Multilocus DNA sequence analysis shows that *Penicillium biourgeianum* is a distinct species closely related to *P. brevicompactum* and *P. olsonii*

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*Penicillium brevicompactum* and other isolates with the compact, complex conidiogenous apparatus typical of the species were sequenced in one ribosomal and two protein coding regions. The aligned DNA sequences were analyzed by maximum parsimony and the data from different loci were tested for compatibility using the partition homogeneity test. Analysis of each of the three loci revealed three clades corresponding to *P. brevicompactum*, *P. olsonii* and *P. biourgeianum*. Using the phylogenetic species concept and the genetic isolation of the clades, *P. hagemi*, *P. patrismei*, *P. stoloniferum* and *P. griseobrunneum* are all synonyms of *P. brevicompactum*. *P. volgaense* is a synonym of *Penicillium olsonii*, while *P. biourgeianum* is a distinct species closely related to *P. brevicompactum* and *P. olsonii*. Phenotypic distinctions between the species are mostly based on colony characteristics such as colour. *P. bialowiezenze*, often treated as a synonym of *P. brevicompactum*, is most closely related to *P. polonicum*.

**INTRODUCTION**

Recently I obtained a number of *Penicillium* strains newly isolated by Donald T. Wicklow from decaying fungal fruit bodies, and initial examination of the identity of the isolates based on the ITS and large subunit rDNA sequences suggested that some of them represented undescribed taxa. In an effort to verify the novelty of the newly collected isolates, I began examining type cultures, where available, of *Penicillium* species, including those that have been relegated to synonymy by one or more recent authorities (Raper & Thom 1949, Pitt 1980, Ramirez 1982).

One particular group of isolates appeared to be a new species and its DNA sequence was most closely related to that of *P. brevicompactum*. Accordingly, several of the species that have been assigned to synonymy with *P. brevicompactum* were sequenced and compared to the putative new species. Because prior study has shown that *P. olsonii* is also closely related to *P. brevicompactum*, isolates representing that species were also included in this study.

Single locus sequencing is useful for rapidly finding the phylogenetic placement of isolates (Peterson 2000), but it does not show the genetic isolation of isolates that is required in the phylogenetic species concept (Taylor *et al.* 1999). In order to investigate the species boundaries of the isolates near *P. brevicompactum*, multiple loci were sequenced. The data were analyzed using maximum parsimony and the partition homogeneity test to establish whether the putative new species was supported by congruent trees from all loci.

**MATERIALS AND METHODS**

The isolation or history of each isolate used in this study is listed in Table 1. For phenotypic examinations, isolates were cultured using the media and growth conditions specified by Pitt (1980). The Ridgway (1912) colour guide was used to assess colours and give them names. Light microscopic examinations were made on specimens teased apart in a drop of 0.5% Kodak photoflo. Pictures of specimens were taken with a Kodak 420B digital camera either with close-up lenses or by attachment of the camera to a Zeiss light microscope. Scanning electron microscopy was performed on samples fixed overnight in osmium tetroxide, dehydrated in a series of acetone rinses of increasing concentration, critical-point dried, and sputter coated with gold-palladium, in a JEOL scanning electron microscope following the procedure of Peterson (1992). Digital images from both sources were fitted into composite plates using Adobe Photoshop 6.01.

Cultures for use in DNA extraction were grown on malt extract agar (MEA) slopes for 5–7 d. Biomass was scraped from the surface, along with small amounts of agar, and placed in a 15 ml disposable screw-cap
Table 1. Origin or history of the *Penicillium* isolates used in this study.

<table>
<thead>
<tr>
<th>NRRL no.</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>865</td>
<td>Poland: Forest soil. Received 1928, from Westerdijk, Baarn, Holland. Zaleski’s ‘type’ culture of <em>Penicillium biouregiunum</em>.</td>
</tr>
<tr>
<td>862</td>
<td>Unknown: Received from da Fonseca, as <em>Penicillium pataum</em> from the Bainier collection, 1922.</td>
</tr>
<tr>
<td>2013</td>
<td>Germany: Nuremberg. Isolated from mushroom spawn. Received from CBS, 1948.</td>
</tr>
<tr>
<td>28149</td>
<td>USA: Isolated July 1997 by D. T. Wicklow, from a dead agaric on logs collected by H. D. Thiers.</td>
</tr>
<tr>
<td>32187</td>
<td>Panama: Isolated from textile sample, by W. H. Weston, 1944.</td>
</tr>
<tr>
<td>32239</td>
<td>USA: California: Isolated from a <em>Sequoia</em> cone scale by J. G. N. Davidson, 1971.</td>
</tr>
<tr>
<td>32240</td>
<td>USA: Maryland: Bethesda. Received from K. J. Kwon-Chung, 1971.</td>
</tr>
</tbody>
</table>

**Penicillium biouregiunum** Bainier 1927

- Received Jan. 1946 as *P. brevicompactum* Dierckx from CBS. Ex-neotype.
- 860 USA: Vermont. Isolated from maple sugar by Charles Thom, 1908.
- 864 Unknown: Received from Biorge as his culture number 42, 1922.
- 866 Poland: Soil under conifers. Received 1928, from Westerdijk, Baarn, The Netherlands. Zaleski’s ‘type’ culture of *P. hagemii* Zaleski 1927.
- 867 Unknown: Received 1924, from Biorge (no. 148). Ex-neotype culture of *P. griseobrunneum* Dierckx.
- 868 Poland: Soil under conifers. Received 1928, from Westerdijk, Baarn, The Netherlands. Zaleski’s ‘type’ culture of *P. patris-mei* Zaleski 1927.

**Penicillium brevicompactum** Dierckx 1901

- 2011 Received Feb. 1946 as *P. brevicompactum* Dierckx from CBS. Ex-neotype.
- 2011 USA: Vermont. Received as IMI 31200.

**Penicillium olsonii** Bainier & Sartory 1912

- 5267 Received as *P. stoloniferum* ATCC 14586.
- 5916 Received as *P. stoloniferum* from H. A. Wood, Boyce Thompson Institute, Yonkers, NY.
- 6446 Russia: Soil isolate. Received from L. A. Beljakova, Moscow as BKM f-1127, ex-type of *P. volgaense* Beljakova & Mil’ko 1972.
- 28496 Unknown: Received as CBS 349.61, 1969.

Centrifuge tube along with 1.5 ml of breaking buffer (50 mM KCI, 10 mM EDTA, 1% sarcosine, pH 8.0) and ca 1 g glass beads (0.5 mm diam). Phenol:chloroform (1:1) was added in volume equal to the buffer and the cell walls were broken by vortex mixing for 45–60 s. The organic phase was removed by low speed centrifugation (ca 2000 g for 5 min). The aqueous phase was precipitated by addition of 0.1 vol of sodium acetate (pH 6.0, 3 m) and 1.3 vol of 95% ethanol. Precipitate was collected by centrifugation in a micro-centrifuge for 5 min (ca 10 000 g). DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and DNA was bound to silica matrix in the presence of concentrated sodium iodide (GeneClean, Qbiogen, CA). DNA was dissolved in TE and kept at −10 °C until used.

DNA from the ITS-LSU rDNA (ID region) fragment was amplified using the procedure specified by...
Peterson et al. (2003). Calmodulin fragments were amplified as detailed by Peterson et al. (2001) with the universal 3′ primer CF4 (5′-tttYtgcatacRagYtggcag) and a series of 5′ primers developed and used for different taxa, CF1B (5′-gccgactcctgcaag), CF1C (5′-gaagaaagctgctcgag), CF1D (5′-caggtgctgctgcaag), CF1E (5′-gctgctgctgctgcaag), CFM (5′-gacagggctgctcgag) and CFMR (5′-actcctgctgctgcaag) were used as internal primers for sequencing. Elongation factor 1-α fragments were amplified using the universal 3′ primer EF6 (5′-cttStYcraRcccttgacca) and 5′ primers EF1a (5′-aagaaagctgctgcaac), EF1b (5′-ccatccacctgctgctgta), EF1c (5′-tgctgctgctgcaagcag), EF1d (5′-gccgactcttgactgaa) and EF1e (5′-caggtgcctgctgcaag). EFM (5′-tgggRgycacRaccnga) and Efmr (5′-gcNgYtgcRaaYttcca) were used as internal sequencing primers. Reaction buffer was standard as described by White et al. (1990) and the thermal profile was 96 °C-2 min followed by 42 cycles of 96 °C-30 s, 51 °C-30 s, 72 °C-90 s and a final elongation reaction of 5 min at 72 °C. Sequencing reactions were carried out using ABI dye terminator chemistry and the manufacturer’s protocol and sequences were read on an ABI 3100 or 3730 sequencer.

DNA sequence data were aligned using CLUSTALV (Thompson, Higgins & Gibson 1994) followed by visual inspection and correction using a text editor. Trees were calculated using maximum parsimony in PAUP* 4.0b10 (Swofford 2003), specifying random addition order (10 replications) and treating gaps as missing data. Bootstrap analysis was performed using maximum parsimony criterion in 1000 replicate samples. The partition homogeneity test was performed with uninformative sites excluded and 10 000 replicates. *Penicillium canescens* was chosen as the outgroup species on the basis of published trees (Peterson 2000). Tree diagrams produced by PAUP* were visualized using TREEVIEW 1.6.6 (Page 1996) and prepared for publication using CorelDraw version 9.

**RESULTS**

The ID region DNA sequences clustered into three main groups identified as *Penicillium brevicompactum*, *P. olsonii* and *P. biourgeianum* (Fig. 1). Among the isolates in the *P. brevicompactum* lineage, three isolates had sequences that differed from the ex-type strain at a single base position and one isolate differed from type at two base positions. Among *P. biourgeianum* isolates one isolated differed from the ex-type at a single base position, and one isolate differed at five base positions. Among the *P. olsonii* isolates, isolates had no more than a single base change in the coding region and among *P. brevicompactum* isolates, two had single base changes in the amino acid coding region and a single isolate had two coding region substitutions. In *P. biourgeianum*, isolates differed by as many as four base substitutions in the coding region and among *P. olsonii* isolates, isolates had no more than a single base change in the coding region. The majority of substitutions occurred in the intron regions of this gene. Introns were inserted in the gene after amino acid codon 20, between bases 1 and 2 of codon 26 and between bases 1 and 2 of codon 68 (using the *Aspergillus oryzae* sequence in GenBank D44468 as reference). Predicted amino acid sequences were nearly uniform with NRRL 32577 and NRRL 910 each having a single substitution, and NRRL 2012 and NRRL 2302 with two amino acid substitutions each. Isolates of *P. brevicompactum* formed a clade supported by 99% of the bootstrap samples, *P. biourgeianum* isolates
formed a clade supported by 97% of the bootstrap samples, and *P. olsonii* isolates formed a clade supported by 100% of the bootstrap samples.

The aligned translation elongation factor 1-α DNA sequences formed three lineages (Fig. 3) that corresponded to the three species. The proportions of bootstrap samples supporting the clades were *P. olsonii* 100%, *P. brevicompactum* 72%, and all except one isolate of *P. biourgeianum* 72%. DNA sequence differences in the coding regions were scant in *P. olsonii* with two isolates each having one substitution, in *P. brevicompactum* two isolate share six coding region substitutions while other isolates had one or two substitutions, and *P. biourgeianum* displayed two to four substitutions in the coding region. Introns occurred after the codon for amino acid 45 and between bases 1 and 2 of the codon for amino acid 92 (based on the amino acid sequence of *A. oryzae* GenBank AB007770). Most sequence differences between species were found in the intron regions and those differences were of such magnitude that the homology of bases in interspecific alignment is questionable.

In order to determine the congruence of these trees, the partition homogeneity test (PHT) included in PAUP* was used. The test statistic values were $P=0.0848$ for the calmodulin/tef 1-α dataset with introns removed, $P=0.1965$ for the calmodulin/ITS-lsu rDNA dataset, and $P=0.6049$ for the tef 1-α/ITS-lsu rDNA dataset. The test values suggest that the differences in the trees are due to random chance rather than differences in the underlying phylogeny. Figs 2–3 show the differences between the tef 1-α and calmodulin data sets. The calmodulin data set places *P. biourgeianum* and *P. brevicompactum* as sister taxa, while the tef 1-α data set places *P. biourgeianum* and *P. olsonii* as sister taxa. The test statistic $P=0.0178$ when the PHT was run on these data sets with introns included. Introns were excluded because of the questionable nature of the alignments.

Some cultures of *P. biourgeianum* were grown and observed under the system Pitt (1980) suggests for identification. Colonies (Fig. 4) grown 7 d on CYA (Czapek’s yeast extract agar) at 25 °C attain 20–29 mm diam, and are velutinous and sulcate with a 1–2 mm white border around the margin on the agar surface. Colonies are deep bluish grey–green (R-XLII) with white secondary growth over parts of the colony. Clear exudate is present but soluble pigments are absent. Sporulation is heavy and the colony reverse is cream in colour. Colonies grown 7 d on MEA at 25 °C are velutinous and plane attaining 15–20 mm diam, and with a 1 mm white border at the margin on the agar surface. Colony colour is Russian green (R-XLVII) with a pale to ecru olive (R-XXX) reverse (which is influenced
by conidial development). Both exudate and soluble pigments are absent. Sporulation is heavy. 

*P. biourgeianum* did not grow, nor did the conidia germinate at 37 °C. When incubated at 5 °C, micro-colonies formed. On G25N medium colonies attained 16–17 mm diam and appeared similar to the CYA colonies. 

Conidiophores (Figs 5–6) are 350–2–3 (–4) μm and smooth arising from basal hyphae producing a complex crowded conidiogenous apparatus apically (Figs 7–8) with conidiogenous cells (phialides) 6–8 × 2.5–3 μm and crowded. Metulae, the cells subtending the phialides, are 12–15 × 3–4 μm. Conidia (Figs 9–10) are spherical, 2.5–3.5 μm diam with a small proportion ellipsoidal and are occasionally seen in short chains (Fig. 9).

**DISCUSSION**

Parsimony analysis of the DNA sequence data from each locus produced three terminal clades containing isolates of *Penicillium brevicompactum*, *P. olsonii* and *P. biourgeianum*, respectively. In most cases (Figs 1–3), bootstrap values strongly support these taxa. The isolates assigned to each species are the same in the analysis of each of the three loci, and the clades are well separated from each other, suggesting that there is no mixis occurring between isolates of the different species. Under the phylogenetic species concept (Taylor et al. 1999) *P. brevicompactum*, *P. olsonii* and *P. biourgeianum* are each distinct species (Table 2).

The PHT results show, from a statistical viewpoint, that any differences in the parsimony trees from each of the three loci are no greater than one might expect from random differences generated in the evolution of the species. In the case of the two protein coding genes, the signals were significantly different when the intron sequences were included in the PHT analysis, but not significantly different when the introns were excluded from the analysis. In examining the intron sequence alignment, differences are so great between species that...
there is no certain way to determine the homology of the bases in the alignment. The disjunction of these sequences could result from our failure to sample all lineages in the *P. brevicompactum* clade. Alternatively, intron sequences may evolve by methods other than genetic drift. In some cases, introns are known to be mobile genetic elements (Moran, DeBerardinis & Kazazian 1999) but the function of most introns is unknown as is their mechanism of evolution. Given our lack of understanding of intron evolution, and the observable disjunctions of the intron data, it seemed reasonable (Balter 1997) to exclude intron sequences from the PHT analysis.

*P. olsonii* was described by Bainier & Sartory (1912) from a molding banana. Thom (1930) relied on the original description plus a single culture sent to him from Jamaica by C. G. Hansford to assess the validity of the species. *P. olsonii* was accepted by Thom with some small reservations because he had seen only one isolate. Raper & Thom (1949) relied on the observations of Thom (1930) and accepted the species. Pitt (1980) accepted the species as a rare but distinct species, especially the compact complex penicillus. Thom (1930) accepted it because the conidia were more ellipsoidal and the colonies more velutinous than in *P. brevicompactum*. Culture NRRL 866 was derived from Thom 4733.68, and is an ex-type culture of the species. Genetically (Figs 1–3) this culture shows little to distinguish it as a species separate from *P. brevicompactum*. The DNA sequence data (Figs 1–3) show that there is no genetic basis for maintaining the species.

*P. griseobrunneum* also bears a marked resemblance to *P. brevicompactum* in growth rates, colours and especially the compact complex penicillus. Thom (1930) accepted it because the conidia were more ellipsoidal and the colonies more velutinous than in *P. brevicompactum*. Culture NRRL 867 was derived from Thom 4733.68, and is an ex-type culture of the species. Genetically (Figs 1–3) this culture shows no significant difference from *P. brevicompactum*. On the basis of phenotypic and genetic similarity, it is considered a synonym of *P. brevicompactum*.

*P. bialowiezenze* was also placed in the ‘Brevi-compacta’ by Thom (1930). This species resembles

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**Table 2. Phenotypic characters of Penicillium brevicompactum, *P. olsonii*, and *P. biourgeianum***

<table>
<thead>
<tr>
<th></th>
<th><em>P. brevicompactum</em></th>
<th><em>P. olsonii</em></th>
<th><em>P. biourgeianum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CYA colony 25 °diam</td>
<td>20–30</td>
<td>30–40</td>
<td>20–23</td>
</tr>
<tr>
<td>colour</td>
<td>tea green</td>
<td>greyish-green</td>
<td>grey-green (gnaphalium)</td>
</tr>
<tr>
<td>reverse</td>
<td>pale to reddish brown</td>
<td>yellow to yellow brown</td>
<td>cream</td>
</tr>
<tr>
<td>exudate</td>
<td>usual, pale to reddish brown</td>
<td>unusual, clear when present</td>
<td>variable presence, clear</td>
</tr>
<tr>
<td>soluble pigments</td>
<td>Variable presence, reddish</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>MEA colony 25 °diam</td>
<td>12–20</td>
<td>25–35</td>
<td>15–16</td>
</tr>
<tr>
<td>reverse</td>
<td>pale to brown</td>
<td>pale to dull yellow brown</td>
<td>pale to ecru-olive</td>
</tr>
<tr>
<td>CYA 5 ° micro-colony</td>
<td>to 4 mm</td>
<td>germination to micro-colony</td>
<td>microcolony</td>
</tr>
<tr>
<td>CYA 37 °</td>
<td>no growth</td>
<td>no growth</td>
<td>no growth</td>
</tr>
<tr>
<td>Microscopic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>conidiophore</td>
<td>500–800 × 4–6 μm</td>
<td>500–2000 × 4–6 μm</td>
<td>350 × 3–4 μm</td>
</tr>
<tr>
<td>metulae</td>
<td>9–12(–15) × 3–5 μm</td>
<td>10–12 × 3–4 μm</td>
<td>12–15 × 3–4 μm</td>
</tr>
<tr>
<td>phialides</td>
<td>6–9 × 2–2.5 μm</td>
<td>9–10 × 2.2–2.5 μm</td>
<td>6–8 × 2.5–3 μm</td>
</tr>
<tr>
<td>conidia</td>
<td>ellipsoidal 2.5–3.5 × 2.5</td>
<td>ellipsoidal 3–4 × 2.5–3.0 μm</td>
<td>globose 2.5–3.5</td>
</tr>
</tbody>
</table>

Data compiled from this study, Pitt (1980), and Raper & Thom (1949).

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*P. brevicompactum* in its production of a compact and complex penicillus structure, but differs in colony characteristics (Thom 1930). Thom’s culture 5010.4 (= NRRL 863) was accepted by Thom as the type of the species. The ID region DNA sequence from NRRL 863 is identical to the sequence from the ex-type culture of *P. polonicum*. On these bases, *P. biourgeianum* is considered a synonym of *Penicillium polonicum*. Pitt (1980) incorrectly lists NRRL 862, NRRL 863, NRRL 2011 and Thom 4733.21 as subcultures of a single original isolate, Biourge no. 42. NRRL 863 derives from Thom culture, 5010.4 (also see Table 1).

Thom received a culture from the ex-type strain of *P. patrismei* and accessioned it as 5010.29 (= NRRL 868). Although Zaleski (1927) described the species with conidia smaller than those of *P. brevicompactum*, Thom’s (1930) observations suggested little difference between *P. patrismei* and *P. brevicompactum*. Genetically (Figs 1–3), the species is indistinguishable from *P. brevicompactum* and is a synonym of the earlier species.

*P. biourgeianum* is represented in NRRL as Thom 5010.5 (= NRRL 865), the ex-type isolate. Thom observed it and distinguished it from *P. brevicompactum* on the basis of the olive-green to grey-green colony colour, orange colour of the colony reverse and slightly smaller conidia. Genetically (Figs 1–3), *P. biourgeianum* is a member of one of the three clades that phylogenetically segregate cultures from Thom’s (1930) concept of ‘Brevi-compacta’. Because it is genetically isolated from *P. brevicompactum* and *P.olsonii*, it must be treated as a distinct species.

In observations of many cultures of *P. brevicompactum* and *P. biourgeianum*, it was quite clear that the colony colour of *P. biourgeianum* cultures is a much lighter shade than that typically present in *P. brevicompactum*. Culture NRRL 28149, isolated from a decaying mushroom (Table 1) was originally thought to represent a new species and sequences from that isolate were deposited in GenBank under a laboratory name that has never been formally published or typified, and as the fungus is clearly not distinguishable from *P. biourgeianum*, the designation should be disregarded.

*P. biourgeianum* cultures grow at rates within the ranges listed by Pitt (1980), but at the lowest end of the range. Colony colours are typically in the pale end of the spectrum described by Pitt, and the colony reverse colour is cream or pale on CYA vs the reddish brown considered typical by Pitt, and brownish green (ecru-olive) on MEA vs pale or brown observed by Pitt. Growth of *P. biourgeianum* on G25N is at the low end of the *P. brevicompactum* range listed by Pitt, and growth at 5 °C and 37 °C are the same for both species. The penicillus and conidia of *P. biourgeianum* are typical of those ascribed to *P. brevicompactum*. *P.olsonii* cultures appear to grow faster than either *P. brevicompactum* or *P. biourgeianum*, soluble pigments are absent on CYA and the colony reverse on CYA is yellow to yellow-brown. Pale colony reverse and lack of soluble pigments in *P. biourgeianum* vs *P. brevicompactum* with reddish brown reverse and soluble pigments of the same colour, may serve to distinguish the two species, but further analysis of genetically characterized isolates is essential to assess its reliability.

**REFERENCES**


*Corresponding editor:* D. L. Hawksworth