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Agromyces subbeticus sp. nov., isolated from a cave in southern Spain

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An actinomycete, strain Z33^T, was isolated from a cyanobacterial biofilm in the Cave of Bats, near Zuheros (Cordoba, southern Spain). 16S rRNA gene sequence analysis showed that strain Z33^T formed a distinct phyletic line within the genus *Agromyces*. This isolate could be readily distinguished from representatives of all recognized *Agromyces* species on the basis of a broad range of phenotypic characteristics and DNA–DNA relatedness data. Genotypic and phenotypic properties indicate that strain Z33^T represents a novel species, for which the name *Agromyces subbeticus* sp. nov. is proposed. The type strain is Z33^T (=HKI 0340^T=DSM 16689^T=NCIMB 14025^T).

Since the description of the genus *Agromyces* by Gledhill & Casida (1969) with the type species *Agromyces ramosus*, the number of recognized species in the genus has increased to 16, and studies have indicated that these are widely distributed in nature and might play a significant role in a variety of ecosystems. *Agromyces cerinus* subsp. *cerinus* and *Agromyces cerinus* subsp. *nitratus* were described by Zgurskaya *et al.* (1992), *Agromyces mediolanus* by Suzuki *et al.* (1996), *Agromyces luteolus*, *Agromyces rhizospherae* and *Agromyces bracchium* by Takeuchi & Hatano (2001), *Agromyces aurantiacus* by Li *et al.* (2003), *Agromyces albus* by Dorofeeva *et al.* (2003) and *Agromyces ulmi* by Rivas *et al.* (2004). A reclassification of *Agromyces fucosus* subsp. *fucosus* and *Agromyces fucosus* subsp. *hippuratus* as *Agromyces fucosus* and *Agromyces hippuratus*, respectively, was proposed by Ortiz-Martinez *et al.* (2004). Studies on the diversity and role of *Agromyces* species in hypogean environments resulted in the description of two novel species, *Agromyces salentinus* and *Agromyces neolithicus*, which were isolated from an Italian cave (Jurado *et al.*, 2005a), and three novel species, *Agromyces italicus*, *Agromyces humatus* and *Agromyces lapidis* (Jurado *et al.*, 2005b), originating from Roman catacombs.

The aim of the present study was to determine the taxonomic position of a further cave isolate, strain Z33^T. On the basis of the results presented below, strain Z33^T is considered to represent a novel species of the genus *Agromyces*.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Z33^T is AY737778.

Strain Z33^T was isolated from a blue–grey cyanobacterial biofilm covering the walls of the Cave of Bats (Zuheros, Cordoba, southern Spain) on PY-BHI medium (Yokota *et al.*, 1993) at 28 °C. Laboratory cultivation of strain Z33^T was performed on medium 79 (OM79) (Prauser & Falta, 1968; Jurado *et al.*, 2005a).

All experimental methods used in this study are as described by Jurado *et al.* (2005a). The range of pH for growth was established using liquid OM79 medium adjusted to initial pH values of 4–11 with either 1 M HCl or 20 % (w/v) Na_2CO_3 solution and incubated at 28 °C for up to 10 days.

The following type strains were used as references for comparative studies: *A. fucosus* IMET 11529^T, *A. ramosus* IMET 11027^T, *A. albus* VKM 1800^T, *A. cerinus* subsp. *nitratus* IMET 11532^T and *A. cerinus* subsp. *cerinus* IMET 11525^T.

Morphological and physiological traits are summarized under the species description below and in Table 1. Chemotaxonomic characteristics are given in Table 2.

16S rRNA gene sequence analysis showed that strain Z33^T had closest phylogenetic relationships to *A. fucosus* (97·6 % sequence similarity), *A. ramosus* (95·3 %), *A. albus* (95·1 %), *A. cerinus* subsp. *cerinus* (95·0 %) and *A. cerinus* subsp. *nitratus* (92·1 %). Sequence alignment was performed using the software suite ARB (Ludwig *et al.*, 2004). Alignment was manually edited considering the expected sequence secondary structure. An unrooted phylogenetic tree was constructed by the neighbour-joining method through the ARB suite. The tree topology obtained was reconstructed by quartet-puzzling using the program TREEPUZZLE (Strimmer & von Haeseler, 1996) available within the ARB package. The quartet-puzzling tree represented a consensus tree showing

Table 1. Characteristics that differentiate strain Z33^T from its closest relatives within the genus *Agromyces*

Taxa: 1, Z33^T; 2, *A. fucosus*; 3, *A. ramosus*; 4, *A. albus*; 5, *A. cerinus* subsp. *nitratus*; 6, *A. cerinus* subsp. *cerinus*. −, Negative; +, positive; (+), weakly positive; +/−, variable; *, delayed; ND, not determined; tr, trace. Data from this study unless indicated.

Characteristic	1	2	3†	4	5	6†
Colony colour‡	Y	Y	W	W	Y	Y
DNA G+C content (mol%)	71·2	70·6	68·9	69·0	70·9	70·5
Growth at 37 °C	tr	+	+	+	(+)*	ND
Decomposition or hydrolysis of:						
Casein	+	+	−	+	+	−
Gelatin	+/−	+	−	+	−	−
Hippurate	+	+	+	+	+	−
Hypoxanthine	+	+	−	−	+	(+)
Tyrosine	+	+	−	−	+	+
Urea	−	−	−	+	−	−
Xanthine	+	+	−	−	−	−
Biochemical tests						
Nitrate reduction	−	−	+/−	−	+	−
Catalase reaction	+	+	−	+	+	+
Oxidase test	+/−	+	−	+	+/−	+
Microaerophilic growth	+	+	+	−	+	−[a]
Acid production from (API 50 CH B/E):						
Adonitol	−	−	−[b]	+	−	−[b]
Amygdalin	+	+	+[b]	−	+	+[b]
L-Arabinose	+	+	+	+	−	−
Cellobiose	+	+	−	−	+	+
D-Fucose	−	−	−[b]	(+)	−	−[b]
L-Fucose	+	+	+[b]	−	+	(+)[b]
Galactose	+	+	−	(+)	+	+
D-Glucose	+	+	−	(+)	+	+
Glycerol	+	+	+	−	+	+
Inulin	+	(+)	+	−	−	−
Lactose	(+)	−	−	−	−	+
Methyl α-D-mannoside	+	+	−[b]	−	−	−[b]
Methyl α-D-glucoside	−	+	−[b]	−	−	−[b]
Maltose	+	+	−	+	+	+
Mannitol	+	−	(+)	+	−	−
Mannose	+	+	−	(+)	+	+
Melibiose	−	−	−[b]	(+)	−	−[b]
N-Acetylglucosamine	+/−	+	+[b]	−	+	−[b]
D-Raffinose	+	+	+	+*	−	−
Ribose	−	−	−	+*	−	−
Salicin	+	+	−	−	+	+
Sucrose	+	+	+	(+)	−	+
Trehalose	−	(+)*	−	−	−	−
D-Xylose	+	−	−	+*	−	−
Utilization of:						
Aconitate	−	−	−	−	−	+
Citrate	−	−	−	−	−	+
Malate	+	−	+	+	−	+
Succinate	+	−	+	+	−	−
Enzyme assays (API ZYM)						
α-Galactosidase	−	−	−[b]	−	+	+[b]
β-Galactosidase	+	+	−[b]	+	+	+[b]
α-Glucosidase	+	+	−[b]	+	+	+[b]

Table 1. cont.

Characteristic	1	2	3†	4	5	6†
N-Acetyl-β-glucosaminidase	+	+	−[b]	+	−	−[b]
α-Fucosidase	−	−	−[b]	−	+	−[b]
Antibiotic sensitivity						
Ampicillin (10 μg)	−	−	+	+	−	+
Ciprofloxacin (5 μg)	+	+	+	+	−	+/−
Kanamycin (30 μg)	−	+	+	+	+	+
Nalidixic acid (30 μg)	−	−	−[b]	+	−	−[b]
Novobiocin (5 μg)	+	+	−[b]	+	+	+[b]
Penicillin G (10 IU)	−	−	+	−	−	−
Polymyxin B (300 IU)	−	−	+	+	+	+/−
Tetracycline (30 μg)	+	+	−[b]	+	+	+[b]

†Data from Groth *et al.* (1996) except where indicated by: *a*, data from Dorofeeva *et al.* (2003); *b*, data from this study.
‡W, White; Y, yellow.

well-supported branching, and was based on 1000 puzzling trials. The reliability value of each internal branch indicates as a percentage how often the corresponding cluster was found. Fig. 1 shows the proposed phylogenetic relationships between the members of the genus *Agromyces* and strain Z33^T.

The DNA G+C content of strain Z33^T was 71·2 mol%, which is in agreement with the values observed within the genus *Agromyces* (68·9–72·8 mol%; Dorofeeva *et al.*, 2003). The degree of DNA–DNA relatedness between the *Agromyces* type strains investigated and strain Z33^T was determined by two independent methods: the DNA–DNA hybridization method described by Ziemke *et al.* (1998) and by measuring the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m) as described by Gonzalez & Saiz-Jimenez (2005). These studies revealed significant differences between strain Z33^T and its closest phylogenetic neighbours within the genus *Agromyces*. Hybridization experiments revealed DNA–DNA relatedness levels of $\leqslant$66 % and a ΔT_m of $\geqslant$5·8 °C (equivalent to a

Table 2. Chemotaxonomic characteristics of strain Z33^T

Abbreviations: Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; DAB, diaminobutyric acid; DPG, diphosphatidylglycerol; GL, unknown glycolipid; PG, phosphatidylglycerol; PL, unknown phospholipid.

Characteristic	Strain Z33^T
Whole-cell sugars	Rha, Glc, Gal, Man
Cell-wall amino acids	DAB, Glu, Gly, Ala
Major menaquinones	MK-12, MK-13 (58 : 35)
Polar lipids	DPG, PG, PL, 2GL
Acyl type	Acetyl
Fatty acid composition (%)	
iso-$C_{15:0}$	16·3
anteiso-$C_{15:0}$	45·6
iso-$C_{16:0}$	12·4
$C_{16:0}$	1·01
iso-$C_{17:0}$	3·57
anteiso-$C_{17:0}$	20·4

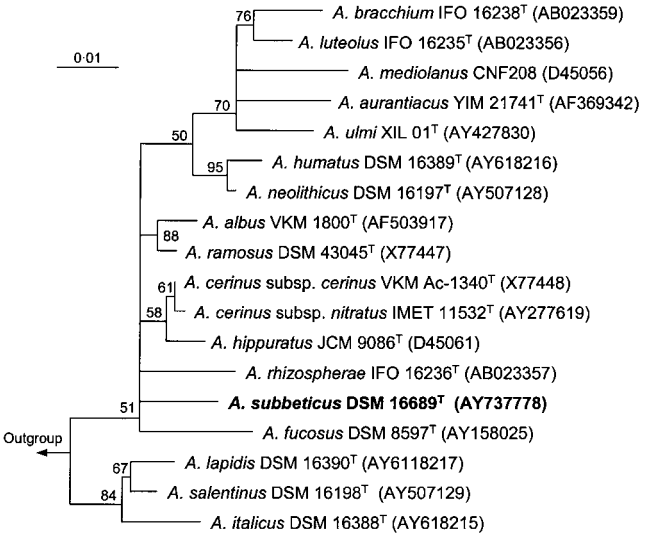

Fig. 1. Phylogenetic tree showing the proposed relationships between the type strains of *Agromyces* species and the studied strain Z33^T. Reliability values are given at branching points as percentages from 1000 trials. *Corynebacterium* sp. QSSC3-5 (GenBank accession number AF170740) was used as the outgroup (not shown). Strain names and accession numbers (in parentheses) for the species represented in the tree are shown. Bar, 0·01 nucleotide substitutions per site.

DNA–DNA relatedness level of approximately 51 %, as proposed by Rosselló-Mora & Amann, 2001). These values were below the 70 % DNA–DNA relatedness and above the 5 °C ΔT_m recommended as cut-off points for the delineation of species (Wayne *et al.*, 1987). These results indicate that strain Z33^T shows enough genomic coherence and low enough levels of DNA–DNA relatedness to its closest relatives to be considered as a novel species (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002).

The genotypic and phenotypic characteristics of strain Z33^T are consistent with its classification in the genus *Agromyces* (Gledhill & Casida, 1969; Zgurskaya *et al.*, 1992).

Strain Z33^T can be readily distinguished from representatives of recognized *Agromyces* species on the basis of a number of phenotypic properties (Table 1). DNA–DNA relatedness data support the differentiation between strain Z33^T and its closest relatives within the genus *Agromyces*. Based on the results of this polyphasic approach, we suggest the studied isolate represents a novel species of the genus *Agromyces*, for which the name *Agromyces subbeticus* sp. nov. is proposed.

Description of *Agromyces subbeticus* sp. nov.

Agromyces subbeticus (sub.be′ti.cus. N.L. masc. adj. *subbeticus* referring to the Subbetic Mountain Range, southern Spain, where the Cave of Bats is located).

Gram-positive, aerobic and microaerophilic actinomycete. Cells form branching hyphae (0·3–0·5 μm in width by 2·5–4·0 μm in length) that break up into irregular rod-shaped and diphtheroid fragments. Colonies are circular, convex, smooth and intense yellow. Colony diameter is about 1 mm. Growth occurs between 6 and 37 °C (optimally at 28 °C) and at pH values between 5 and 9·5. Growth occurs at up to 4 % NaCl. It hydrolyses aesculin and starch, but not adenine or Tween 80. Utilizes acetate, but not benzoate or DL-tartrate. Produces H_2S. Indole, Voges–Proskauer and methyl red tests are negative. It produces acid from starch, arbutin, aesculin, D-fructose, glycogen and rhamnose, but not from DL-arabitol, dulcitol, 2-ketogluconate, 5-ketogluconate, erythritol, β-gentiobiose, gluconate, inositol, D-lyxose, melezitose, sorbitol, L-sorbose, D-tagatose, D-turanose, L-xylose, methyl β-xyloside or xylitol. It produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase, but not lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase or α-mannosidase. It is sensitive to chloramphenicol (30 μg), imipenem (10 μg), ofloxacin (10 μg), oxytetracycline hydrochloride (30 μg), rifampicin (5 μg), streptomycin (10 μg) and vancomycin hydrochloride (30 μg), but not to lincomycin (2 μg), methicillin (5 μg), norfloxacin (10 μg), nalidixic acid (30 μg) or sulfonamide (200 μg). Additional phenotypic characteristics are given in Table 1. Whole-cell sugars are rhamnose, glucose, galactose and mannose. Cell-wall amino acids are diaminobutyric acid, glutamic acid, glycine and alanine. Major menaquinones are MK-12 and MK-13. Polar lipids are diphosphatidylglycerol, an unknown phospholipid and two unknown glycolipids. Acyl type is acetyl. Major fatty acids are anteiso-$C_{15:0}$ (45·6 %), anteiso-$C_{17:0}$ (20·4 %), iso-$C_{15:0}$ (16·3 %), iso-$C_{16:0}$ (12·4 %), iso-$C_{17:0}$ (3·57 %) and $C_{16:0}$ (1·01). G+C content of the DNA is 71·2 mol%.

The type strain, Z33^T (=HKI 0340^T=DSM 16689^T=NCIMB 14025^T), was isolated from a blue–grey cyanobacterial biofilm covering the walls of the Cave of Bats, Zuheros, Cordoba, southern Spain.

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