PCR-RFLP of ITS rDNA for the rapid identification of *Penicillium* subgenus *Biverticillium* species

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Summary RFLP of ITS rDNA is proposed as a useful tool for molecular identification of the most common species of biverticillate penicillia. 60 isolates were analysed representing 13 species and 21 unique sequences were produced. The combination of five restriction enzymes was successful in separating 12 species. However, the variety *Penicillium purpurogenum* var. *rubrisclerotium* remained indistinguishable from *Penicillium funiculosum*. *P. funiculosum* appeared as the most confused species, being mis-identified with *Penicillium miniolutum* and *Penicillium pinophilum,* which were originally part of the species, and with *P. purpurogenum* perhaps because of the common production of red pigment. *Penicillium variabile* was difficult to investigate as introns were found on half of the isolates. *Penicillium piceum, Penicillium rugulosum, Penicillium loliense, Penicillium erythromellis* and *P. purpurogenum* were homogeneous from molecular and morphological positions and corresponded to a well circumscribed taxon. Furthermore, intraspecific variability was evidenced within *P. pinophilum* and *P. funiculosum.* The ex-type isolate of *P. funiculosum* produced a unique pattern. The method is sensitive, rapid and inexpensive and can be used for isolate identification of the biverticillate species. It is recommended particularly when many isolates have to be authentificated prior to analysis for phylogenetic assessment or population genetics.

Key words *Penicillium,* Biverticillate penicillia, Rapid identification, PCR-RFLP

Biverticillate penicillia include many species which affect humans. This includes (a) *Penicillium marneffei* the fatal pathogen of immunocompromised people, (b) mycotoxigenic species contaminating food, (c) species used in biotechnology to produce metabolites or enzymes, and (d) tester strains for biodegradation abilities. They are primarily associated with soil or moist decaying vegetation [19] and most can degrade different forms of cellulose (e.g. *Penicillium variabile, Penicillium rubrum, Penicillium rugulosum, Penicillium funiculosum, Penicillium purpurogenum* and *Penicillium minioluteum*). Some can degrade other macromolecules such as starch and pectin [2,5]. Related to the above, they can be high producers of cellulase, dextranase, and ß-glucosidase. *P. funiculosum* and *Penicillium pinophilum* are used as tester strains for the biodeterioration of papers, wall covering adhesives, paints and textiles. Biverticillate penicillia are usually uncommon in foods, however six species are listed in [19] indicating they occur. Most are reported from cereals, beans and nuts. Mycotoxin production is documented for *P. funiculosum* (patulin), *P. rubrum* (rubratoxin), *P. rugulosum* and *P. variabile* (both rugulosin) and *Penicillium islandicum* (cyclochlorotine, islanditoxin, luteoskyrin and erythoskyrin). *Penicillium piceum, P. purpurogenum* and *P. rugulosum* are also reported as human pathogens [4].

Biverticillate penicillia are considered to be a homogeneous subgenus, with regards to their phenotype, teleomorphic states and ecology. The most readily recognized feature is the biverticillate and symmetrical penicillus, with metulae in appressed or divergent verticils bearing typical acerose phialides tapering to narrow apical pores. Colony pigmentation is of taxonomic value and growth rate on G25N is highly correlated with penicillus type, with the colony diameter never exceeding 10 mm [19]. Confusion concerning *P. funiculosum* and related species was clarified after morphological examination of numerous isolates in comparison to reference material by Van Reenen-Hoekstra et al. [27]. However, the identification of *Penicillium* species using phenotypic characters remains subjective when identifying strains individually and disconnected from the context of other representative strains. Moreover, when subcultured several times or maintained for years in culture collections, some strains may loose typical morphology and became impossible to recognize. Consequently a more relevant and applicable taxonomy is required for the genus. Solutions have been proposed for the subgenus *Penicillium* especially with regards to myco-

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toxin production [16] and a polyphasic taxonomy is now available [7]. On the basis of 18S rDNA sequences, the genus *Penicillium* was demonstrated to be polyphyletic. Two clades were identified, one including *Talaromyces* species with *Penicillium* anamorphs of the subgenus *Biverticillium*, while the other clade contains *Eupenicillium* species and *Penicillium* anamorphs from subgenera *Penicillium*, *Furcatum* and *Aspergilloides* [14]. ITS and partial large subunit rDNA were useful in providing an infrageneric phylogenetic structure of the genus [17]. But for the purpose of species delimitation, ITS was demonstrated to be invariant in subgenus *Penicillium* [1,24,25]. ß-tubulin, successfully tested on a preliminary set of species in sect. *Viridicata* [23], were further used for the complete investigation of the subgenus *Penicillium* [22].

The present study was engaged to search for molecular characters to assist with the rapid identification of the major species in subgenus *Biverticillium,* following the methodology used for *Phaeoacremonium* [6]. This approach is part of an on-going programme of authentification of all the *Penicillium* isolates deposited in the Laboratoire Cryptogamie Paris collection (LCP), with a view to conform to the high quality standards recommanded for Biological Resources Centres.

Materials and methods

Isolates. Isolates included in this study (Table 1) represent 13 of the most common species described in *Penicillium* subgenus *Biverticillium*. Several isolates were investigated for each species and compared to the type specimen or to strains considered as typical of the species. Some isolates were obtained from CBS (Centraalbureau voor Schimmelcultures), IMI (International Mycological Institute), MUCL (Mycothèque de l'Université Catholique de Louvain-La-Neuve) and CMPG (Collection de Mycologie de Pharmacie de Grenoble) culture collections and are now maintained at the culture collection of the Muséum National d'Histoire Naturelle (LCP).

Culture conditions. Cultures were grown at 25 °C for seven days according to the standardized regime described by Pitt [18]. The three standard media, Malt Extract Agar (MEA), Czapek Yeast Extract Agar (CYA) and 25% Glycerol Nitrate Agar (G25N), are described in [18]. Cultures were also grown at 5 °C and 37 °C for CYA.

Phenotypic studies. Colony morphologies and growth were examined on all plates at seven days, and general morphological features were observed microscopically from MEA.

DNA extractions, PCR amplifications and sequencing. Genomic DNA was extracted from fresh mycelium grown on MA (Malt Extract 2%, Agar 2%) for two days using a CTAB (hexadecyltrimethylammonium bromide) micropreparation method [21]. PCR was performed in 50 µl reactions, using 25 µl of template DNAs, 1.25 units of Taq DNA polymerase (Q-BIOgene, Illkirch, France), 5 µl of 10X Taq DNA Polymerase buffer, 5 µl of 50% glycerol, 2 µl of 5 mM dNTPs (Eurogentec, Seraing, Belgium), 2 µl of each 10 µM primer and 50-100 ng template DNA. The oligonucleotide primer set ITS4 and ITS5 [28] was used to amplify the ITS region of the rDNA. Amplifications were performed on a Perkin Elmer Cetus thermal cycler model 2400 using the following parameters: a 4 min step at 94 °C, followed by 30 cycles of 15 s at 94 °C, 15 s at 50 °C and 20 s at 72 °C, and then a final 2 min extension step at 72 °C. DNA sequencing was performed on both strands using the ABI PRISM TM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with amplification primer sets and internal primers ITS2 and ITS3 for the ITS region [28]. Sequencing assays were analysed on an automated DNA Sequencer ABI PRISM 377. The nucleotide sequences were aligned using the BioEdit programme.

Restriction enzyme digestions. Full restriction maps of the ITS sequences were defined using the BioEdit programme for the type strains of the different species. The combination of five enzymes, amongst the most common, *Ava* I, *Ava* II, *Hae* III, *Hha* I and *Rsa* I was necessary to differentiate the species under study (Table 2, a). The different restriction patterns expected were defined and codified in table 2 (b) and the patterns observed for all the isolates examined are reported in Table 1. Aliquots of 10 µl of the amplified DNAs were digested with two units of *Ava* I, *Ava* II, *Hae* III, *Hha* I (Qbiogène, Illkirch, France) and *Rsa* I (Fermentas/Euromedex, Mundolsheim, France) following the manufacturer's directions. All the enzymes gave complete digestion of DNAs in the PCR buffer, making it unnecessary to purify the DNA prior to digestion. The restriction fragments were separated on 2% "small fragments" agarose gels (Eurogentec), stained with ethidium bromide (10 µg/µl) and photographed under UV light. The molecular size markers were the "Superladder-low 20 bp or 100 bp Ladder" (Eurogentec).

Restriction profiles analysis. Restriction profiles were compared based on the molecular weight of the bands correlated to the presence or absence of restriction sites (deduced from the theoretical restriction profiles obtained from ITS sequences of reference isolates, described in table 2). Bands were scored by direct visual observation as present (1), absent (0), uncertain or missing (?) then encoded in a data matrix. Unweighted pair group cluster analysis using arithmetic means (UPGMA) was used to examine the isolate identification with reference to ex-type strains of the *Penicillium* subgenus *Biverticillium* species.

Results

PCR amplification of the ITS region resulted in a single product of approximately 620 bp for all isolates. Exception were LCP 90.2602, LCP 00.4464, FRR 1868, CMPG 610 and CMPG 244 which produced a fragment of 1000 bp, including an intron in the ITS1 (data not shown). The PCR fragments from 60 isolates were digested with five restriction enzymes: *Ava I, Ava II, Hae III, Hha I* and *Rsa I*. An example of representative patterns obtained with *Hha* I for the different species is shown on figure 1. Bands less than 50 bp in size were difficult to visualise under the electrophoresis conditions used.

The restriction method successfully identified most of the species under study *Penicillium aculeatum, Penicillium erythromellis, P. islandicum, P. minioluteum, P. piceum, P. purpurogenum, P. rubrum, P. rugulosum, P. variabile* and *Penicillium verruculosum*. An intraspecific variability was observed within *P. funiculosum* and *P. pinophilum. P. purpurogenum* var. *rubrisclerotium* was indistinguishable of the predominant genotype observed for *P. funiculosum.*

Among the 60 isolates analysed, the identification of 35 was confirmed by molecular data in relation to morphological characters. Twenty one were mis-identified. Five were impossible to analyse by this method because of the presence of introns in ITS1, possibly containing supplementary restriction sites which modify the restriction patterns expected. Mis-identifications were principally observed within (a) *P. funiculosum*, (b) *P. minioluteum,* (c) *P. pinophilum* and (d) *P. aculeatum* which were con-

Table 1. *Penicillium* isolates used in this study. Restriction patterns and identifications deduced from this analysis.

Collection no.	Received as	Substrate	Origin	GenBank no.	Ava I	Ava II	Hae I	Hha I	Rsa I	Actual identity
FRR 1290	*P. variabile* Sopp	Sandstone	Tasmania, Australia	AY393736	A	C	E	E	B	*P. variabile*
LCP 78.3162	*P. variabile* Sopp	Plasterboard	Lille, France		A	C	E	E	B	*P. variabile*
FRR 1055	*P. variabile* Sopp	Unrecorded source	California	L14507	C	C	J	E	B	*P. variabile*
FRR 1339	*P. islandicum* Sopp	Animal food	Australia	L14504	A	O	E	D	B	*P. islandicum*
CBS 642.68	*P. minioluteum* Dierckx	Unrecorded source	Unrecorded source	AY213674	B	G	L	H	B	*P. minioluteum*
MUCL 28666	*P. minioluteum* Dierckx	Unrecorded source	Unrecorded source		B	G	L	H	B	*P. minioluteum*
CBS 996.72	*P. funiculosum* Thom	Jute sugar bag	Netherlands		B	G	L	H	B	*P. minioluteum*
LCP 96.3962	*P. variabile* Sopp	Lemon sauce	Apt, France		B	O	L	H	B	*P. minioluteum*
LCP 03.4583	*P. variabile* Sopp	Moraine soil	Svalbard, Norway		B	G	L	H	B	*P. minioluteum*
LCP 04.5205	*Penicillium* sp.	Fresco	France		B	G	L	H	B	*P. minioluteum*
LCP 71.2101	*P. minioluteum* Dierckx	Laboratory contaminant	Paris, France		B	G	L	H	B	*P. minioluteum*
BSM 102-C-18	*P. piceum* Raper et Fennell			AY787846	A	C	K	F	B	*P. piceum*
LCP 70.2082	*P. piceum* Raper et Fennell	Animal food	Unrecorded source		A	C	K	F	B	*P. piceum*
LCP 89.3573	*P. piceum* Raper et Fennell	Soil under strawberry plants	Jouy-en-Josas, France		A	C	K	F	B	*P. piceum*
CMPG 358	*P. piceum* Raper et Fennell	Incubator floor	Ploufragan, France		A	C	K	F	B	*P. piceum*
LCP 03.4582	*P. pinophilum* Hedgcock	Moraine soil	Svalbard, Norway		A	C	K	F	B	*P. piceum*
MUCL 38543	*P. pinophilum* Hedgcock	Polyvinyl chloride	France		C	B	A	A	A	*P. pinophilum*
Unknown	*P. pinophilum* Hedgcock			AF176660	C	B	A	A	A	*P. pinophilum*
LCP 56.1698	*P. funiculosum* Thom	Sand	Cote de Siam, Vietnam		C	A	A	A	A	*P. pinophilum*
LCP 56.1627	*P. funiculosum* Thom	Soil	Algeria		C	A	A	A	A	*P. pinophilum*
IMI 211742	*P. funiculosum* Thom	Unrecorded source	Massachusetts, USA		C	B	A	A	A	*P. pinophilum*
LCP 90.2604	*P. pinophilum* Hedgcock	Soil	Bahamas		A	B	A	A	A	*P. pinophilum*
CMPG 563	*P. pinophilum* Hedgcock	Flour	Grenoble, France		A	A	A	A	A	*P. pinophilum*
CMPG 505	*P. pinophilum* Hedgcock	Alluvium soil	Cormoran cave, France		A	A	A	A	A	*P. pinophilum*
CMPG 1507	*P. pinophilum* Hedgcock	Water from dam	Rabat, Maroc		A	A	A	A	A	*P. pinophilum*
LCP 88.3569	*P. pinophilum* Hedgcock	Grazing soil	Uruguay		A	B	A	A	A	*P. pinophilum*
LCP 86.3433	*P. verruculosum* Peyronel	Soil	Yaounde, Cameroon		A	A	A	A	A	*P. pinophilum*
MUCL 29233	*P. pinophilum* Hedgcock	Soil under *Zea mais*	Virginia, USA		A	A	A	A	A	*P. pinophilum*
IMI 216901	*P. loliense* Pitt	*Lolium* sp.	New Zealand		A	C	I	N	A	*P. loliense*
LCP 81.2762	*P. loliense* Pitt	Soil	Uruguay		A	C	I	N	B	*P. loliense*
FRR 1898	*P. erythromellis* Hocking	Soil	Australia		Intron	Intron	Intron	Intron	Intron	*P. erythromellis*
LCP 73.2253	*P. islandicum* Sopp	Desertic soil	Egypt		D	D	I	G	A	*P. erythromellis*
LCP 93.3589	*P. erythromellis* Hocking	Soil	Malaysia		D	D	I	G	B	*P. erythromellis*
LCP 94.3727	*P. erythromellis* Hocking	Soil	Malaysia		D	D	I	G	B	*P. erythromellis*
MUCL 38968	*P. funiculosum* Thom	*Lagenaria vulgaris*	India		B	B	I	A	A	*P. funiculosum*
LCP 84.3383	*P. purpurogenum* var. *rubrisclerotium* Thom	Kolatier leave	Ivory Coast		A	A	A	A	A	*P. purpurogenum* var. *rubrisclerotium*
LCP 00.4464	*P. purpurogenum* var. *rubrisclerotium* Thom	Soil from Parana delta	Argentina		Intron	Intron	Intron	Intron	Intron	*P. purpurogenum* var. *rubrisclerotium*
LCP 78.3189	*P. purpurogenum* var. *rubrisclerotium* Thom	Caroube pod	Crete, Greece		A	A	A	A	A	*P. funiculosum*
CMPG 567	*P. funiculosum* Thom	Flour	Grenoble, France		A	A	A	A	A	*P. funiculosum*
CMPG 821	*P. funiculosum* Thom	Incubator floor	Ploufragan, France		A	A	A	A	A	*P. funiculosum*
CMPG 177	*P. purpurogenum* Stoll	Dead spider	Cormoran cave, France		A	A	A	A	A	*P. funiculosum*
CMPG 253	*P. purpurogenum* Stoll	Walnut	St Marcellin, France		A	A	A	A	A	*P. funiculosum*
LCP 87.3516	*P. funiculosum* Thom	Gladiolus stem	Poitiers, France		A	A	N	A	A	*P. funiculosum*/*P. rubrum*
FRR 1823	*P. funiculosum* Thom	[illegible]	Australia	L14503	A	A	A	A	A	*P. funiculosum*
CMPG 334	*P. purpurogenum* Stoll	Atmosphere	Meylan, France		A	A	A	A	A	*P. funiculosum*
CMPG 811	*P. purpurogenum* Stoll	Crab shell	Malmö, Sweden		A	A	A	A	A	*P. funiculosum*
CMPG 757	*P. minioluteum* Dierckx	Crab shell	Malmö, Sweden		A	A	A	A	A	*P. funiculosum*
CMPG 527	*P. minioluteum* Dierckx	Alluvium soil	Cormoran cave, France		A	A	A	A	A	*P. funiculosum*
CMPG 556	*P. minioluteum* Dierckx	Flour	Grenoble, France		A	A	A	A	A	*P. funiculosum*
CMPG 84	*P. minioluteum* Dierckx	Soil	Toulouse, France		A	A	A	A	A	*P. funiculosum*
FRR 1714	*P. rubrum* Stoll			L14509	A	A	B	A	A	*P. rubrum*
LCP 04.4941	*P. funiculosum* Thom	Silicone	France		A	A	I	A	A	*P. rubrum*
MUCL 31201	*P. rugulosum* Thom	Tuber of *Solanum tuberosum*	Connecticut, USA		A	I	[illegible]	C	B	*P. rugulosum*
CMPG 516	*P. rugulosum* Thom	Limestone	Cormoran cave, France		A	F	H	O	B	*P. rugulosum*
LCP 83.1920	*P. rugulosum* Thom	Sand	Mont des Récollets, France		A	F	H	O	B	*P. rugulosum*
LCP 96.3957	*P. rugulosum* Thom	Cosmetic	Chevilly La Rue, France		A	F	H	O	B	*P. rugulosum*
DAOM 215361	*P. rugulosum* Thom	Building timber	Canada	AY787845	A	I	[illegible]	C	B	*P. rugulosum*
FRR 1061	*P. purpurogenum* Stoll	Unrecorded source	Ceylon	AY393726	B	D	F	I	B	*P. purpurogenum*
LCP 90.2600	*P. purpurogenum* Stoll	Soil	Grand Bahamas		B	D	F	I	B	*P. purpurogenum*
LCP 92.2806	*P. purpurogenum* Stoll	Lecithine	Unrecorded source		B	D	F	I	B	*P. purpurogenum*
LCP 92.2929	*P. purpurogenum* Stoll	Natural zeolite	Paris, France		B	D	F	I	B	*P. purpurogenum*
LCP 04.5204	*P. purpurogenum* Stoll	Soil	Svalbard, Norway		B	D	F	I	B	*P. purpurogenum*
CMPG 776	*P. purpurogenum* Stoll	Domestic waste	Grenoble, France		B	O	F	I	B	*P. purpurogenum*
LCP 95.4196	*P. purpurogenum* Stoll	Honey	China		B	D	F	I	B	*P. purpurogenum*
NRRL 2129	*P. aculeatum* Raper & Fennell	Textile	Florida, USA	AF033397	A	B	A	A	B	*P. aculeatum*
MUCL 38603 (= NRRL 2130)	*P. aculeatum* Raper & Fennell	Textile	Florida, USA		A	B	A	A	B	*P. aculeatum*
LCP 56.1697	*P. verruculosum* Peyronel	Sandy soil	Angkor, Cambodia		A	B	A	A	B	*P. aculeatum*
LCP 57.1353	*P. verruculosum* Peyronel	Unrecorded source	Unrecorded source		A	B	A	A	B	*P. aculeatum*
ATCC 62396	*P. verruculosum* Peyronel	*Eucalyptus marginata*	Australia	AF510496	C	B	C	A	A	*P. verruculosum*
MUCL 38731	*P. verruculosum* Peyronel	Soil	Texas, USA		C	B	C	A	A	*P. verruculosum*
LCP 90.2602	*P. variabile* Sopp	Soil	Bahamas		Intron	Intron	Intron	Intron	Intron	?
CMPG 610	*P. variabile* Sopp	Fruit juice bottle cap	Marseille, France		Intron	Intron	Intron	Intron	Intron	?
CMPG 244	*P. variabile* Sopp	Walnut	St Marcellin, France		Intron	Intron	Intron	Intron	Intron	?

[T] ex-type isolate; MUCL 29235 type of *P. purpurogenum* var. *rubrisclerotium* Thom.
[G] Sequences obtained from GenBank.
[S] Sequences produced in the laboratory for this study.
[N] Not unique profile.

Table 2. Theoretical restriction patterns deduced from ITS rDNA sequences of *Penicillium* species analysed in this study (a). Profiles (A, B, C, ...) are detailed for each enzyme (b).

(a) Restriction patterns of ITS rDNA for the species under study.

Species	*Ava* I	*Ava* II	*Hae* III	*Hha* I	*Rsa* I
P. aculeatum AF033397	A	B	A	A	B
P. erythromellis LCP 93 3691	D	D	I	G	B
P. funiculosum L14503	A	A	A	A	A
P. funiculosum MUCL 38968	B	B	B	A	A
P. islandicum L14504	A	C	E	D	B
P. minioluteum AY213674	B	G	J	H	B
P. piceum AY787846	A	C	K	F	B
P. pinophilum AF176660	C	B	A	A	A
P. purpurogenum AY393726	B	D	F	I	B
P. purpurogenum var. *rubrisclerotium* LCP 84 3383	A	A	A	A	A
P. rubrum L14505	A	A	B	A	A
P. rugulosum AY787845	A	F	H	C	B
P. variabile AY393736	A	C	E	E	B
P. variabile L14507	C	C	D	E	B
P. verruculosum AF510496	C	B	C	A	A

(b) Detailed profiles obtained with the five enzymes used. Restriction sites are numbered from 1 to 10 (depending on the enzyme) according to the 5' - 3'direction af the DNA fragment. For each profile, the presence of the site (1) and the expected length of the fragments produced after digestion are mentioned.

Ava I		*1*		*2*		*3*		*4*	
A	155-165	1	270	1	43	1	27	1	107-137
B	139-168	1		314-325		1	27	1	107-129
C		427		1	43	1	27	1	107-124
D			470			1	27	1	107

Ava II		*1*		*2*		*3*		*4*		*5*		*6*		*7*	
A			420					1	11	1	33	1		140	
B			420					1	11	1	34	1	39	1	100
C					465/474							1		139/156	
D	40	1	12	1		353		1	19	1	36	1		160	
E		154				1	263	1	18	1	35	1		141	
F		168				1			305			1		168	
G	77			1		353		1	19	1	36	1		139	

Hae III		*1*		*2*		*3*		*4*		*5*		*6*		*7*		*8*		*9*		*10*	
A	65	1	9	1	45	1	10	1		34		1					445				
B	63	1	9	1	45	1			44			1					445				
C	66	1		54		1	10	1		32							440				
D		118				1	10	1	5	1	28	1					459				
E		118				1	10	1	5	1	28	1			376			1		84	
F		122				1			44			1	357	1	14	1			81		
G		125				1								504							
H		123				1	10	1	5	1					415					1	88
I		112				1			46			1					446				
J	64	1		58		1			45			1	356	1	14	1			87		
K		123				1	10	1		37		1					443				

Hha I		*1*		*2*		*3*		*4*		*5*		*6*		*7*		*8*		*9*	
A	164-170			1	12	1	167	1						255-260					
B	179			1	12	1	169	1				177				1		90	
C	175			1	12	1	166	1			111			1	60	1		117	
D	166			1	12	1	166	1	55	1	17	1	38	1	61	1		106	
E	166			1		178		1		72		1	38	1	61	1	35	1	70
F	176			1		178		1			111			1			148		
G		172				1	167	1						265					
H	157	1	14	1	14	1	165	1						274					
I	136	1	14	1	12	1	168	1	55	1					235				

Rsa I Uncut : A, cut at site 215 : B

fused with (a) *P. purpurogenum* and *P. minioluteum*, (b) *P. funiculosum* and *P. variabile*, (c) *P. funiculosum* and *P. verruculosum,* (d) *P. verruculosum* respectively. The other species were correctly identified.

Divergent patterns were observed within the species:

– *P. pinophilum,* where half of the isolates were ABAAA and not CBAAA, which was the pattern expected from the GenBank reference AF176660 and observed in the ex-type isolate. This was because of a mutation from nucleotide T to C restoring the first *Ava* I site (position 156) present in the profile A. The ex-type of *P. purpurogenum* var. *rubrisclerotium* MUCL 29225, and *P. funiculosum* IMI 211.742 were ABAAA and were actually *P. pinophilum* as indicated in [27].

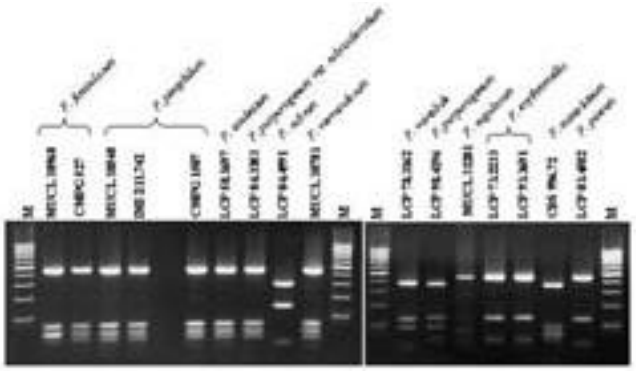

Figure 1. ITS restriction profiles of some *Penicillium* isolates using *Hae* III with reference to type strains. M: 1000bp molecular marker.

– *P. funiculosum*, where the ex type isolate MUCL 38968, was a BBBAA pattern, whereas all other isolates had the AAAAA pattern deduced from the sequence of FRR 1823 which was recognized as representative of the species by Pitt [14]. The isolates LCP 78.3189 and 84.3383 which were identified as *P. purpurogenum* var. *rubrisclerotium* shared the majority pattern of *P. funiculosum*. Only 84.3383 still produce numerous dark reddish brown sclerotia typical of the variety. The isolate LCP 87.3516 exhibited a unique pattern with *Hae* III which remained undetermined (N in table 1) because the sequencing on both strands failed. This isolate will be treated subsequently using the ß-tubulin gene to estimate the intraspecific variability within the *P. funiculosum* species.

The phenogram (Figure 2) produced by UPGMA analysis of the ITS rDNA restriction patterns data of isolates listed in table 1 (isolates showing introns were not included) demonstrates species assignation and distributes the species into three clusters: 1) *P. funiculosum, P. purpurogenum* var. *rubrisclerotium, P. rubrum, P. pinophilum, P. aculeatum* and *P. verruculosum*, 2) *P. minioluteum, P. purpurogenum* and *P. erythromellis*, and 3) *P. piceum, P. loliense, P. rugulosum, P. variabile* and *P. islandicum*.

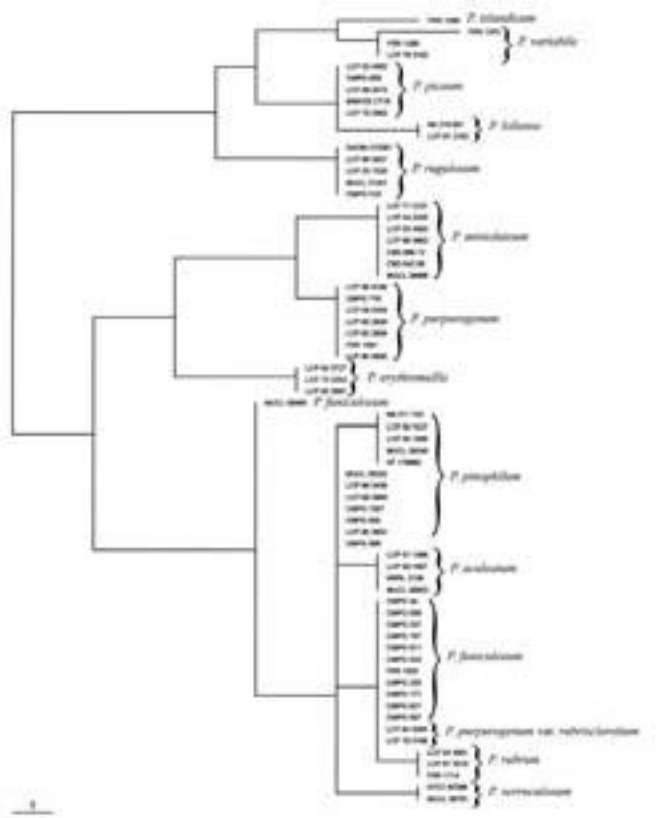

Figure 2. Phenogram produced by UPGMA cluster analysis based on ITS rDNA PCR-RFLP data to compare and identify isolates of *Penicillium* subgenus *Biverticillium*. Horizontal branches of the tree are drawn to scale to reflect genetic distance according to restriction sites matching (1 = one differing character).

Discussion

Reliable identifications are crucial for all scientific work using biological material. Penicillia are particularly difficult to identify on the base of classical characters [16], and an effective concept of morpho-species can be acquired only when all the species are compared together, using more than one isolate of each species and in comparison to reference material. The ITS rDNA based PCR-RFLP assay developed in this study successfully differentiated the 12 biverticillate penicillia species analysed, although some apparent discrepancies were observed.

Among the taxa analysed, the variety *P. purpurogenum* var. *rubrisclerotium* was indistinguishable from *P. funiculosum*. However, it would have been expected to be similar to *P. purpurogenum* or to *P. pinophilum*. Thom [26] described this variety as differing from the typical description of the species by the production of dark-red sclerotia. When Pitt observed the original material [18], sclerotia were not produced and the fungus was typical of *P. pinophilum* and so this variety was reduced in synonymy with this species. Van Reenen-Hoekstra et al. [27] reported that nine isolates were unique morphologically and chemically and a new species was suggested. This will be evaluated by sequencing more isolates in our laboratory in future research.

P. rubrum which was synonymised with *P. purpurogenum* by Pitt [18] shows a distinct pattern AABAA, confirming the morphological and chemical differences observed by Van Reenen-Hoekstra et al. [27]. The isolates observed by these authors [27] could usefully be investigated before any further re-assessments are made.

P. variabile was not fully explored in the present study as half of the isolates contained introns. In addition, two were apparently mis-identified as *P. minioluteum*. Further work will be undertaken in this often encountered group [18]. Introns are common in SSU rRNA of fungi [9] and ß-tubulin gene will be used preferably.

P. funiculosum sensu lato [26] included *P. minioluteum* and *P. pinophilum*, which are now considered to be separate [18,27]. This partition is confirmed by the distinct profiles evidenced in the present study (i.e. AAAAA for *P. funiculosum*, BGJHB for *P. minioluteum* and C/ABAAA for *P. pinophilum*).

Further, this assay detected intraspecific genetic variability at different levels. Sporadic variations were observed in some isolates within the *P. funiculosum* species for example, and two sub-species groups were apparent in *P. pinophilum*. Further work is necessary to understand the meaning of this variability and more isolates will be sequenced by the use of ITS and ß-tubulin genes.

During this study, we found well circumscribed taxa by molecular and morphological methods: *P. piceum, P. rugulosum, P. loliense, P. erythromellis* and *P. purpurogenum*. The distinction between *P. aculeatum* and *P. verruculosum* remains unclear perhaps because very few isolates were investigated. *P. aculeatum* is an uncom-

mon species showing a close morphological affinity with *P. verruculosum* [18].

In conclusion, this assay is a routine, sensitive, rapid and reliable alternative to morphological identification of these fungi, providing that their subgenus affiliation was previously obtained by morphological observation, as also recommended in [16]. It could be very useful in studies comparing a large sample of isolates, where sequencing cannot reasonably be undertaken. For identification of a single isolate sequencing is preferable. However, reference sequences in ITS rDNA and ß-tubulin are not available for most penicillia species in public databases. Complete tubulin data are restricted to subgenus *Penicillium*. Furthermore, as a number of deposited sequences originate from material which was not taxonomically authenticated, blast analysis within GenBank have to be carefully interpreted. In connection with this, Hawksworth encouraged authors of sequences to verify identifications by specialists and to deposit voucher specimens or permanently preserved cultures in public reference collections [10].

This present work indicated the imperative to examine many isolates for systematic revisions, particularly for investigating phylogenies. This is required because of possible mis-identifications or contaminations, and also studies have revealed cryptic species within fungi where morphologically identical taxa are demonstrated to be different genetically, e.g. human pathogens [11-13], plant pathogens [15] and toxigenic fungi [3,8].

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