Morphological, biochemical and molecular characteristics of *Metarhizium anisopliae* and *M. flavoviride*

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Twenty-four isolates of *Metarhizium anisopliae* and *M. flavoviride*, screened for virulence to desert locust (*Schistocerca gregaria*), were characterized by conidial morphology, API ZYM, isoenzyme electrophoresis and RFLP analysis of *Hae*III digests on agarose minigels. Conidial morphology showed considerable overlap in measurements between the two species. However the API ZYM method showed that all isolates of *M. anisopliae* differed from those of *M. flavoviride* by possessing α -fucosidase activity. Isoenzyme electrophoresis clearly distinguished the two species by catalase and acid phosphatase banding patterns. *M. anisopliae* had a much simpler RFLP banding pattern than *M. flavoviride*, which showed > 20 bands, but individual isolates of both species had unique patterns. The methods used could all contribute to a unique 'fingerprint' for isolates of both species including *M. flavoviride* IMI 330189, which has been under intensive study for biopesticide development against locusts and grasshoppers.

Introduction

The genus *Metarhizium* is, since the works of Tulloch (1976) and Rombach *et al.* (1987), generally accepted as including just three species: *Metarhizium anisopliae* (Metschnikov) Sorokin, *M. flavoviride* (W. Gams & Rozsypal) and *M. album* Petch. *M. anisopliae* is isolated most often from insects in a wide range of orders (Veen, 1968). The other two species have apparently more limited patterns of occurrence and distribution (Rombach *et al.* 1986, 1987), but collections have not been comprehensive enough to determine if this apparent rarity is genuine. The taxonomy of the genus is still causing controversy with, for example, three new species recently described from China: *M. cylindrospora* Q.-t. Chen & H.-l. Guo, *M. guizhouense* Q.-t. Chen & H.-l. Guo and *M. pingshaense* Q.-t. Chen & H.-l. Guo (Guo *et al.*, 1986). These species have not been studied critically by other workers. Guo *et al.* (1986) accepted *M. anisopliae* but not *M. flavoviride* or *M. album*.

The genus has a world-wide distribution (Brady, 1979a) and *M. anisopliae* has been used extensively in experimental systems for the biological control of a wide variety of insect pests (Gillespie, 1988). Records of *M. flavoviride* are less numerous than those for *M. anisopliae*

(e.g. Humber & Soper, 1986; R. J. Milner, personal communication) although *M. flavoviride* probably has as wide a distribution as *M. anisopliae*. During a recent programme to develop entomopathogenic Deuteromycotina as mycoinsecticides for control of locusts and grasshoppers (Prior *et al.*, 1992), numerous isolates of *M. flavoviride* were recovered from acridoid hosts in Africa.

Isolates of *Metarhizium* spp. have widely differing host ranges (Ferron *et al.*, 1972). Cultural characteristics have been used to separate some populations of *M. anisopliae* (Yip *et al.*, 1992), but individual isolates could not always be distinguished by these methods. Yip *et al.* (1992) note that adequate and accurate separation of strains of *Metarhizium* spp. will become increasingly important as interest in their exploitation for pest control intensifies. A unique 'fingerprint' is required for individual genotypes, for two reasons: to provide commercial organizations with the ability to identify their isolate for patenting and registration purposes, and to enable monitoring of the environmental impact of a particular isolate following its use as a mycopesticide. In the latter case if any side-effects are suspected, such as infection in non-target organisms, the added isolate must be distinguishable from the very large number of morphologically identical, indigenous isolates already present.

This paper reports the characterization, by biochemical and molecular methods, of 24 isolates of *Metarhizium*

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Table 1. Cultures used in study

Strain no*	Received as	Host	Host family	Country of origin
ARSEF 324	<i>Metarhizium anisopliae</i>	<i>Austracris guttulosa</i>	Orthoptera: Acrididae	Australia
ARSEF 435	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 436	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 439	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 440	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 441	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 442	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 443	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 444	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 445	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 446	<i>M. anisopliae</i>	<i>Teleogryllus commodus</i>	Orthoptera: Gryllidae	Australia
ARSEF 727	<i>M. anisopliae</i>	Unid. tettigoniid	Orthoptera: Tettigoniidae	Brazil
ARSEF 728	<i>M. anisopliae</i>	Unid. orthopteran	Orthoptera	Brazil
ARSEF 1009	<i>M. anisopliae</i>	<i>Orinebius kanetataki</i>	Orthoptera: Gryllidae	Japan
IIBC 190-574	<i>M. anisopliae</i>	<i>Acrotylus humbertianus</i>	Orthoptera: Acrididae	Pakistan
IMI 047038	<i>M. anisopliae</i>	<i>Tomaspis saccharina</i>	Hemiptera: Cercopidae	Venezuela
IMI 152222	<i>M. anisopliae</i>	<i>Myllocerus discolor</i>	Coleoptera: Curculionidae	India
IMI 168777ii	<i>M. anisopliae</i>	<i>Schistocerca gregaria</i>	Orthoptera: Acrididae	Ethiopia
IMI 298059	<i>M. anisopliae</i>	<i>Scapanes australis</i>	Coleoptera: Scarabaeidae	Papua New Guinea
IMI 298061	<i>M. anisopliae</i>	<i>Brontispa longissima</i>	Coleoptera: Hispididae	Papua New Guinea
ARSEF 2023	<i>M. flavoviride</i>	Unidentified acridid	Orthoptera: Acrididae	Galapagos Is
IMI 324673	<i>M. flavoviride</i>	<i>Zonocerus variegatus</i>	Orthoptera: Pyrgomorphidae	Tanzania
IMI 330189	<i>M. flavoviride</i>	<i>Ornithacris cavroisi</i>	Orthoptera: Acrididae	Niger
IMI 333138	<i>M. flavoviride</i>	<i>Perkinsiella saccharicida</i>	Homoptera: Delphacidae	Mauritius

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spp. screened for virulence to the desert locust, *Schistocerca gregaria* Forskål (Prior *et al.*, 1992). These isolates originated mainly from orthopteran hosts, both within and outside the geographic range of *S. gregaria*, but some coleopteran and homopteran isolates were also screened and are included here. Isolates were classified using both conventional morphology and biochemical and molecular methods in an attempt to determine clear distinctions both between species and between genotypes at the sub-varietal level.

Methods

Isolates. Cultures of the 24 isolates of *Metarhizium anisopliae* and *M. flavoviride* (Table 1) were received on Molisch's agar (Speare, 1920) and maintained at 4 °C on slopes of malt extract agar (Smith & Onions, 1983).

Growth conditions. For morphological examination cultures were grown on potato carrot agar (PCA) (Smith & Onions, 1983) at 21 ± 2 °C. Slide preparations of mature spores from 28 d cultures were mounted in lactofuchsin for detailed examination of conidial morphology and size.

For other procedures cultures were grown in glucose/yeast extract liquid medium (GYM; Mugnai *et al.*, 1989). Cultures for the API ZYM test kits were grown in stationary culture at 25 °C for 14 d, and cultures for isoenzyme and DNA determinations were grown for 60 h on an orbital shaker at 27 °C.

API ZYM method. Culture fluid was collected from the stationary grown cultures and used as a direct inoculum in the API ZYM test kit

(bioMérieux). Test strips were inoculated with 50 µl culture fluid in each well, and incubated for 4 h at 35 °C. Enzyme activity was determined by colour reactions after adding the API ZYM reagents A and B. Initial test strips were inoculated in duplicate.

Isoenzyme electrophoresis. Fungal mycelium was harvested from liquid cultures by filtration with a Buchner funnel, and washed in Tris/glycine buffer (0.3% Tris, 1.44% (w/v) glycine, pH 8.3). Mycelium was freeze-dried by cooling at 1 °C min⁻¹ to -45 °C and drying for 24 h at 2.2 mbar (Edwards Minifast 3400). The dried mycelium was broken by grinding in a mortar and pestle. The ground mycelium (100–200 mg) was resuspended in approximately 1 ml Tris/glycine buffer in a 1.5 ml tube and clarified by centrifugation in an angle rotor in a Sigma 2K15 refrigerated bench centrifuge at 4 °C at 13000 g for 40 min. The resulting supernatant was collected and total protein content determined by the Lowry method. Electrophoresis was carried out in 10% (w/v) polyacrylamide gels for acid phosphatase, and 8.2% (w/v) polyacrylamide gels for catalase. Samples were loaded onto 10 × 10 × 0.1 cm gels to give a loading of 150 µg protein in each lane. Electrophoresis was carried out at 2.5 mA cm⁻¹ in a discontinuous system with a Tris/glycine tank buffer and a Tris/citrate (1.37% (w/v) Tris, 0.0525% citric acid) gel buffer. Acid phosphatase isoenzymes were stained according to the method of Shaw & Prasad (1970) and catalase isoenzymes were stained according to Woodbury *et al.* (1971).

RFLP methods. Fungal mycelium was harvested and disrupted as for isoenzyme extraction. Total cellular DNA was extracted from the powdered freeze-dried mycelium according to the method of Raeder & Broda (1985). DNA samples were checked and quantified against known concentrations of λ phage DNA in a 1% (w/v) agarose minigel. DNA samples were digested overnight with *Hae*III (Sigma) according to manufacturer's conditions and 5–10 µg samples were separated in a 0.7% agarose gel at 5 V cm⁻¹ in Tris/borate/EDTA buffer (0.605%

Table 2. *Conidial measurements*

Strain no.	Length (µm)		Width (µm)	
	Minimum	Maximum	Minimum	Maximum
324	6.5	8.4	2.0	3.2
435	5.2	6.5	2.3	3.2
436	5.6	8.0	1.6	3.2
439	5.6	6.4	1.6	2.4
440	4.5	7.1	2.0	3.2
441	5.8	7.1	2.0	3.2
442	5.0	7.2	1.6	2.4
443	5.2	6.5	2.3	2.9
444	5.8	7.1	2.0	3.2
445	4.8	7.2	1.6	2.4
446	4.8	6.8	1.8	2.6
727	5.6	8.0	1.6	2.4
728	5.8	7.1	2.0	2.6
1009	6.4	8.0	1.6	2.0
I90-574	6.4	8.0	1.6	2.4
047038	6.5	8.4	2.0	2.9
152222	5.2	7.1	2.0	2.6
168777ii	5.8	7.1	2.0	2.6
298059	5.8	7.1	2.0	2.6
298061	5.5	7.1	2.0	2.6
2023	7.1	9.1	2.9	3.9
324673	5.2	6.5	2.3	2.9
330189	5.2	6.5	2.6	3.2
333138	6.4	8.0	1.6	2.8

Tris, 0.3085% boric acid, 0.0372% EDTA). A sample of λ phage DNA was included with each set of digestions and on each gel as an internal control of the restriction. Gels were stained for 45–60 min in 0.5 µg ethidium bromide ml⁻¹ and photographed on Polaroid 667 and 665 film on a UV transilluminator.

Reproducibility. Reproducibility of results was investigated in two ways. For API ZYM and isoenzyme determination, five strains were tested 7 months after the original study. DNA restriction patterns were tested by repeating the extraction, digestion and electrophoresis on later subcultures of stock culture lines.

Results

Morphology

Light-microscope examination of the isolates listed in Table 1 revealed morphology according with the descriptions of the species under which they were deposited, notable exceptions being IMI 333138 which had a spore shape more typical of *M. anisopliae*. Two isolates of *M. flavoviride*, IMI 324673 and IMI 330189, had shorter conidia and might therefore be placed in the var. *minus* (Table 2). ARSEF 324 had morphology closer to *M. flavoviride* but the dark-green colour of the spore mass was more typical of *M. anisopliae*. All the *M. anisopliae*

Table 3. *API ZYM activities of 14 d culture fluids from Metarhizium species*

Strain no.	API ZYM reaction*																			
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
ARSEF 324	+	+	+	—	—	—	—	—	—	+	+	—	+	+	—	w	+	—	—	
ARSEF 435	w	+	+	—	—	—	—	—	—	+	+	+	+	+	w	+	+	—	w	
ARSEF 436	+	+	+	w	—	—	—	—	w	+	+	+	+	+	—	+	+	—	+	
ARSEF 439	w	+	+	w	—	—	—	—	—	+	+	w	+	+	—	+	+	—	+	
ARSEF 440	w	+	+	w	—	—	—	—	—	+	+	w	+	+	—	+	+	—	+	
ARSEF 441	w	+	+	—	—	—	—	—	w	+	+	+	+	+	—	+	+	—	+	
ARSEF 442	w	+	+	w	—	—	—	—	—	+	+	—	+	+	—	+	+	—	+	
ARSEF 443	—	+	+	w	—	—	—	—	—	+	+	—	+	+	w	+	+	—	+	
ARSEF 444	w	+	+	—	—	—	—	—	—	+	+	+	+	+	—	+	+	—	+	
ARSEF 445	+	+	+	w	—	—	—	—	—	+	+	+	+	+	—	+	+	—	+	
ARSEF 446	w	+	+	w	—	—	—	—	—	+	+	+	+	+	—	+	+	—	+	
ARSEF 727	w	+	+	—	—	—	—	—	—	+	+	+	+	+	—	+	+	—	+	
ARSEF 728	w	+	+	w	—	—	—	—	—	+	+	+	+	+	—	+	+	—	+	
ARSEF 1009	w	+	+	w	—	—	—	—	w	+	+	+	+	+	+	+	+	—	+	
IIBC 190-574	—	+	+	—	—	—	—	—	—	+	+	w	+	w	—	+	+	—	+	
IMI 047038	+	+	+	—	w	—	—	—	—	+	+	+	+	—	w	+	+	—	+	
IMI 152222	w	+	+	w	—	—	—	w	w	+	+	w	+	+	+	+	+	—	+	
IMI 168777ii	+	+	+	—	—	—	—	—	—	+	+	+	+	+	—	+	+	—	+	
IMI 298059	w	+	+	w	—	—	—	—	—	+	+	w	+	—	—	+	+	—	+	
IMI 298061	w	+	+	+	w	—	—	—	—	+	+	—	+	+	w	+	+	—	+	
ARSEF 2023	w	+	w	—	—	—	—	w	w	+	+	+	+	+	+	+	+	—	—	
IMI 324673	w	+	+	w	—	—	—	—	—	+	+	—	+	+	+	+	+	—	—	
IMI 330189	w	+	w	w	—	—	—	—	—	+	+	w	+	—	—	—	+	—	—	
IMI 333138	w	+	+	—	—	—	—	—	—	+	+	+	+	+	—	+	+	—	—	

* 2, Alkaline phosphatase; 3, esterase (C4); 4, esterase/lipase (C8); 5, lipase (C14); 6, leucine arylamidase; 7, valine arylamidase; 8, cystine arylamidase; 9, trypsin; 10, chymotrypsin; 11, acid phosphatase; 12, naphthol-AS-BI-phosphohydrolase; 13, α -galactosidase; 14, β -galactosidase; 15, β -glucuronidase; 16, α -glucosidase; 17, β -glucosidase; 18, *N*-acetyl- β -glucosaminidase; 19, α -mannosidase; 20, α -fucosidase.

+, reaction of 4 or 5 on manufacturer's scale; —, reaction of 0 on manufacturer's scale; w, reaction of 1–3 on manufacturer's scale.

Fig. 1. Diagrammatic representation of catalase isoenzymes. Lane 1 IMI 16877ii (*M. anisopliae*), lane 2 ARSEF 444 (*M. anisopliae*), lane 3 ARSEF 727 (*M. anisopliae*), lane 4 IIBC 190-574 (*M. anisopliae*), lane 5 ARSEF 2023 (*M. flavoviride*), lane 6 ARSEF 449 (*M. anisopliae*).

strains examined fitted var. *anisopliae* and none belonged in var. *majus*.

API ZYM method

The results of the API ZYM test strips from the 14 d culture fluids are given in Table 3. In addition, some determinations were made on 7 d culture fluids; however, these showed much lower levels of enzyme activity and were poorly reproducible (data not shown). All isolates showed broadly similar reactions after 14 d, although a few isolates differed in their β -glucuronidase, α -glucosidase and β -glucosidase activities. All isolates received as *M. flavoviride* lacked α -fucosidase activity, in addition to strain ARSEF 324, received as *M. anisopliae*. These isolates also showed unique individual API ZYM profiles.

Isoenzyme electrophoresis

Electrophoresis of catalase isoenzymes gave four distinct patterns, designated as A1, A2, B1 and B2. Isolates received as *M. anisopliae* showed either one band (pattern

Fig. 2. Diagrammatic representation of acid phosphatase isoenzymes. Lane numbering as Fig. 1.

A2) or two bands (pattern A1). The A2 pattern top band was common to all strains with some showing a second lower band (A1). Isolates received as *M. flavoviride* showed a top band which did not migrate as far as that in *M. anisopliae* (B2), and some isolates showed a second lower band of the same R_F as *M. anisopliae* (B1). A diagrammatic representation of patterns for six isolates is given in Fig. 1. Isolate ARSEF 324 gave a single band in the same position as that from *M. flavoviride* samples (B2) (data not shown).

Electrophoresis of acid phosphatase isoenzymes gave two distinct patterns (Fig. 2). All samples from *M. anisopliae* showed two major bands near the top of the gel (pattern C). Samples from *M. flavoviride* showed a single area of diffuse staining approximately equivalent to the two bands from *M. anisopliae*, although distinct bands were not seen (pattern D). In addition, in some gel runs *M. anisopliae* samples showed two further bands nearer the middle of the gel (data not shown). Strains received as *M. flavoviride* also sometimes showed two further bands which, when present, had different R_F values than those from *M. anisopliae*. Isolate ARSEF 324 gave a diffusely stained area similar to other isolates of *M. flavoviride*.

Fig. 3. Examples of *Hae*III restriction of total DNA. Lane 1 λ phage (size markers), lane 2 ARSEF 443 (*M. anisopliae*), lane 3 IIBC 190-574 (*M. anisopliae*), lane 4 IMI 047038 (*M. anisopliae*), lane 5 ARSEF 324 (*M. flavoviride*).

RFLP methods

RFLP analysis from *Hae*III digests of total cellular DNA from 11 representative isolates (IMI 047038, 168777ii, 298061, 324673, 330189, IIBC 190-574, ARSEF 324, 435, 443, 1009, 2023) showed two overall patterns (Fig. 3). *Hae*III digests of the total DNA from isolates of *M. anisopliae* showed simple patterns of only a few bands. There was considerable similarity between different isolates, although patterns were unique to each isolate studied. The digests from the isolates of *M. flavoviride* were considerably more complex, consisting of 20 or more bands. The complex patterns shared some bands in common with the simple *M. anisopliae* patterns but were otherwise quite distinct from them. Isolate ARSEF 324 showed a complex DNA pattern, similar to those from *M. flavoviride*.

Reproducibility

The reproducibility of the above results was generally good. The only discrepancies seen in API ZYM patterns involved weak reactions. Isoenzyme patterns were found to be the same on repeating the work although the lower acid phosphatase bands were variable in that they sometimes stained weakly or not at all. Similarly, the possession of the lower catalase band, which was not seen in all isolates, was slightly variable. These differences are presumably due to relative concentrations of the different enzyme components, but do not interfere with the main diagnostic criteria.

Discussion

Morphology

Conidial measurements have been the critical taxonomic character for species differentiation in the genus *Metarhizium* (Gams & Rozsypal, 1973; Tulloch, 1976; Brady, 1979*a, b*; Rombach *et al.*, 1986); however, published conidial dimensions for *M. anisopliae* and *M. flavoviride* overlap. It is obvious therefore that the spore measurements alone do not precisely distinguish the two species. Furthermore work by Kamat *et al.* (1952) indicated that spore size in *M. anisopliae* was dependent on the culture medium employed. Neither is colony colour reliable, although different authors usually refer to *M. flavoviride* as having either pale-green, greyish yellow-green or olivaceous buff colonies. *M. anisopliae* has been described as having yellowish green, olivaceous, dark-herbage green, pink or vinaceous buff (Brady, 1979*a*). ARSEF 324, with typical colour of *M. anisopliae*, comes out with *M. flavoviride* according to other criteria used in the current study.

Biochemical methods

Although enzyme expression will be dependent on culture conditions, the API ZYM strip provided a single enzyme that differentiated between the species. α -Fucosidase is an enzyme that cleaves α bonds to the deoxy-sugar fucose. In the API ZYM kit this is present as a naphthyl substituted fucopyranoside, where the free naphthyl group is subsequently detected with the API ZYM B reagent. Substituted deoxy-sugars are found in a variety of places in nature, including bacterial cell walls and plant glycosides. The overall API ZYM patterns from the strains changed considerably between 7 and 14 d culture. Few polysaccharide-degrading enzymes were seen during active growth, and so gave negative reactions at 7 d. However, they were commonly detected once the culture had become glucose-limited. In addition to the species separation, it was possible to distinguish

between individual isolates of *M. flavoviride* from the API ZYM results, although not between isolates of *M. anisopliae*. This may be due to the wider distribution of the *M. flavoviride* isolates studied, as identical profiles were often shown by isolates of *M. anisopliae* from the same host and geographic location.

The catalase isoenzyme patterns included a common lower band between isolates of the two species. This may indicate a close genetic relatedness between the two species. However, further characterization of the enzyme bands and ultimately protein sequence data would be needed to confirm this.

Molecular methods

A number of papers have reported the use of RFLPs derived solely from restricted DNA, i.e. without the use of a probe, and some enzymes such as *EcoRI*, *HindIII* and *EcoRV* can show distinct bands within a 'smear' of total DNA (Coddington *et al.*, 1987). Enzymes such as *HaeIII* which recognize four base sequences consisting of only guanine and cytosine, appear to cut fungal chromosomal DNA into fragments of less than 1 kb, while cutting mitochondrial DNA less frequently to leave larger fragments (Marriott *et al.*, 1984; Cooley, 1992). It is presumed in this study that the majority of the bands seen are therefore derived from mitochondrial DNA. However, this cannot be stated categorically, particularly with regard to *M. flavoviride*. The bands from the *M. anisopliae* strains are overall very similar, and most differences between strains can be accounted for by relatively minor changes in restriction sites. The difference between these strains and those of *M. flavoviride* is less clear. Digestion of DNA from *M. flavoviride* isolates gives what appears to be a partial digest. The total size of the fragments seen in the *M. flavoviride* strains is over 100 kb, which is large for a fungal mitochondrion. The *M. flavoviride* strains contain the common bands seen in *M. anisopliae* in addition to other bands. There are a number of possible explanations for this including differential methylation, contaminating components, or the presence of more than one mitochondrial type. However, this pattern is representative of the taxon and so an exact explanation is not required for the practical purpose of separating the two species.

The three approaches used in this study – API ZYM, isoenzyme analysis and *HaeIII* RFLP determinations – were each able to distinguish between isolates received as *M. anisopliae* and *M. flavoviride*, and in some cases appeared to characterize individual isolates. The one discrepancy is ARSEF 324, which has consistently shown biochemical attributes and an RFLP pattern in agreement with *M. flavoviride*. Morphologically this strain showed colony features in agreement with *M. anisopliae*,

although it appeared closer to *M. flavoviride* from microscopic observation. This isolate was received from the USDA-ARS Collection of Entomopathogenic Fungi and was included in a recent study of isoenzymes in *M. anisopliae* (St Leger *et al.*, 1992). The dendrogram from that study shows two loose outgroups of 10 isolates, nine of which were labelled as *M. flavoviride* or varieties of that species, ARSEF 324 being the tenth isolate. In view of this, and the results reported here, it seems likely that this isolate is a member of *M. flavoviride*.

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