Fungal DNA barcoding in action: Two novel psychrotolerant species of *Penicillium* isolated from alpine soil

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INTRODUCTION

The mould genus *Penicillium* includes about 250 species; subgenus *Penicillium*, the most economically important part of the genus, includes 58 species. These species cause food spoilage (apples, oranges, garlic etc.), produce toxins in grain, but others are beneficial and are used in industry. Mould cheeses such as Roquefort, Brie, Camembert or Stilton are produced by some of these species, and *P. chrysogenum* is the commercial source of the antibiotic penicillin.

A recent taxonomic monograph of subgenus *Penicillium* (Samson & Frisvad 2004), with partial ß-tubulin sequences (*BenA*) used as the primary molecular marker, enabled the first feasibility study of *Cox1*-based DNA barcoding in fungi, recently published by Seifert *et al*. (2007). That paper showed that about 2/3 of the species of *Penicillium* subgenus *Penicillium* possess unique *Cox1* DNA barcodes. Here, two recently discovered species of this group are described based on unique morphological traits, extrolite (i.e. secondary metabolite) production and multiple gene sequencing.

MOLECULAR METHODS

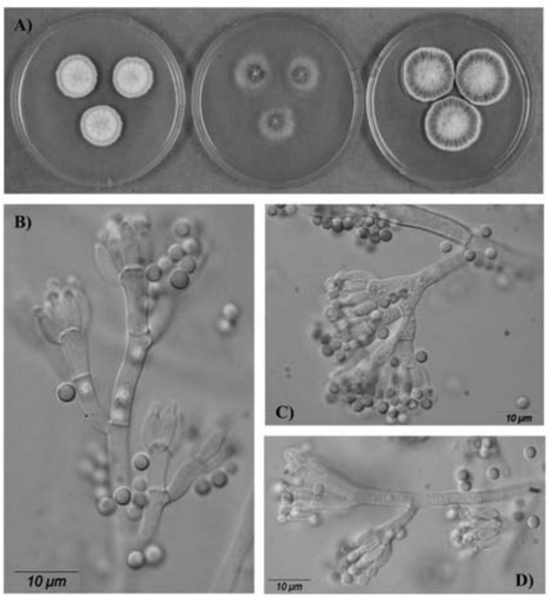
For morphological and metabolic characterization, strains were grown on Czapek yeast autolysate agar (CYA), Blakeslee's malt extract agar (MEA), yeast extract sucrose agar (YES), creatine sucrose agar (CREA) and oatmeal agar (OA) by incubating in the dark at 20 and 25°C.

The complete ITS and 5.8S rRNA genes were amplified and sequenced using the primers ITS5 and ITS4, with ITS2 and ITS3 primers used for cycle sequencing when necessary (White et al. 1990). The *Cox1* gene was amplified and sequenced using primers PenF1 and PenR1 (Seifert *et al*. 2007). Exons 3-6 of *BenA* were amplified using primers T1, T10 and T224 or T222 (O'Donnell & Cigelnik), and sequenced using primers Bt2a and Bt2b (Glass & Donaldson 1995). Consensus sequences were assembled using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI, USA).

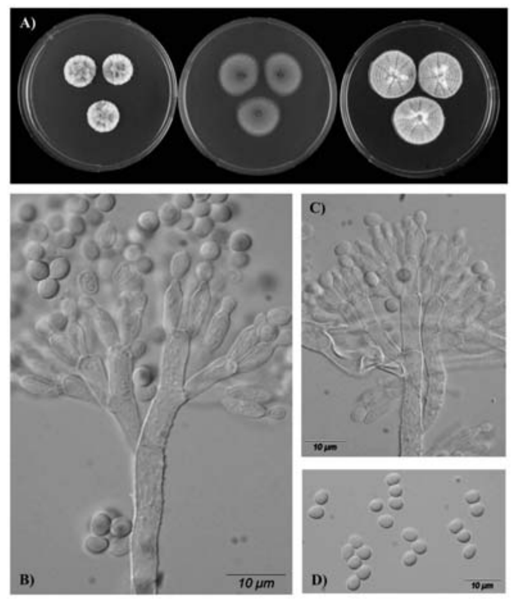
CHEMISTRY

HPLC analyses were performed following the methods of Frisvad & Thrane (1987, 1993) as modified by Smedsgaard (1997). Comparative data on UV spectra of the fungal metabolites are listed by Frisvad & Thrane (1993).

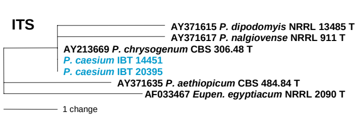
Strains of both new species produced consistent profiles of secondary metabolites. Several characteristic extrolites remain unidentified, and may represent novel chemistry. Strains of the new species *P. aquamarinum* consistently produced chaetoglobosin A and C, compactin, ML-236A, solistatin, solistatinol and roquefortine C and D. Additional characteristic extrolites include several members in the avrainvillamide/aspergamide biosynthetic family and two unidentified metabolites designated met K and territ. Strains of the new species *P. caesium* consistently produced several Raistrich phenols and two unidentified metabolites designated YELLO-1506 and bevan.



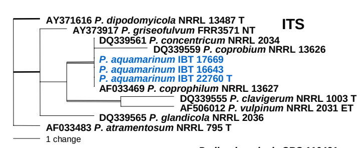
A, Colony morphology of *Penicillium caesium* after 7 days growth at 20 °C (from left to right: CYA, MEA and YES)-. **B**-**D**, *P. caesium* conidiophores and conidia.



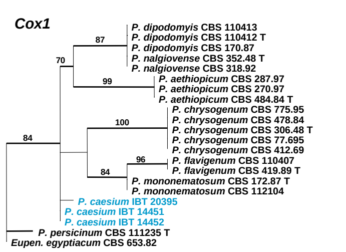
A, Colony morphology of *Penicillium aquamarinum* after 7 days growth at 20 °C (from left to right: CYA, MEA and YES)-. **B**,**C**, *P. aquamarinum* conidiophores and conidia.

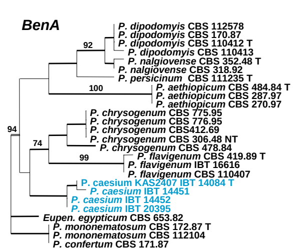


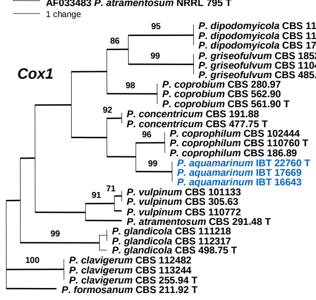
	ITS	CO1	BenA
Inf. chars.	9*	20	34
MPTs	1	9	22
Steps	10	29	53
CI	1.000	0.690	0.755
RI	1.000	0.909	0.906
RC	1.000	0.627	0.684
HI	0.000	0.310	0.245

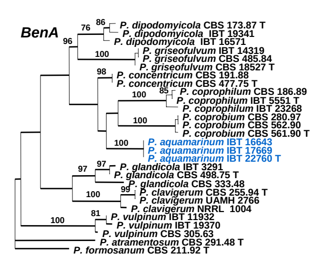


	ITS	CO1	BenA
Inf. chars	9	45	116
MPTs	33	4	6
Steps	16	86	247
CI	0.625	0.580	0.676
RI	0.647	0.845	0.829
RC	0.404	0.481	0.560
HI	0.375	0.430	0.324









Gene trees and tree statistics for the internal transcribed spacer (ITS), rRNA operon, and partial cytochrome oxidase 1 (*Cox1*) and beta-tubulin (*BenA*) gene sequences for the species complexes including the two new species *Penicillium caesium* (left) *and Penicillium aquamarinum* (right). Branches in bold occur in the strict consensus of the most parsimonious trees (MPTs), and the double lined branches occur in more than 75% of the MPTs. Ex-type cultures are indicated with T. *For the ITS analysis, all unique and informative characters were included, rather than just informative characters.

CONCLUSIONS

- The *Cox1* sequences of *P. aquamarinum* were invariant, whereas a single substitution was found among strains of *P. caesium.*
- The phylogenies for the species complexes including the two species with *Cox1* and *BenA* were slightly discordant, perhaps unsurprising when comparing mitochondrial and nuclear genes.
- Both *Cox1* and *BenA* provided superior resolution to the internal transcribed spacer of the rDNA (ITS).
- This is its first use of *Cox1* as part of the recognition and characterization of undescribed fungal species.

REFERENCES & ACKNOWLEDGMENTS

Glass N.L. and Donaldson G.C., 1995, Appl. Env. Microbiology 61 (4):1323–1330.
Frisvad JC, Samson RA (2004) Stud. Mycol. 49:1–174.
Frisvad, J. C. & Thrane, U. (1987). J. Chromatogr. 404: 195–214.
Frisvad, J. C. & Thrane, U. (1993). In Chromatography of Mycotoxins. Techniques and Applications, pp. 253–372, Journal Chromatography Library, vol. 54. Edited by V. Betina. Amsterdam: Elsevier.
O'Donnell, K. & Cigelnik, E. (1997). Mol. Phylogenet. Evol. 7: 103–116.
Seifert, K. A., R. A. Samson, J. R. deWaard, J. Houbraken, C. A. Lévesque, J.-M. Moncalvo, G. Louis-Seize, P. D.N. Hebert. 2007. Proc. Nat. Acad. Sci. 104: 3901–3906.
Smedsgaard, J. (1997). J. Chromatogr. A 760: 264–270.
White, T. J., Bruns, T., Lee, S. & Taylor, J. W. (1990). In PCR Protocols: a Guide to Methods and Applications, pp. 315–322. Edited by M. A. Innis, R. H. Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.

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