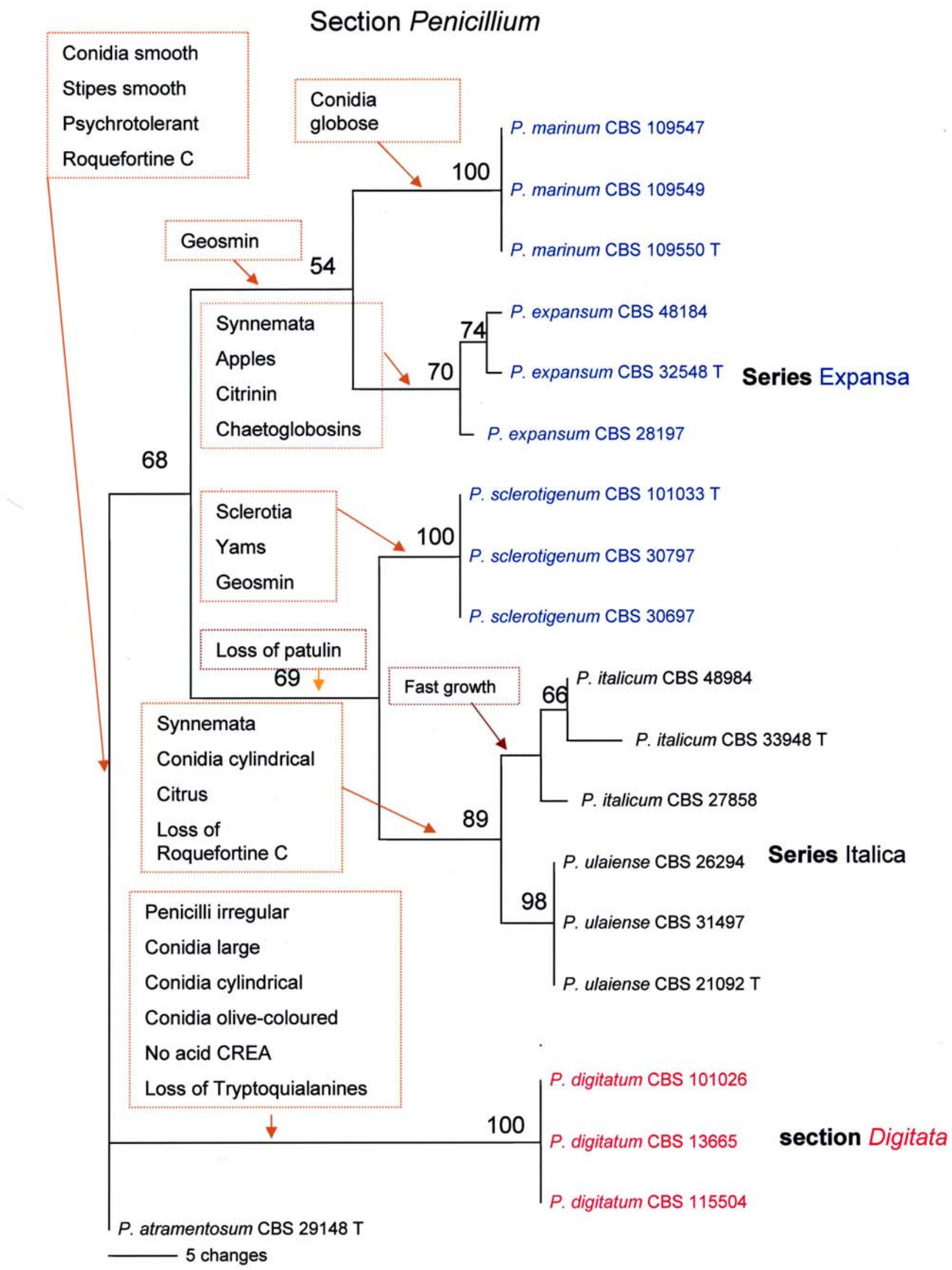

Fig. 6. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Digitata,* sect. *Penicillium*, ser. *Italica* and *Expansa,* including all sequenced strains. Single most parsimonious tree of 96 steps based on a heuristic search with *P. atramentosum* as the outgroup. The numbers represent bootstrap percentages > 50 %. (CI= 0.792 RI= 0.904 RC= 0.716, HI= 0.208). Ex-type cultures are indicated with T.

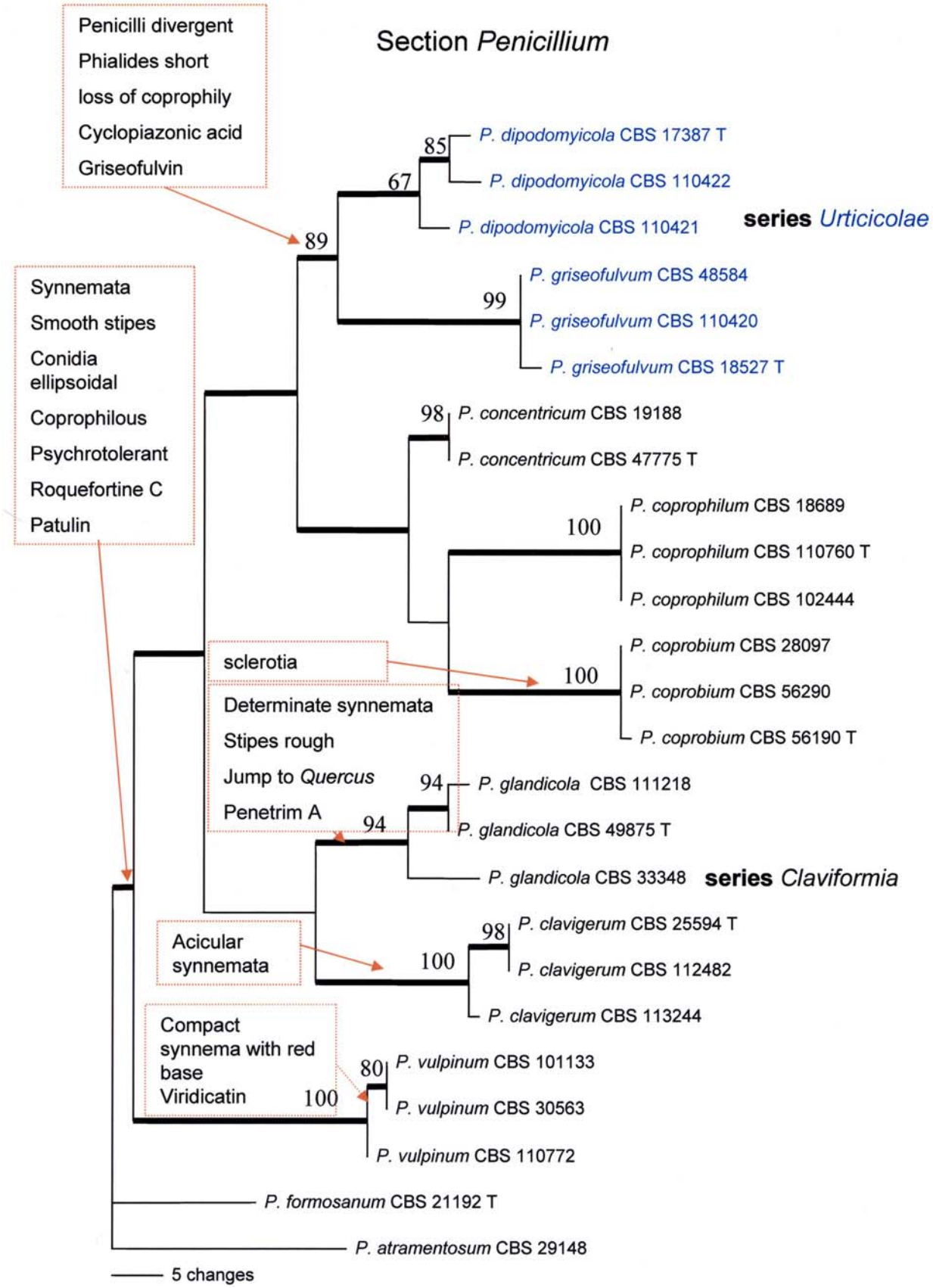

Fig. 7. β-ubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Penicillium*, ser. *Urticicolae* and *Claviformia,* including all sequenced strains. One of four equally most parsimonious trees of 195 steps based on a heuristic search with *P. atramentosum* as outgroup. The branches in bold occur in 100% of the equally most parsimonious trees. The numbers represent bootstrap percentages > 50 %. (CI= 0.687 RI= 0.841 RC= 0.578, HI= 0.313). Ex-type cultures are indicated with T.

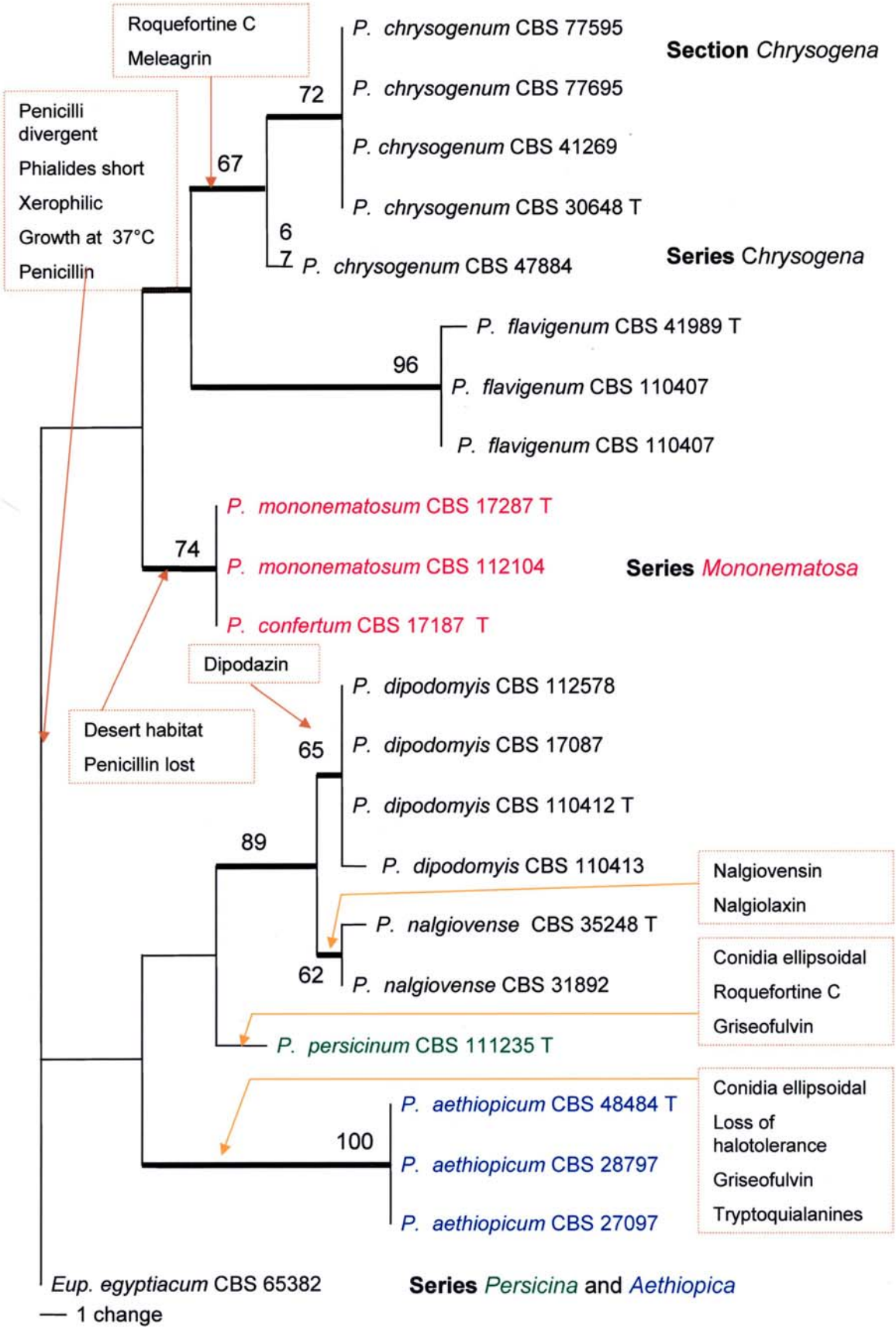

Fig. 8. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Chrysogena,* including all sequenced strains. One of the 39 equally most parsimonious trees of 44 steps based on a heuristic search with *Eup. egyptiacum* as outgroup. The branches in bold occur in 100% of the equally most parsimonious trees. The numbers represent bootstrap percentages > 50 %. (CI=0.795, RI= 0.920, RC= 0.732, HI= 0.205). Ex-type cultures are indicated with T.

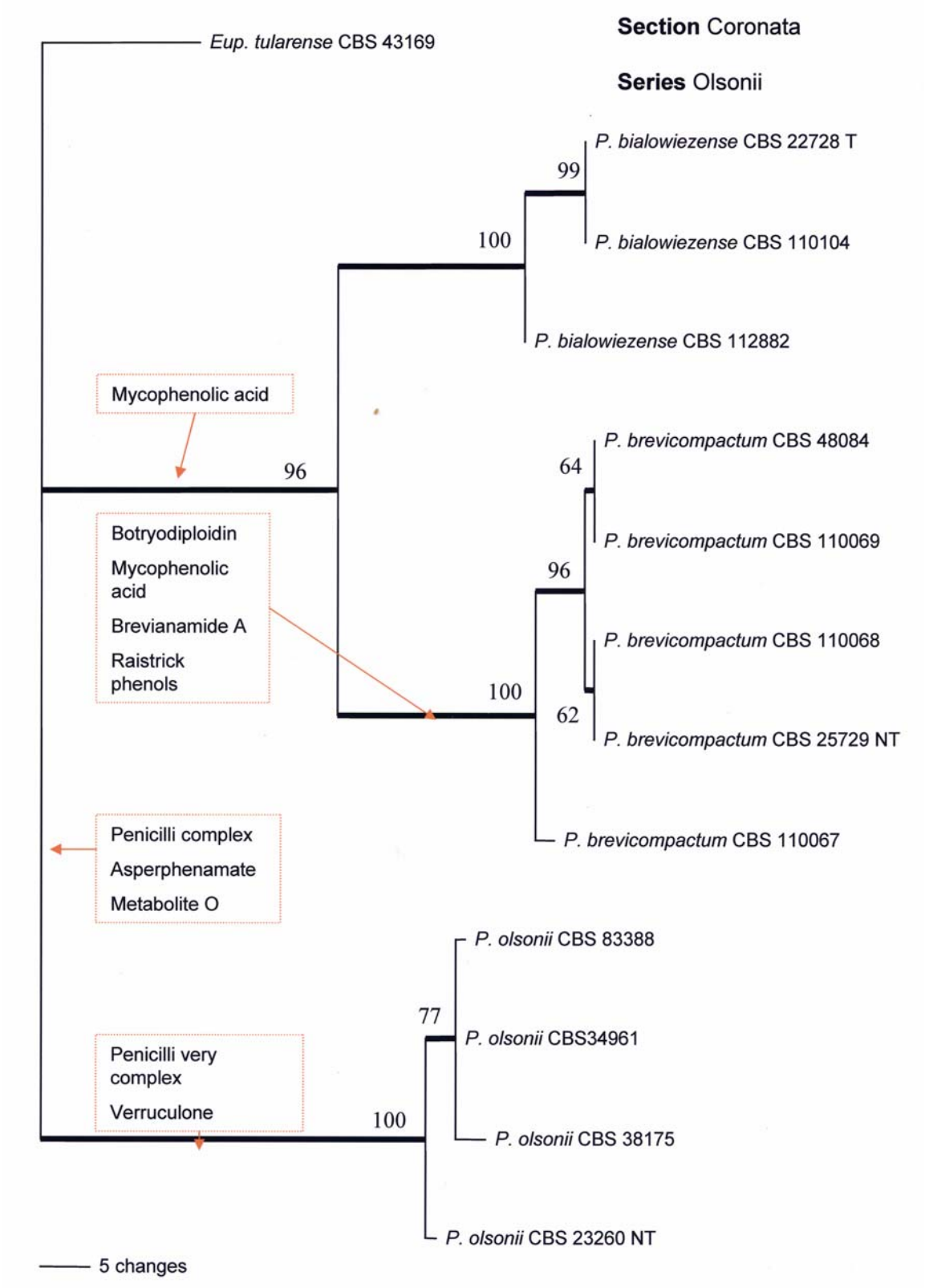

Fig. 9. β-Tubulin gene tree of *Penicillium* subgenus *Penicillium*, sect. *Coronata*, ser. *Olsonii,* including all sequenced strains. One of two equally most parsimonious trees of 147 steps based on a heuristic with *Eup. tularense* as outgroup. The branches in bold are 100 % in the 70% majority-rule consensus of equally most parsimonious trees. The numbers represent bootstrap percentages > 50 %. (CI= 0.912 RI= 0.961 RC= 0.876, HI= 0.088). Ex-type cultures are indicated with T.

Section *Coronata*

In Fig.1, sect. *Coronata* was a well-supported monophyletic group with a bootstrap support value of 82% at the base of the tree. The length of the branch leading into the section, and the length of the branches separating the species, suggested that this section and these species are generally distantly related to each other and to the other sections of subgenus *Penicillium*. The three species of sect. *Coronata* were all well-supported with 100% bootstrap support each (Fig. 9). *Penicillium olsonii* was basal in the section, with the sibling species *P. bialowiezense* and *P. brevicompactum* forming a well-supported clade (96%). The divergence among isolates of the species in this section was higher than within species of other sections.

Discussion

In this study, partial β-tubulin sequences of 180 strains representing all known species of *Penicillium* subgenus *Penicillium* were determined in order to evaluate the infra-subgeneric classification proposed by Frisvad & Samson (2004), and to assess their utility as species markers. Parsimony analysis of data sets containing representative sequences of each species were used to establish the broad phylogeny of the whole subgenus, then a series of more specific analyses were used to examine the phylogenetic structure of particular clades. In general, parsimony analysis of β-tubulin alignments provided good support for the proposed classification into sections and series, but the topology did not receive strong bootstrap support except for in some series. Where there were conflicts between the cladification suggested by the phylogeny, and the classification proposed by Frisvad & Samson (2004), constraint analyses confirmed that the topology of the most parsimonious trees (MPTs) was rather 'soft'. Enforced monophylies resulting in trees as much a 4 steps longer than the MPTs were accepted by the Kishino-Hasegawa test. Therefore, a phylogenetically robust phylogeny of subgenus *Penicillium* will require the study of other genes, or longer β-tubulin sequences. Despite this limitation, the partial β-tubulin sequences were excellent markers at the species level, providing species specific sequences for 50 of 58 species. Infrageneric cladification and species concepts are discussed at length below.

Most molecular taxonomic studies of fungi have employed parts of the ribosomal operon. In *Penicillium,* Skouboe *et al*. (1996) showed that ribosomal internal transcribed spacer (ITS) sequences were relatively invariant in subgenus *Penicillium,* with only 29 variable sites providing a poorly resolved phylogram. For example, the phenotypically distinct *P. solitum* and *P. discolor* (ser. *Solita*) had no differences in the ITS1-5.8S-ITS2 region (Skouboe *et al*., 2000). Members of ser. *Roqueforti* had species specific ITS sequences (with 13 variable sites within the complex) (Boysen *et al*., 1996). The exploration of ribosomal DNA sequences was continued by Peterson (2000), who combined ITS and partial large subunit (D1 and D2 domains) sequences. This demonstrated the infrageneric phylogenetic structure of the entire genus, allowing phylogenetic evaluation of the infrageneric taxonomy proposed by Pitt (1979). Subgenus *Penicillium,* the focus of the present study, was identified as a monophyletic group by Skouboe *et al*. (1996) and Peterson (2000). There is evidence that certain species traditionally classified in *Penicillium* subgenus *Furcatum*, including *P. lanosum* (as *P. kojigenum*), *P. raistrickii*, *P. soppii, P. swiecickii* and *P. matriti* and some Eupenicillia such as *E. crustaceum*, *E. egyptiacum*, *E. baarnense* could also be included as part of a large well-supported clade (Peterson 2000, Peterson *et al*., 2003) within subgenus *Penicillium*. Even though these species have similar branching patterns to species of *Penicillium* subgenus *Penicillium*, they were not included in the monograph of the subgenus by Frisvad & Samson (2004) and we have not included them in the present molecular study. In addition these species show different extrolite patterns.

These analyses were the basis for considering subgenus *Penicillium* a monophyletic group in this study, and provided the springboard for the β-tubulin analysis presented here. Preliminary studies by Seifert & Louis-Seize (2000) of some species here included in sect. *Viridicata* demonstrated the utility of β-tubulin sequences for revealing phylogenetic relationships and providing molecular support for phenotypically based species concepts. In general, the overall relationships suggested by the ITS were similar to those suggested by β-tubulin, but the β-tubulin analyses had more resolution in the terminal branches. Even though there was little bootstrap support for most species in the ITS study by Skouboe *et al.* (1996, 2000) and the rDNA study by Peterson (2000), the small sequence differences found were in agreement with our results in most cases.

Since its initial use in phylogenetic studies of *Epichloe* (Schardl *et al.,* 1997), β-tubulin sequences have been used as putative species markers or for phylogenetic analysis in a variety of ascomycete and hyphomycete genera, including *Neocosmospora* and *Gibberella* (anamorphs *Fusarium,* O'Donnell *et al*. 1998, 2004), *Calonectria* (anamorphs *Cylindrocladium*, Schoch *et al*., 2001), *Neonectria* (anamorphs *Cylindrocarpon*, Seifert *et al*. 2003), *Bionectria* (anamorphs *Clonostachys*, Schroers, 2001), *Phaeoacremonium* (Dupont *et al*., 2002), *Botryosphaeria* (Slippers *et al*., 2004), *Ophiostoma* (various anamorphs, Jacobs and Kirisits, 2003), *Stachybotrys* (Andersen *et al*., 2003), *Aspergillus* (Geiser *et al*., 1998; Peterson 2001), *Ascochyta*

(Fatehi *et al*., 2003), *Sphaerophorus* (Hognabba and Wedin, 2003), *Pseudocercospora* (Beilharz and Cunnington, 2003), and *Parmelia* (Molina *et al*., 2004). Despite its demonstrated utility in these genera, problems with multiple gene copies and failure of primers in some groups restrict its applicability. Furthermore, the sometimes highly variable introns at the 5' end of the most frequently sequenced part of the β-tubulin gene can make alignment even across a genus a serious challenge for a computer, resulting in an alignment almost impossible to evaluate using the human eye. Fortunately, our data set for subgenus *Penicillium* is free of paralogs, and the taxon is sufficiently homogenous that reasonable alignments could be assembled with confidence.

The β-tubulin sequences generated in this study were intended to serve two functions. First, we hoped to find phylogenetic support for the phenotypically based classification of sections and series proposed by Frisvad & Samson (2004). Second, we wanted to derive DNA sequence data that would complement the species concepts suggested by phenotypic data, and facilitate species discovery and identification, and eventually the development of molecular diagnostics. The cladification presented in Figs. 1-9 is generally complimentary to the classification of species into sections and series in subgenus *Penicillium* proposed by Frisvad & Samson (2004) (Table 2) and is discussed in detail below.

The limitations of a single gene phylogeny for producing conclusive evidence for a well-resolved and supported phylogeny are clear in our analysis. Our decision to emphasize collecting sequence data from a large set of strains (180) required that we focus on a single gene. Our phylograms sometimes lack satisfactory bootstrap support for clades representing sections or series in the Frisvad & Samson (2004) classification. Questions of monophyly of these higher taxa cannot be conclusively settled because of these limitations. The constraint analyses we ran to test the monophyly of groups that were in conflict between the classification and the cladification did not reject these alternative hypotheses. Sequencing and analysis of additional genes, such as calmodulin (Peterson 2001, 2004), elongation factor 1-alpha (Peterson 2004), and ribosomal polymerase B2 (Seifert *et al*., unpublished), should be explored. The recent assertion by Rokas *et al*. (2003) that analysis of as many as thirty genes may be necessary to obtain a fully resolved, completely supported phylogeny of a group of eight yeast species is certainly sobering in the context of a speciose taxon like subgenus *Penicillium*.

Analysis of sections and series

The taxonomic classification of the largest section of subgenus *Penicillium*, sect. *Viridicata* was generally supported. Three series were monophyletic, and the monophyly of ser. *Camemberti, Solita* and *Corymbifera* were not evident in the most parsimonious trees, but not rejected by constraint analyses (Figs. 1-4). Even for those clades lacking strong bootstrap support, we identified several phenotypic characters that are consistent with the cladification. The phylogram for the mostly grain- or seed-borne species in ser. *Viridicata* was similar to that for many of the same species presented by Seifert & Louis-Seize (2000). The addition of *P. tricolor* to the data set confirms its phylogenetic position in this series. Seifert & Louis-Seize (2000) did not include this species in their analysis because only unalignable β-tubulin paralogs were amplified and sequenced at that time.

The lipid- and protein-loving species in ser. *Camemberti* and *Solita* have several phenotypic characters (e.g. strong growth on CREA, large conidia) that support the branch that distinguishes them from the other series (Fig. 3). However, both series as defined by Frisvad & Samson (2004) include species whose classification could not be confirmed or rejected by the β-tubulin gene tree. *Penicillium cavernicola*, included by Frisvad & Samson (2004) in ser. *Solita,* appeared more closely related to ser. *Camemberti* in Fig. 3, but there was no bootstrap or strict consensus support for this alternative classification. Based on strict consensus but not bootstrap support, *P. crustosum* was closer to ser. *Solita* than its proposed classification in ser. *Camemberti*, but constraint analyses suggested that either relationship was possible. Earlier studies using rDNA sequences indicated that *P. crustosum* was closely related to *P. camemberti* and *P. commune* (Skouboe *et al*., 1996, 2000; Peterson, 2000). The production of viridicatins and ability to rot apples is shared by *P. solitum* and *P. crustosum*, but *P. crustosum* also has phenotypic similarities to *P. expansum* (growth rate, apple rot).

As delimited by Frisvad & Samson (2004), ser. *Corymbifera* appeared phylogenetically dispersed in the β-tubulin analysis. This series, united by the tendency of the species to attack plant bulbs and formation of feathery synnemata, was also differentiated from the other series by its smaller conidia and weak growth on CREA. However, the results suggesting polyphyly for this series were not particularly strong. A more focussed multigene phylogeny may be necessary to conclusively prove or reject the monophyly of this series.

Section *Roqueforti* ser. *Roqueforti* was defined by a long list of phenotypic characters and had a completely resolved, well-supported species phylogeny (bootstrap support, 98%, Fig. 1, see also Fig. 5). The β-tubulin analysis was consistent with the ITS analysis of the same group of species by Skouboe *et al.* (1996) and Boysen *et al*. (1996). According to Fig. 1, ser. *Roqueforti* is a sister group to *Eupenicillium osmophilum*, with which it shares few

phenotypic characters except the ability, widespread in the subgenus, to produce roquefortine C. Given the phenotypic similarities to species in ser. *Urticicolae*, *Expansa* and *Claviformia*, ser. *Roqueforti* may eventually be shown to be more closely related to these series. Most of these species are able to grow on creatine as the sole nitrogen source, and produce patulin and roquefortine C. However, the coprophilous species in ser. *Claviformia* compete well in this alkaline habitat and grow along with the associated alkali-tolerant bacteria. In contrast, species in ser. *Roqueforti* appear to have co-evolved with lactic acid bacteria and tolerate lactic acid bacteria (and yeast) products such as lactic acid, carbon dioxide and ethanol.

The phylogeny suggested for sect. *Penicillium* provided good support for the species recognized by Frisvad & Samson (2004), but variable support for the proposed infra-section classification (Figs. 6, 7). One problem was the position of *P. sclerotigenum*, which was classified in subgenus *Furcatum* by Pitt (1979). This species has some affinities with ser. *Expansa* by its high growth rate, plant rot, production of roquefortine C and patulin, and was therefore placed by Frisvad & Samson (2004) in series *Expansa.* According to the β-tubulin phylogeny, however, it may be more appropriately classified with the citrus-loving Penicillia *P. italicum* and *P. ulaiensis*. Despite this, *P. sclerotigenum* shares very few phenotypic features with the species included in ser. *Italica*.

Series *Claviformia* comprises mostly synnematous species, many of which occur on dung. In Fig. 7, ser. *Claviformia* appeared to be paraphyletic with ser. *Urticicolae* derived within it, although this was strongly supported by strict consensus but not bootstrap. However, the phylogeny supports some of the ecological hypotheses of Frisvad (1998). Perhaps the primarily soil-borne species *P. griseofulvum* was derived from coprophilous fungi associated with rodent dung. Its closest relative, *P. dipodomyicola*, is found primarily in seed caches of desert rats. Similarly, perhaps *P. glandicola*, generally associated with *Quercus* (especially acorns), was derived from a fungus that originally lived on the pellets of a rodent collecting these seeds.

The phylogenetic structure of sect. *Chrysogena* was disrupted by ser. *Mononematosa* and *Persicina*, which rendered ser. *Chrysogena* polyphyletic (Fig. 8). The backbone of this phylogram, which represents an ecologically and phenotypically distinctive group of species, was not well-supported by bootstrap analysis although strict consensus support was relatively strong. Isozyme data indicated that the four species in ser. *Chrysogena* are closely related (Banke *et al*., 1997), with *P. confertum* a possible outgroup. Isozyme analyses suggested close relationships between *P. chrysogenum* and *P. flavigenum,* and *P. dipodomyis* and *P. nalgiovense*, which were confirmed by the β-tubulin data presented here and extrolite profiles. While *P. confertum* shares the production of roquefortine C, meleagrin and secalonic acid with *P. chrysogenum* and *P. flavigenum*, *P. mononematosum* has no extrolites in common with any species of ser. *Chrysogena* (Frisvad and Samson, 2004). *Penicillium confertum* and *P. mononematosum* should be examined to determine whether they produce penicillin, which is common to all species in ser. *Chrysogena*.

Section *Coronata* was the phylogenetically most distant section included in subgenus *Penicillium*, and includes species that are themselves also phylogenetically rather distant from one another (Fig. 9). The position of this group of species at the base of subgenus *Penicillium* was clear in the ITS-D1/D2 analysis by Peterson (2000). However, the structure of this clade suggested either that several species remain undiscovered, or the possibility that it might be pulled into subgenus *Penicillium* by long branch attraction. Perhaps the discovery and phylogenetic analysis of additional taxa will demonstrate that sect. *Coronata* should be classified with another subgenus, or be regarded as a subgenus on its own.

Species concepts

The β-tubulin sequence analysis provided excellent support for the species concepts adopted by Frisvad & Samson (2004), with a few exceptions. The species that were problematic were:

a) *P. freii* and *P. neoechinulatum*, which had identical sequences (Fig. 2);

b) *P. viridicatum*, which despite some sequence differences among its strains, was not phylogenetically distinct from *P. freii/P. neoechinulatum* (Fig. 2);

c) *P. camemberti* and *P. caseifulvum*, which had identical sequences (Fig. 3);

d) *P. commune*, which had strains that were either paraphyletic with or identical to *P. camemberti* and *P. caseifulvum* (Fig. 3);

e) *P. nordicum*, which was paraphyletic because *P. verrucosum* was derived within it, and

f) *P. confertum,* represented by one strain with an identical sequence to the two strains of *P. mononematosum* (Fig. 8).

The three species *P. freii*, *P. neoechinulatum* and *P. viridicatum* are easily separated by conidium ornamentation, conidium colour and extrolite profiles (Frisvad & Samson 2004). Additional similarities are the production of the closely related diketopiperazines extrolites aurantiamine and viridamine. A synonymy of these three species cannot be considered based on the available data and it is clear that β-tubulin lacks sufficient resolution to support the phenotype-based classification. More genes should be sequenced in order to determine whether a DNA-based

phylogenetic species concept can be applied to these species.

The lack of consistent sequence differences between *P. camemberti* and *P. caseifulvum* could be a reflection of the hypothesis that they are two domesticated derivatives of *P. commune* (Polonelli *et al.* 1987). However, they differ in macroscopic morphology and extrolite profiles and can be regarded as distinct species. Perhaps *P. caseifulvum* has a silent gene cluster for cyclopiazonic acid, a characteristic metabolite of *P. camemberti,* but this extrolite has never been detected in that species.

The few sequence differences between *P. verrucosum* and *P. nordicum* were in agreement with the results of Castella *et al*. (2002), who also found few sequence differences based on ITS1-5.8S-ITS2. However, they found a clear distinction between *P. verrucosum* and *P. nordicum* based on RAPD and AFLP patterns. *Penicillium nordicum* itself presents an interesting situation for additional work. Originally, Frisvad & Samson (2004) had considered the possibility that the two variants evident in Fig. 4 were different species. One variant of *P. nordicum* was mostly found on salted lumpfish roe and produced the extrolite lumpidin (Larsen *et al*., 2001a, b). Multigene analysis will be necessary to determine whether this possibility warrants further consideration.

Only one strain of *P. confertum* is available, but it differs fom the related *P. mononematosum* by its less complicated penicilli and thin often sinuous conidophore stipes. Furthermore it produces asteltoxin and meleagrin which are absent in *P. mononematosum.*

Apart from these examples, all other species were monophyletic in our phylogenetic analyses. Branch lengths leading into the species were variable in length, from 1-40 bp substitutions or indels, being particularly short in some taxa (eg. sect. *Viridicata*, Fig. 2-4), where the longest branches were about 5 bp leading into *P. venetum* and *P. allii*), to very long in sect. *Coronata* (Fig. 8). In general, the longer supporting branches also had highest bootstrap support.

With sampling usually limited to three or four strains per species, only preliminary observations can be made on infraspecific variation in the β-tubulin allele with a species. Many species were invariant, including *P. aethiopicum*, *P. allii, P. aurantiogriseum, P. concentricum, P. coprophilum, P. cyclopium, P. digitatum, P. echinulatum, P. hirsutum, P. melanoconidium, P. mononematosum, P. sclerotigenum, P. tricolor, P. tulipae, P. verrucosum* and *Eupenicillium osmophilum*. In many species, only one bp substitution was noted among the strains, including *P. albocoremium, P. cavernicola, P. coprobium, P. dipodomyis, P. hordei, P. nordicum, P. palitans, P. paneum, P. polonicum, P. thymicola* and *P. venetum*. Other species generally had 2-4 substitutions, and only for *P. dipodomyicola, P. expansum, P. flavigenum, P. glandicola, P. italicum, P. nalgiovense, P. olsonii* and *P. viridicatum* did each strain have a unique sequence. The most divergent species were in sect. *Coronata*, where 6-8 bp differences were seen among the three strains of *P. bialowiezense* and five strains of *P. brevicompactum* sequenced.

As noted above, in this study DNA sequences were used to evaluate and support phenotypic species concept for *Penicillium* subgenus *Penicillium* employed by Frisvad & Samson (2004). We have not attempted to apply a DNA-based phylogenetic species concept but advocate a concept based on ecological and phenotypic characters, including traditional morphological and cultural characters, as well as extrolite profiles. This is in contrast to the study by O´Donnell *et al*. (2004), for example, which divided *Fusarium graminearum* into nine DNA-based phylogenetic species based on concordance among eight genes, but with no phenotypic characters to support them. This kind of approach has not yet been applied to a great extent in *Penicillium*. Peterson (2004) recovered the same species groups in sect. *Olsonii* that are shown here in Fig. 8, and demonstrated phylogenetic species concepts using a three gene phylogeny that did not include β-tubulin.

The connection to *Eupenicillium*

Pitt (1979) recognized four subgenera in *Penicillium* and these have been used often in later taxonomic studies. Both extrolite data (Frisvad and Filtenborg, 1989; 1990 a, b; Frisvad *et al.*, 1990; Samson *et al.*, 1989) and DNA sequence data (Peterson, 1993; LoBuglio *et al.*, 1993; 1994; Berbee *et al.*, 1995) indicate that the subgenera *Aspergilloides* and *Furcatum* are related to *Eupenicillium javanicum* and related soil-borne forms, while *E. egyptiacum* and *P. gladioli* are related to subgenus *Penicillium.* Subgenus *Biverticillium* is very different from all those supraspecific taxa and belongs to the ascomycete genus *Talaromyces* (Pitt, 1979; Frisvad *et al.*, 1990; Peterson, 1993; LoBuglio *et al.*, 1993, Berbee *et al.*, 1995).

The data presented here and those of Peterson (2000) suggest that *E. crustaceum* and *E. egyptiacum* are closely related phylogenetically to sect. *Chrysogena, Roqueforti, Urticicolae* or *Claviformia*. Some phenotypic characters indicate that *E. egyptiacum, E. crustaceum* and *E. osmophilum* are most closely related to sect. *Chrysogena*. They resemble sect. *Chrysogena* in their relative xerophily, and by the production of similar extrolites. *Eupenicillium crustaceum* CBS 344.61 and CBS 581.67 both produce fumitremorgins and verrucologen, in common with *P. mononematosum*; the latter strain also produces andrastin A in common with *P. mononematosum* and *P. confertum* from ser.

Mononematosa. *Eupenicillium crustaceum* CBS 344.61 produces roquefortine C in common with *P. confertum, P. chrysogenum* and *P. flavigenum*. *E. egyptiacum* CBS 244.32 and CBS 458.72 produce secalonic acid D in common with *P. chrysogenum* and *P. flavigenum*. Given the desert habitat of those Eupenicillia and ser. *Mononematosa*, we believe that these will eventually be shown to be sister groups.

Acknowledgements
We are grateful to John Bissett and Sarah Hambleton for presubmission reviews of this manuscript. Some sequences were kindly provided by Gerry Louis-Seize.

References

Andersen B, Nielsen KF, Thrane U, Szaro T, Taylor JW, Jarvis BB (2003) Molecular and phenotypic descriptions of *Stachybotrys chlorohalonata* sp. nov. and two chemotypes of *Stachybotrys chartarum* found in water–damaged buildings. *Mycologia* **95**: 1227-1238.

Banke S, Frisvad JC, Rosendahl S (1997) Taxonomy of *Penicillium chrysogenum* and related xerophilic species, based on isozyme analysis. *Mycological Research* **101**: 617-624.

Berbee ML, Yoshiomura A, Sugiyama J, Taylor JW (1995) Is *Penicillium* monophyletic? An evaluation of phylogeny in the family Trichocomaceae from 18S, 5.8S and ITS ribosomal data. *Mycologia* **87**: 210-222.

Beilharz V, Cunnington J (2003) Two closely related species of *Pseudocercospora* on unrelated host families from south-eastern Australia. *Mycological Research* **107**: 445-451.

Bridge PD, Hawksworth DL, Kozakiewicz Z, Onions AHS, Paterson RRM, Sackin MJ, Sneath PHA (1989a) A reappraisal of the terverticillate Penicillia using biochemical, physiological and morphological features I. Numerical taxonomy. *Journal of General Microbiology* **135**: 2941-2966.

Bridge PD, Hawksworth DL, Kozakiewicz Z, Onions AHS, Paterson RRM, Sackin MJ (1989b) A reappraisal of the terverticillate Penicillia using biochemical, physiological and morphological features II. Identification. *Journal of General Microbiology* **135**: 2967-2978.

Castella G, Larsen TO, Cabañes J, Schmidt H, Alboresi A, Niessen L, Färber P, Geisen R (2002) Molecular characterization of ochratoxin A producing strains of the genus *Penicillium*. *Systematic and Applied Microbiology* **25**: 74-83.

Dupont J, Magnin S, Cesari C, Gatiga M (2002) ITS and beta-tubulin markers help delinieate *Phaeoacremonium* species, and the occurrence of *P. parasiticum* in grapewine disease in Argentina. *Mycological Research* **106**: 1143-1150.

Fatehi J, Bridge PD, Punithalingam E (2003) Molecular relatedness within the *Ascochyta pinodes*-complex. *Mycopathologia* **156**: 317-327.

Frisvad JC (1989) The connection between the Penicillia and Aspergilli and mycotoxins with special emphasis on misidentified isolates. *Archives of Environmental Contamination and Toxicology* **18**: 452-467,

Frisvad JC (1998) *Secondary metabolites and species models in* Penciillium *and* Aspergillus. Dr. Techn. Thesis. Technical University of Denmark, Kgs. Lyngby, Denmark.

Frisvad JC, Filtenborg O (1990b) Secondary metabolites as consistent criteria in *Penicillium* taxonomy and a synoptic key to *Penicillium* subgenus *Penicillium*. In: *Modern concepts in* Penicillium *and* Aspergillus *classification* (Samson RA, Pitt JI, eds.). Plenum Press, New York: 373-384.

Frisvad JC, Filtenborg O (1983) Classification of terverticillate Penicillia based on profiles of mycotoxins and other secondary metabolites. *Applied and Environmental Microbiology* **46**: 1301-1310.

Frisvad JC, Samson RA (2004) Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Studies in Mycology (Utrecht)* **49**: 1-173.

Geiser DM, Frisvad JC, Taylor JW (1998) Evolutionary relationships in *Aspergillus* section *Fumigati* inferred from partial β-tubulin sequences. *Mycologia* **90**: 831-845.

Glass N L, Donaldson G C (1995) Development of Primer Sets Designed for Use with the PCR To Amplify Conserved Genes from Filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Hillis D M, Bull J J (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182–192.

Hognabba F, Wedin M (2003) Molecular phylogeny of the *Sphaerophorus* species complex. *Cladistics* **19**: 224-232.

Jacobs K, Kirisits T (2003) *Ophiostoma kryptum* sp. nov. from *Larix decidua* and *Picea abies* in Europe, similar to *O. minus*. *Mycological Research* **107**: 1231-1242.

Larsen TO, Frisvad JC (1995a) Characterization of volatile metabolites from 47 *Penicillium* taxa. *Mycological Research* **99**: 1151-1166.

Larsen TO, Frisvad JC (1995b) Chemosystematics of fungi in genus *Penicillium* based on profiles of volatile metabolites. *Mycological Research* **99**: 1167-1174.

Larsen TO, Svendsen A, Smedsgaard J (2001a) Biochemical characterization of ochratoxin A-producing strains of the genus *Penicillium*. *Applied and Environmental Microbiology* **67**: 3620-3635.

Larsen TO, Petersen BO, Duus JØ (2001b) Lumpidin, a novel biomarker of some ochratoxin A producing Penicillia. *Journal of Agricultural and Food Chemistry* **49**: 5081-5084.

Liu, YJ, Whelen, S, Hall, BD (1999) Phylogenetic relationships among Ascomycetes: Evidence from an RNA Polymerase II Subunit. *Molecular and Biological Evolution* **16**: 1799-1808.

LoBuglio K, Pitt JI, Taylor JW (1993) Phylogenetic analysis of two ribosomal DNA regions indicates multiple independent losses of a sexual *Talaromyces* state among asexual *Penicillium* species in subgenus *Biverticillium*. *Mycologia* **85**: 592-604.

LoBuglio KF, Pitt JI, Taylor JW (1994) Independent origins of the synnematous *Penicillium* species, *P.*

duclauxii, *P. clavigerum* and *P. vulpinum*, as assessed by two ribosomal DNA regions. *Mycological Research* **98**: 250-256.

Molina MD, Crespo A, Blanco O, Lumbsch HT, Hawksworth DL (2004) Phylogenetic relationships and species concepts in *Parmelia* s.str. (Parmeliaceae) inferred from nuclear ITS rDNA and beta-tubulin sequences. *Lichenologist* **36**: 37-54.

O´Donnell K, Cigelnik E, Nirenberg H (1998) Molecular systematics and phylogeography of the *Gibberella fujikori* complex. *Mycologia* **90**: 465-493.

O´Donnell K, Ward TJ, Geiser DM, Kistler HC, Aoki T (2004) Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenitically distinct species within the *Fusarium graminearum* clade. *Fungal genetics and Biology* **41**: 600-623.

Peterson SW (1993) Molecular genetic assessment of relatedness of *Penicillium* subgenus *Penicillium*. In: *The fungal holomorph: mitotic, meotic and pleomorphic speciation in fungal systematics* (Reynolds DR, Taylor JW, eds.), CAB International, Wallingford, UK: 121-128.

Peterson SW (2000) Phylogenetic analysis of *Penicillium* species based on ITS and LSU-rDNA nucleotide sequences. In: *Integration of modern taxonomic methods for* Penicillium *and* Aspergillus *classification* (Samson RA, Pitt JI, eds.), Harwood Academic Publishers, Amsterdam, the Netherlands: 163-178.

Peterson SW (2001) *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. *Mycologia* **93**: 689-703.

Peterson SW (2004) Multilocus DNA sequence analysis shows that *Penicillium biourgeanum* is a distinct species closely related to *P. brevicompactum* and *P. olsonii*. *Mycological Research* **108**: 434-440.

Peterson SW, Pérez J, Vega FE and Infante F (2003) *Penicillium brocae*, a new species associated with the coffee borer in Chiapas, Mexico. *Mycologia* **95**: 141-147.

Pitt JI (1973) An appraisal of identification methods for *Penicillium* species: novel taxonomic criteria based on temperature and water relations. *Mycologia* **65**: 1135-1157.

Pitt JI (1979) *The genus* Penicillium *and its teleomorphic states* Eupenicillium *and* Talaromyces. Academic Press, London, UK.

Pitt JI, Cruickshank RH (1990) Speciation and synonymy in *Penicillium* subgenus *Penicillium* – towards a definitive taxonomy. In: *Modern concepts in* Penicillium *and* Aspergillus *classification* (Samson RA, Pitt, JI, eds.). Plenum Press, New York, USA: 103-119.

Polonelli L, Morace G, Rosa R, Castagnola M, Frisvad JC (1987) Antigenic characterization of *Penicillium camemberti* and related common cheese contaminants. *Applides and Environmental Microbiology* **53**: 872-878.

Ramírez C (1982) *Manual and atlas of the Penicillia*. Elsevier, Amsterdam, the Netherlands.

Raper KB, Thom C (1949) *A manual of the Penicillia*. Williams and Wilkins, Baltimore, USA.

Rokas A, Williams BL, Carroll SB (2003) Genome-scale approaches to resolving incongruence in molecular phylogenies. *Nature* **425**: 798-804.

Samson RA, Stolk AC, Frisvad JC (1989) Two new synnemateous species of *Penicillium*. *Studies inx Mycology (Baarn)* **31**: 133-143.

Samson RA, Stolk AC, Hadlok R (1976) Revision of the subsection fasciculata of *Penicillium* and some allied species. *Studies in Mycology (Baarn)* **11**: 1-47.

Schardl CL, Leuchtmann A, Chung K-R, Penny D, Siegel MR (1997) Coevolution by common descent of fungal symbionts (*Epichloë* spp.) and grass hosts. *Molecular Biology and Evolution* **14**: 133-143.

Schoch CL, Crous PW, Wingfield BD, Wingfield MJ (2001) Phylogeny of *Calonectria* based on comparison of beta-tubulin DNA sequences. *Mycological Research* **105**: 1045-1052.

Schroers HJ (2001) A monograph of *Bionectria* (Ascomycota, Hypocreales, Bionectiaceae) and its *Clonostachys* anamorphs. *Studies in Mycology (Utrecht)* **46**: 1-211.

Seifert KA, Louis-Seize G (2000) Phylogeny and species concepts in the *Penicillium aurantiogriseum* complex as inferred from partial β-tubulin gene DNA sequences. In: *Integration of modern taxonomic methods for* Penicillium *and* Aspergillus *classification* (Samson RA, Pitt JI, eds.). Harwood Academic Publishers, Amsterdam, the Netherlands: 189-198.

Seifert KA, McMullen CR, Yee D, Reeleder RD, Dobinson KF (2003) Molecular differentiation and detection of ginseng-adapted isolates of the root fungus *Cylindrocarpon destructans*. *Phytopathology* **93**: 1533-1542.

Skouboe P, Frisvad JC, Lauritsen D, Boysen M, Taylor JW, Rossen L (1999) Nucleotide sequences from the ITS region of *Penicillium* species. *Mycological Research* **103**: 873-881.

Skouboe P, Taylor JW, Frisvad JC, Lauritsen D, Larsen L, Albæk C, Boysen M, Rossen L (2000) Molecular methods for differentiation of close related *Penicillium* species. In: *Integration of modern taxonomic methods for* Penicillium *and* Aspergillus *classification* (Samson RA, Pitt JI, eds.). Harwood Academic Publishers, Amsterdam, the Netherlands: 179-188.

Skouboe, P., Boysen, M., Pedersen, L.H., Frisvad JC & Rossen, L. (1996) Identification of *Penicillium* species using the internal transcribed spacer (ITS) regions. In: *Fungal identification techniques*, (Rossen L, Rubio V, Dawson MT, Frisvad JC, eds.). European Commission, Brussels, Belgium: 160-164.

Smedsgaard J, Frisvad JC (1996) Terverticillate Penicillia studied by direct electrospray mass spectrometric profiling of crude extracts. I. Chemosystematics. *Biochemical Systematics and Ecology* **25**: 51-64

Smedsgaard J, Frisvad JC (1997) Using direct electrospray mass spectrometry in taxonomy and secondary metabolite profiling of crude fungal extracts. *Journal of Microbiological Methods* **25**: 5-17.

Stolk AC, Samson RA, Frisvad JC, Filtenborg O (1990) The systematics of the terverticillate Penicillia. In: Samson, RA, Pitt JI (eds.): *Modern concepts in* Penicillium *and* Aspergillus *systematics*. Plenum Press, New York, USA: 121-136.

Swofford, D. L. (2000) *PAUP* 4.0: phylogenetic analysis using parsimony*. Sinauer Associates, Sunderland, MA.

Thom C (1930) *The Penicillia*. Williams and Wilkins, Baltimore, USA.

White TJ, Bruns T, Lee S, Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In : Innis MS, Gelfand DH (eds): *PCR protocols: a guide to methods and applications*. (pp. 315-322) Academic Press, New York.

Young C, McMillan L, Telfer E, Scott B (2001) Molecular cloning and genetic analysis of an indole-diterpene gene cluster from *Penicillium paxilli*. *Molecular Microbiology* **39**: 754-764.