

Fleming's penicillin producing strain is not *Penicillium chrysogenum* but *P. rubens*

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Abstract: *Penicillium chrysogenum* is a commonly occurring mould in indoor environments and foods, and has gained much attention for its use in the production of the antibiotic penicillin. Phylogenetic analysis of the most important penicillin producing *P. chrysogenum* isolates revealed the presence of two highly supported clades, and we show here that these two clades represent two species, *P. chrysogenum* and *P. rubens*. These species are phenotypically similar, but extrolite analysis shows that *P. chrysogenum* produces secalonidic acid D and F and/or a metabolite related to lumpidin, while *P. rubens* does not produce these metabolites. Fleming's original penicillin producing strain and the full genome sequenced strain of *P. chrysogenum* are re-identified as *P. rubens*. Furthermore, the well-known claim that Alexander Fleming misidentified the original penicillin producing strain as *P. rubrum* is discussed.

Key words:

Fleming
indoor mycology
phylogeny
taxonomy

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INTRODUCTION

Penicillium chrysogenum is a commonly occurring mould in indoor environments such as dust, indoor air, and damp building materials (Chang *et al.* 1995, Gravesen 1999, Hunter & Lea 1995). Furthermore, *P. chrysogenum* is frequently identified as a food spoilage agent and has gained much attention for its use in the production of the antibiotic penicillin (Samson *et al.* 2010). The taxonomy of this species was studied extensively by Raper & Thom (1949) and they accepted four species in the "*Penicillium chrysogenum* series": *P. chrysogenum*, *P. notatum*, *P. meleagrinum*, and *P. cyaneofulvum*. However, Samson *et al.* (1977) regarded this series as one broad species and did not accept Raper and Thom's "*P. chrysogenum* series". More recently, other species have been included in *Penicillium* series *Chrysogena*, such as *P. flavigenum*, *P. nalgiovense*, and *P. dipodomyis* (Frisvad *et al.* 1987, Banke *et al.* 1997, Frisvad & Samson 2004), and these species are clearly different from *P. chrysogenum* although all species produce penicillin (Frisvad *et al.* 1987, Andersen & Frisvad 1994, Banke *et al.* 1997, Färber & Geisen 1994). Scott *et al.* (2004) studied the taxonomy of *P. chrysogenum* using multilocus sequence typing (MLST) data and showed that it could be subdivided into four clades. However, that study mainly focused on indoor isolates and did not link the synonyms of *P. chrysogenum* to those clades.

Penicillium chrysogenum was described by Thom (1910), but the name *P. chrysogenum* is predated by *P. griseoroseum*, *P. citreoroseum*, and *P. brunneorubrum*, which were described by Dierckx (1901). Pitt (1980) accepted *P. griseoroseum* and *P. chrysogenum*, and synonymized *P. citreoroseum* and *P. brunneorubrum* with *P. griseoroseum*. However, subsequent morphological, physiological and extrolite data showed that these species were conspecific with *P. chrysogenum* (Frisvad & Filtenborg 1989, Banke *et al.* 1997). Since *P. chrysogenum* is a widely used name, Frisvad *et al.* (1990) and Kozakiewicz *et al.* (1992) suggested to preserve the name *P. chrysogenum* and to reject the older name *P. griseoroseum* with its synonyms *P. citreoroseum*, and *P. brunneorubrum*. This proposal was accepted by the Committee for Fungi and Lichens and the name *P. chrysogenum* is currently listed as *nomen conservandum* (McNeill *et al.* 2006).

The aim of this study was to find out whether the two most common clades in *P. chrysogenum sensu lato* (Scott *et al.* 2004) represent one or two species. Towards this aim, we have studied the most important strains in the history of penicillin production by combining extrolite and molecular data. Furthermore, the history of the taxonomy of Fleming's penicillin-producer strain is discussed.

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Table 1. Overview of *Penicillium chrysogenum* and *P. rubens* strains studied.

No.	Other collection no.	Name	Substrate, locality	Remarks
NRRL 820	DTO 100G5 = IBT 4395 = IBT 6067 = IMI 92220	<i>P. chrysogenum</i>	Unrecorded source	Ex-lectotype of <i>P. griseoroseum</i>
CBS 306.48 ^T	NRRL 807 = IBT 5233 = IMI 24314	<i>P. chrysogenum</i>	Cheese, Storrs, Connecticut, USA	Ex-lectotype of <i>P. chrysogenum</i>
DTO 8712		<i>P. chrysogenum</i>	Fungal growth on ceiling of archive, Utrecht, the Netherlands	
DTO 102B4	IBT 26889 = C238	<i>P. chrysogenum</i>	House dust, Wallaceburg, ON, Canada	Representative of group 2 in the study of Scott <i>et al.</i> (2004)
CBS 355.48	NRRL 821 = IBT 4344 = DTO 98D4 = IMI 39759	<i>P. chrysogenum</i>	Branches of <i>Hyssopus</i> , Norway	Ex-type of <i>P. notatum</i>
CBS 197.46	NRRL 832	<i>P. rubens</i>	Must contaminant, Belgium	The strain first used for producing penicillin in submerged culture (Raper & Thom 1949: 368–370)
CBS 205.57	NRRL 824 = IBT 30142 = IMI 015378	<i>P. rubens</i>	Culture contaminant in bacterial culture, UK	Fleming's original penicillin producing strain
DTO 95E9	IBT 30661	<i>P. rubens</i>	Cap of beer bottle, Belgium	
NRRL 792 ^T	DTO 98E8 = IBT 30129	<i>P. rubens</i>	Unrecorded source	Ex-lectotype of <i>P. rubens</i>
CBS 307.48	NRRL 1951 = IBT 5857 = IMI 40233	<i>P. rubens</i>	Mouldy cantaloupe Peoria, Illinois, USA	"Wisconsin strain", parent of most high yielding penicillin producing strains
Wisconsin 54-1255		<i>P. rubens</i>	Mouldy cantaloupe Peoria, Illinois, USA	Full genome sequenced strain

MATERIAL AND METHODS

Strains

An overview of *Penicillium chrysogenum* strains used in this study is given in Table 1. Three point position inoculations of these strains were made in order to study their macromorphology. The strains were inoculated on Czapek yeast extract agar (CYA), yeast extract sucrose agar (YES), creatine agar (CREA) and malt extract agar (MEA) (Oxoid). All media were prepared as described by Samson *et al.* (2010). Colony characteristics were recorded after 7 d of incubation at 25 °C. Microscopic mounts were made from MEA or OA and microscopical features were studied by light microscopy (Olympus BH2 and Zeiss Axioskop 2 Plus).

Molecular analysis

DNA extraction was performed using the Ultraclean Microbial DNA isolation Kit (MoBio, Solana Beach, USA) according to the manufacturer's instructions. Parts of the β -tubulin and calmodulin genes were amplified and sequenced according to the method described by Houbraken *et al.* (2007). A part of the RPB2 locus was amplified using the primer pair RPB2-5F_Pc 5'- GATGACCGTGACCACTTCGG-3' and RPB2-7CR_Pc 7CR 5'- CCCATGGCTTGTGGCCCAT-3'. The alignments were combined and maximum likelihood analysis was performed using RAxML v. 7.2.8. Each dataset was treated as a separate partition. The GTR substitution matrix was used for all three partitions and individual per partition

branch length optimization was carried out (Stamatakis *et al.* 2008). Maximum parsimony analysis was performed as a second measure of branch support. This analysis was run in MEGA5 and bootstrap values were determined using 500 replicates. *Penicillium griseofulvum* CBS 185.27 was selected as an outgroup. Newly generated sequences were deposited in Genbank under JF909924–JF909977.

Extrolite analysis

Strains were grown for 7–14 d at 25 °C on the agar media YES, CYA and MEA prior to extrolite extraction. Extrolites were extracted and analyzed by HPLC-DAD as described by Frisvad & Thrane (1987) and modified by Smedsgaard (1997). Identification was performed by comparison of the UV spectra and retention times of the extrolites with those of purified and chemically characterized compounds present in the collection at the Biocentrum-DTU (Lyngby).

RESULTS

Phylogenetic analysis

The structure of the partitions of the alignment was as follows: the calmodulin partition was 506 basepairs long and had 90 distinct alignment patterns; the RPB2 dataset included 981 characters, and 91 of those had a distinct alignment pattern and the tubulin gene partition was 434 basepairs long, 62 were found to have a distinct alignment pattern. In

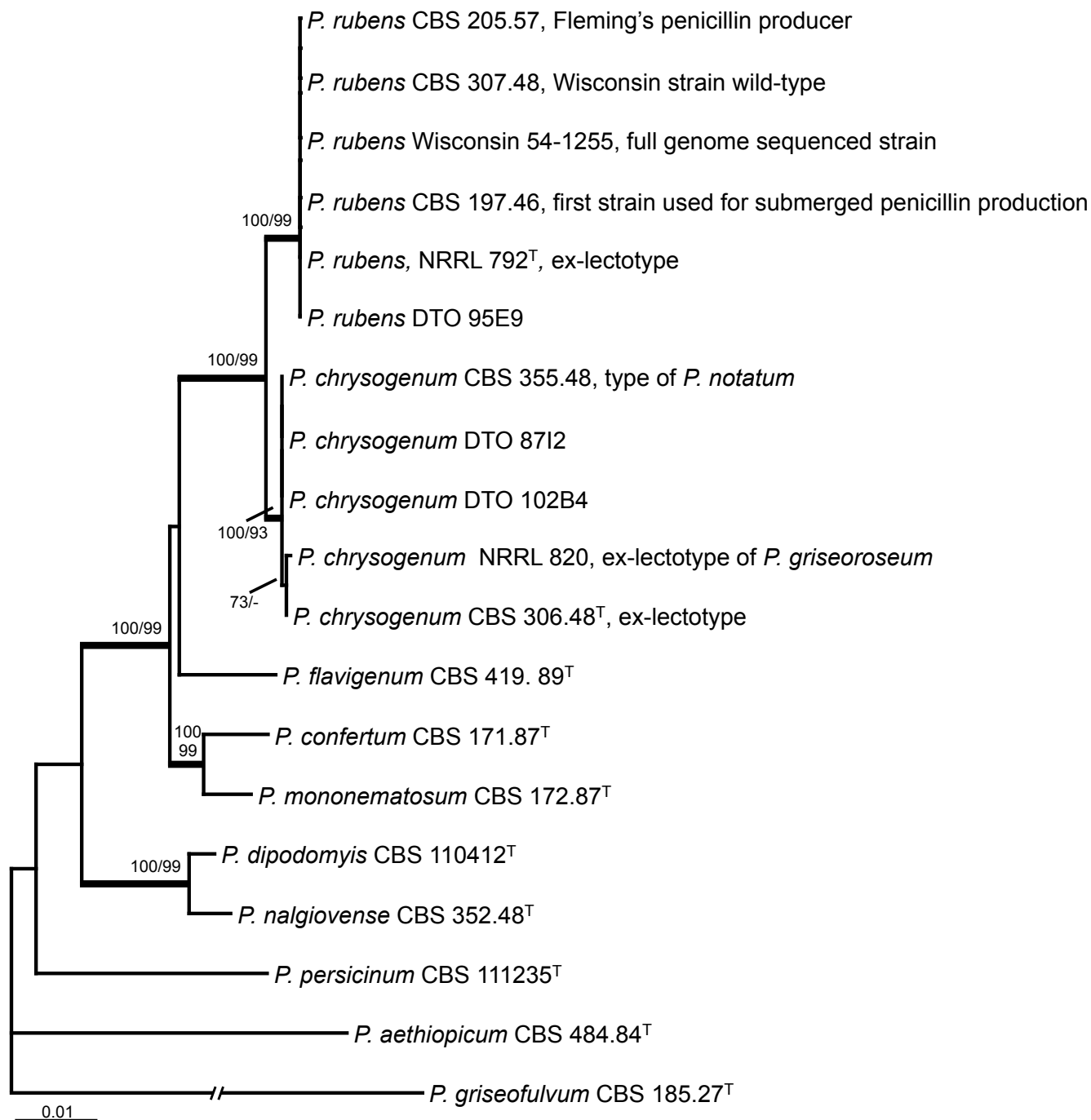


Fig. 1. Best scoring ML tree of combined partial β -tubulin, calmodulin and RPB2 datasets. The bootstrap values of the maximum likelihood analysis (ML, first number) and maximum parsimony analysis (MP, second number) are shown at the nodes. The bootstrap values above 70 % are shown and branches supported less than 70 % in either the MP or ML analysis are indicated with a hyphen.

the maximum parsimony analysis, 118 sites were parsimony informative (3150 MP trees, length = 400, consistency index 0.69, retention index 0.83). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein 1985). The best-scoring maximum likelihood tree is shown in Fig. 1.

Phylogenetic analysis of the combined partial β -tubulin, calmodulin and RPB2 datasets revealed two highly supported clades in *Penicillium chrysogenum sensu lato*. One clade is

centered around the ex-type strain of *P. chrysogenum* (CBS 306.48). Also the ex-type strains of *P. notatum* (CBS 355.48), *P. griseoroseum* (NRRL 820) and several other strains are accommodated in this clade. The other clade is centered around the ex-type strain of *P. rubens* (NRRL 792). Fleming's original penicillin producing strain (CBS 205.57), the wild-type Wisconsin strain isolated from a mouldy cantaloupe (CBS 307.48 = NRRL 1951), the full genome sequenced strain derived from NRRL 1951 (Wisconsin 54-1255) (van den Berg

Table 2. Extrolites produced by *Penicillium chrysogenum* and *P. rubens* strains studied, with a focus on the important strains for the history of penicillin.

No.	Other collection no.	Name	Extrolites
CBS 306.48 ^T	NRRL 807 = IBT 5233	<i>P. chrysogenum</i>	Andrastin A, andrastin B, chrysogine, citreoisocoumarin, cf. lumpidin, meleagrín, penicillin, roquefortine C, secalonic acid D, secalonic acid F, sorbicillins
CBS 355.48	NRRL 821 = IBT 4344 = DTO 98D4	<i>P. chrysogenum</i>	Andrastin B, chrysogine, cf. lumpidin, meleagrín, penicillin, roquefortine C, secalonic acid D, sorbicillins
NRRL 820	DTO 100G5 = IBT 4395 = IBT 6067	<i>P. chrysogenum</i>	Andrastin A, andrastin B, chrysogine, can-741, meleagrín, cf. lumpidin, roquefortine C, sorbicillins
DTO 102B4	IBT 26889 = C 238	<i>P. chrysogenum</i>	Andrastin A, andrastin B, chrysogine, meleagrín, cf. lumpidin, secalonic acid D, secalonic acid F
CBS 205.57	NRRL 824 = IBT 30142	<i>P. rubens</i>	Chrysogine, meleagrín, penicillin, roquefortine C, andrastin A, sorbicillins
NRRL 792 ^T	DTO 98E8 = IBT 30129	<i>P. rubens</i>	Chrysogine, meleagrín, penicillin, cf. roquefortine, sorbicillins
CBS 307.48	NRRL 1951 = IBT 5857	<i>P. rubens</i>	Chrysogine, meleagrín, penicillin, roquefortine C, roquefortine D, sorbicillins
DTO 95E9	IBT 30661	<i>P. rubens</i>	Meleagrín, roquefortine C

et al. 2008), the strain first found to produce satisfactory yields of penicillin when grown submerged (CBS 197.46 = NRRL 832), are accommodated in this clade. These two clades evidently represent two different species, *P. chrysogenum* and *P. rubens*.

Phenotypic and extrolite analysis

Phenotypic examination using macro- and microscopical characters of strains of the *Penicillium rubens* and *P. chrysogenum* clades did not reveal clear differences. The majority of the examined strains showed similar growth characteristics, such as good growth and sporulation on YES agar, weak or moderate growth on CREA with poor acid production and large clear or yellow coloured exudate droplets on MEA and/or CYA (Fig. 2). Three strains had deviating growth characteristics: CBS 355.48 (ex-type culture of *P. notatum*) had smaller colonies on all tested agar media, and NRRL 792 (ex-type culture of *P. rubens*; Fig. 3), and CBS 307.48 (= NRRL 1951, wild-type full genome-sequenced strain) deviated in having reduced sporulation on most agar media.

Extrolite analysis differentiated the *P. chrysogenum* and *P. rubens* clades. An overview of extrolites produced by these species, with emphasis on the important strains in the history of penicillin, is shown in Table 2. These results show that *P. chrysogenum* produces secalonic acid D and F and/or a metabolite related to lumpidin (Larsen et al. 2001), while *P. rubens* does not produce these metabolites.

TAXONOMY

Penicillium chrysogenum Thom, *Bull. Bur. Anim. Ind. USDA* **118**: 58 (1910); nom. cons.

Synonyms: *Penicillium griseoroseum* Dierckx, *Ann. Soc. Sci. Brux.* **25**: 86 (1901); nom. rej.

Penicillium citreoroseum Dierckx, *Ann. Soc. Sci. Brux.* **25**: 86 (1901); nom. rej.

Penicillium brunneorubrum Dierckx, *Ann. Soc. Sci. Brux.* **25**: 88 (1901); nom. rej.

Penicillium notatum Westling, *Ark. Bot.* **11**(1): 95 (1911).

Type: USA: Connecticut: Storrs, isol. ex cheese, 1904, C. Thom (IMI 24314 – typ. cons.).

Penicillium rubens Biourge, *La Cellule* **33**: 265 (1923).

Type: Unrecorded source, *P. Biourge*. CBS H-20595 – **lectotypus hic designatus**; from Biourge (402) to Thom (4733.110) 1930 to NRRL (792) 1949 to CBS 2009; cultures ex-lectotype – NRRL 792 = IBT 30129 = ATCC 9783 = CBS 129667.

Penicillium chrysogenum has, in addition to the species names mentioned above, also various other synonyms (Samson et al. 1977, Samson & Frisvad 2004). These include *P. meleagrimum* Biourge 1923, *P. cyaneofulvum* Biourge 1923, *P. chlorophaeum* Biourge 1923, *P. roseocitreum* 1923, *P. camerunense* Heim 1949 nom. inval., *P. chrysogenum* var. *brevisterigma* Forster nom. inval. 1953, *P. aromaticum* f. *microsporum* Romankova 1955, *P. harmonense* Baghdadi 1968, *P. verrucosum* var. *cyclopium* strain “ananas-olens” Ramírez 1982, and *P. chrysogenum* “mut. *fulvescens*” Takashima et al. ex Ramírez 1982. A more detailed molecular or extrolite analysis is needed to properly place these species in either *P. chrysogenum*, *P. rubens*, or other currently unrecognised species belonging to *P. chrysogenum sensu lato*.

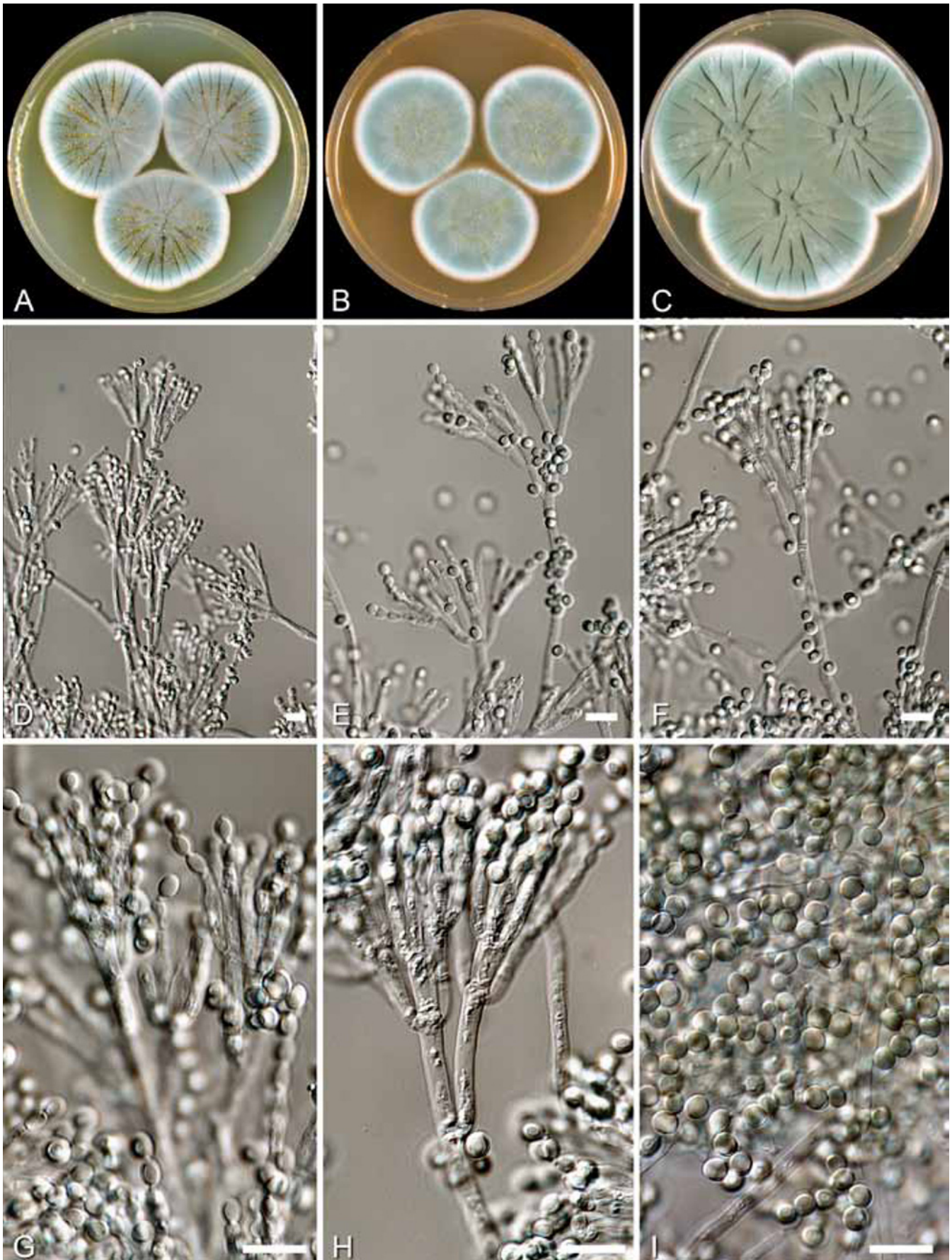


Fig. 2. *Penicillium rubens* CBS 205.57 (Fleming's original penicillin-producer). **A–C.** Colonies 7 d old 25 °C. **A.** CYA. **B.** MEA. **C.** YES. **D–H.** Conidiophores. **I.** Conidia. Bars = 10 µm.

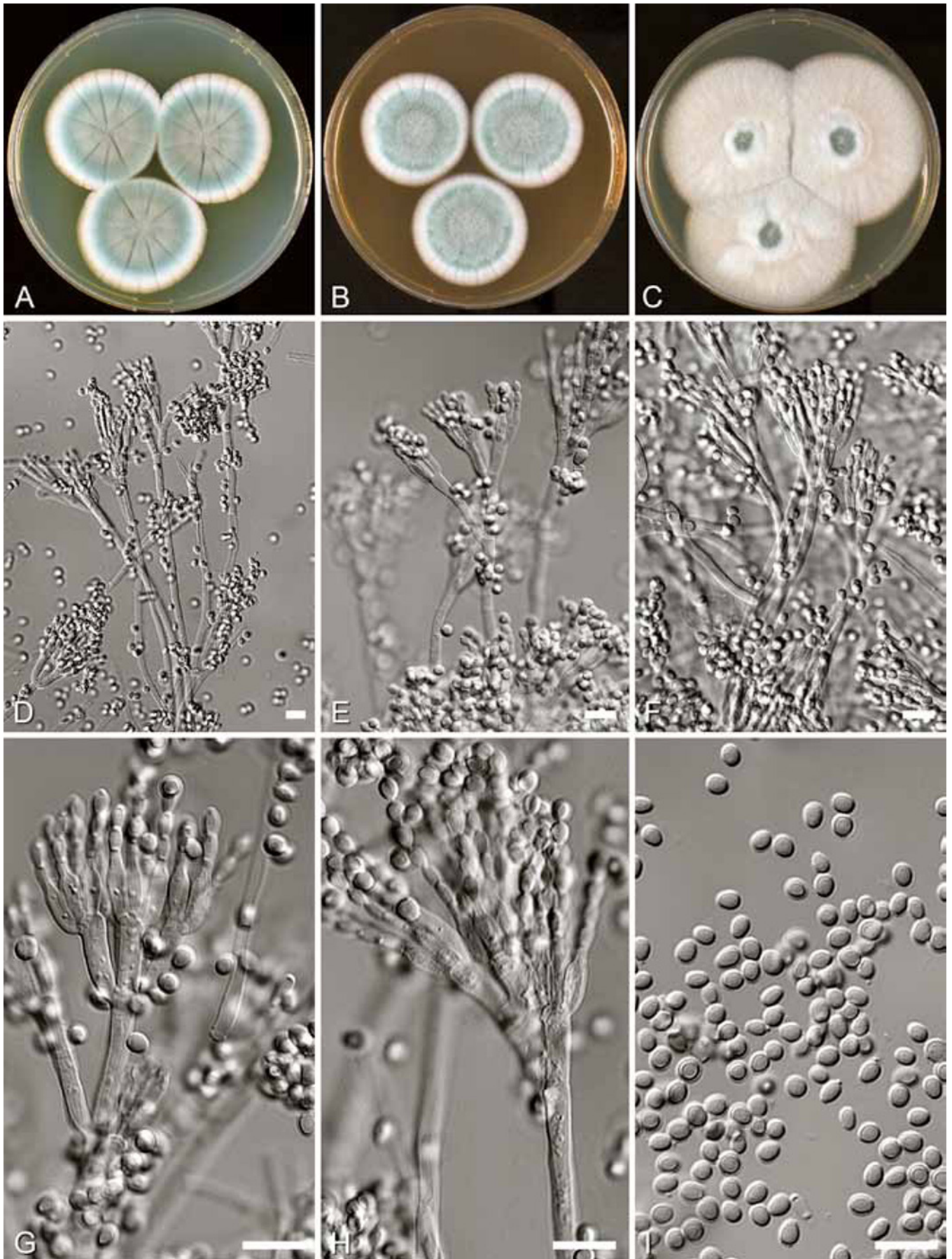


Fig. 3. *Penicillium rubens* NRRL 792. A–C. Colonies 7 d old 25 °C. A. CYA. B. MEA. C. YES. D–H. Conidiophores. I. Conidia. Bars = 10 μm.

DISCUSSION

Identity of Fleming's original penicillin-producing strain

It has long been claimed that Fleming (1929) misidentified the original penicillin-producing strain as "*Penicillium rubrum* Biourge 1923" (Raper & Thom 1949, Lax 2005). However, it was the mycologist, Charles J La Touche (1904–1981), working in a room on the floor below that occupied by Fleming, who actually identified the strain as *Penicillium rubrum*. Our current study shows that the correct name of this strain is *P. rubens*. We discuss two possible scenarios here, which could have led La Touche to his identification of Fleming's strain as *P. rubrum*, both showing that his identification was (rather) accurate:

1. La Touche identified Fleming's strain as *P. rubrum*, but this was a *lapsus* for *P. rubens*. The epithet *rubrum* means "red" and *rubens* "to be red", and thus it is not unlikely that these epithets have been confused. Both yellow and red-brown reverse colours have been observed in *P. chrysogenum sensu lato* and in the true *P. rubrum* (i.e. *P. purpurogenum*) (Pitt 1980). These two species clearly differ in micro-morphology from each other and the mycologist La Touche would have noticed these differences. Pink and vinaceous drab colours are mentioned by Raper & Thom (1949) for strains of *P. meleagrinum* and reddish colours are part of the names of synonyms of *P. chrysogenum sensu lato*, such as *P. citreoroseum*, *P. roseocitreum*, *P. brunneorubrum*, and *P. griseoroseum*.
2. In the other scenario, La Touche correctly identified the strain as *P. rubrum* based on the taxonomic schemes present at that time. In 1929, the most recent monograph on *Penicillium* was that of Biourge (1923), and La Touche would have used this monograph for identification of Fleming's penicillin-producing strain. This is also supported by that monograph being cited in Fleming's report of the original discovery of penicillin (Fleming 1929). *Penicillium rubrum* is listed in Biourge's monograph as "*P. rubrum* (?) Grassberger-Stoll, 1905" and this species was classified in Biourge's subsection "*Les Radiés, Radiata*". This subsection also comprises *P. chrysogenum* and the species *P. griseoroseum*, *P. cyaneofulvum*, *P. brunneorubrum*, *P. notatum*, *P. citreoroseum*, and *P. baculatum*, which are currently all listed as synonyms of *P. chrysogenum*. Examination of Biourge's drawings of "*P. rubrum* (?) Grassberger-Stoll" shows that his definition of *P. rubrum* is very similar to that of *P. chrysogenum* and does not fit in the original concept of *P. rubrum* (Stoll 1904, Pitt 1980). This was also noted by Biourge (1923) and therefore he added the question mark to his description of *P. rubrum*. Furthermore, the close connection between *P. rubrum* Grassberger-Stoll *sensu* Biourge and *P. rubens* was also noted by Thom (1930: 250) who assigned the former species to *P. rubens*. In conclusion, La Touche used Biourge's taxonomic scheme and identified Fleming's strain as *P. rubrum* Grassberger-

Stoll (as defined by Biourge) and this identification was accurate at that time and not incorrect as claimed in literature (Thom 1945, Raper & Thom 1949, Lax 2005). Furthermore, Biourge (1923) wrote in his description of *P. rubrum* that the species was a common laboratory contaminant ("...un animal de laboratoire!"), which could have strengthened La Touche in his idea of having the correct identification, since Fleming's isolate was also a laboratory contaminant.

Name changes of Fleming's penicillin-producing strain

When Charles Thom was about to finish his monograph in 1930, he received the Fleming strain (CBS 205.57 = NRRL 824 = IMI 015378) that had been identified as *Penicillium rubrum*, and he re-identified it as *P. notatum* (Thom 1945). He did not realize that the Fleming isolate was identified by the experienced mycologist Charles La Touche since he wrote: "Not being a mycologist, he [Alexander Fleming] undertook to identify the mold from the literature and selected the name *Penicillium rubrum* Biourge" (Thom 1945). However, the authority name Biourge is not mentioned in Fleming's work and Thom would have been more accurate if he had named it "*Penicillium rubrum* Grassberger-Stoll *sensu* Biourge". Subsequently, Samson *et al.* (1977) broadened the concept of *P. chrysogenum* and placed *P. notatum* in synonymy with the former species. This transfer was later supported in several other works (Pitt 1980, Frisvad & Filtenborg 1989, Banke *et al.* 1997, Samson & Frisvad 2004). From this point on, Fleming's penicillin-producer was identified as *P. chrysogenum*. Our current study shows that the correct name of this strain is *P. rubens*.

Origin of the Fleming strain

Charles La Touche was working with moulds from indoor air and especially those isolated from cobwebs. He isolated hundreds of strains from poorer parts of London and Sheffield (Hare 1970, Kingston 2008) and a large percentage of them were *Penicillium chrysogenum* and *P. rubens*. Furthermore, subsequent research has shown that these might be the most common *Penicillium* in indoor air (Scott *et al.* 2004, Samson *et al.* 2010). La Touche's hypothesis was that these moulds could cause asthma, and this was later confirmed by, for example, Ward *et al.* (2010). It could very well be that one of La Touche's cultures from indoor air contaminated Fleming's plate, since they were working in the same building and Fleming always had his windows shut (Hare 1970). Both *P. rubens* and *P. chrysogenum* produce penicillin, and in that respect it is not surprising that *P. rubens* was present. It is noticeable that the historical important penicillin producers (Wisconsin strain NRRL 1951, Fleming's strain CBS 205.57 = NRRL 824 = IMI 015378, and the strain first used for producing penicillin in submerged conditions CBS 197.46), all proved to be *P. rubens*.

Current taxonomy and implications

Here we recognize the presence of two species in *Penicillium chrysogenum sensu lato*. The presence of four lineages

in *P. chrysogenum* was described by Scott et al. (2004). Scott's lineages 1 and 2 are *P. chrysogenum*, and lineage 4 corresponds to *P. rubens*. The status of lineage 3 is still under investigation. It was suggested by Scott et al. (2004) that lineage 4 (i.e. *P. rubens*) may have a competitive advantage over other lineages to exploit human-associated indoor niches and support for this hypothesis requires investigation of distribution patterns of members of this group in other geographic regions. The full genome sequenced strain derived from NRRL 1951 (van den Berg 2008) is also a *P. rubens*, and this shows that there is as yet no strain of *P. chrysogenum sensu stricto* full genome sequenced.

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