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Agromyces salentinus sp. nov. and *Agromyces neolithicus* sp. nov.

Valme Jurado,[1] Ingrid Groth,[2] Juan M. Gonzalez,[1] Leonila Laiz[1] and Cesareo Saiz-Jimenez[1]

[1]Instituto de Recursos Naturales y Agrobiologia, CSIC, Apartado 1052, 41080 Sevilla, Spain

[2]Hans-Knöll-Institut für Naturstoff-Forschung eV, D-07745 Jena, Germany

Correspondence
Cesareo Saiz-Jimenez
saiz@irnase.csic.es

A polyphasic study was carried out to clarify the taxonomic position of two Gram-positive bacteria isolated from soil samples of the Grotta dei Cervi (Italy), a relatively unexplored hypogean environment. The strains, 20-5^T and 23-23^T, showed phenotypic and phylogenetic characteristics that were consistent with their classification in the genus *Agromyces*. 16S rRNA gene sequence comparisons revealed that the two strains formed distinct phyletic lines within the genus *Agromyces*. Based on 16S rRNA gene sequence similarity, chemotaxonomic data and the results of DNA–DNA relatedness studies, it is proposed that the two isolates represent two novel species of the genus *Agromyces*. Pronounced differences in a broad range of phenotypic characteristics and DNA G+C content distinguished the two strains from each other and from previously described species of the genus *Agromyces*. Two novel species are proposed: *Agromyces salentinus* sp. nov. (type strain, 20-5^T = HKI 0320^T = DSM 16198^T = NCIMB 13990^T) and *Agromyces neolithicus* sp. nov. (type strain, 23-23^T = HKI 0321^T = DSM 16197^T = NCIMB 13989^T).

The genus *Agromyces*, with the type species *Agromyces ramosus*, was established by Gledhill & Casida (1969) for filamentous, nutritionally fastidious, catalase- and oxidase-negative soil isolates. Zgurskaya *et al.* (1992) emended the description of the genus and added two species, each with two subspecies, *Agromyces cerinus* subsp. *cerinus*, *A. cerinus* subsp. *nitratus*, *Agromyces fucosus* subsp. *fucosus* and *A. fucosus* subsp. *hippuratus*, which are characterized by rapid growth on simple media and positive catalase and oxidase reactions. Recently, the reclassification of *A. fucosus* subsp. *hippuratus* as *Agromyces hippuratus* has been proposed (Ortiz-Martinez *et al.*, 2004). Currently, the genus *Agromyces* encompasses ten species, among them *Agromyces mediolanus* (Suzuki *et al.*, 1996), comprising the former misclassified species '*Corynebacterium mediolanum*' and '*Flavobacterium dehydrogenans*' and soil isolates lacking a mycelial growth phase. The remaining recognized species at the time of writing are *Agromyces albus* (Dorofeeva *et al.*, 2003), *Agromyces aurantiacus* (Li *et al.*, 2003), *Agromyces bracchium*, *Agromyces luteolus* and *Agromyces rhizospherae* (Takeuchi & Hatano, 2001).

In this study, two strains, 20-5^T and 23-23^T, are described.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 20-5^T and 23-23^T are AY507129 and AY507128, respectively.

Strain 20-5^T was isolated from a top soil sample collected a few metres from the entrance of Grotta dei Cervi, Porto Badisco, Italy (Laiz *et al.*, 2000) and strain 23-23^T was from a soil sample collected inside the cave. Both strains were isolated using PY-BHI agar (Yokota *et al.*, 1993) incubated at 28 °C. Cells for chemotaxonomic analyses were prepared in OM79 medium (Prauser & Falta, 1968), pH adjusted to 7·5, containing (l^{-1}): 10 g glucose, 10 g Bacto-Peptone (Difco), 2 g casein hydrolysate, 2 g yeast extract and 6 g NaCl. The following type strains were included for comparative studies: *A. fucosus* IMET 11529^T, *A. cerinus* subsp. *cerinus* IMET 11525^T, *A. cerinus* subsp. *nitratus* IMET 11532^T and *A. ramosus* IMET 11027^T.

Cell morphology and cell dimensions were examined using a Zeiss Axioscope 2 phase-contrast microscope equipped with image analysing Axio Vision 2.05 software. Colony morphology of 3- and 14-day-old cultures grown on OM79 was studied using a stereo microscope.

Acid production from a variety of substrates was tested using the API 50 CH system and API 50CHB/E medium (bioMérieux) according to the manufacturer's instructions. Decomposition of adenine, hypoxanthine, xanthine and tyrosine, utilization of organic acids, nitrate reduction, urease activity, catalase and hydrogen sulfide production, hydrolysis of gelatin and Tween 80, methyl red and Voges–Proskauer, and oxidase activity were analysed as reported previously (Groth *et al.*, 1996). Indole production and

hydrolysis of hippurate were studied as recommended by Smibert & Krieg (1994). Casein and starch hydrolysis was examined according to Cowan & Steel (1965). Susceptibility to antibiotics was studied by placing antibiotic discs (Oxoid) on OM79 agar plates that were seeded with suspensions of the test strains. The API ZYM galleries (bioMérieux) were used to study enzymic activities.

Analysis of cell wall amino acids, whole cell sugars, menaquinones, polar lipids and the acyl type was carried out as described by Groth *et al.* (1996). Cellular fatty acid profiles were analysed according to standard methods recently described by Gonzalez *et al.* (2004).

Bacterial DNA was extracted following the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR using the conserved primers 27F (5′-AGA GTT TGA TCC TGG CTC AG) and 1522R (5′-AAG GAG GTG ATC CAG CCG CA). PCR thermal conditions were as follows: 95 °C for 1 min; 35 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 2 min; and a final extension cycle at 72 °C for 10 min. Forward and reverse strands of the amplified DNA fragment were sequenced in an ABI 3700 sequencer (Applied Biosystems). A similarity search was performed using the BLAST algorithm (Altschul *et al.*, 1990) at the NCBI database (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Alignments and phylogenetic relationships were determined by the neighbour-joining method using the ARB software package (Ludwig *et al.*, 1998).

The G+C content of the DNA was determined according to the fluorimetric method described by Gonzalez & Saiz-Jimenez (2002) using thermal denaturation temperature. The degree of DNA–DNA relatedness between the two isolated strains and previously described *Agromyces* species was determined by measuring the divergence between the thermal denaturation midpoint of homoduplex DNA and heteroduplex DNA (ΔT_m), as described by De Ley *et al.* (1970). The approximate degrees of DNA relatedness were calculated following the relationship proposed by Rosselló-Mora & Amann (2001) between ΔT_m and DNA–DNA binding.

It is apparent from both phenotypic and phylogenetic results that strains 20-5^T and 23-23^T are members of the genus *Agromyces*. The comparison of 16S rRNA gene sequences revealed significant differences below the generally accepted species threshold (97 %) (Stackebrandt & Goebel, 1994) between the two isolates and all previously described species of the genus *Agromyces* ($\leqslant$96 % similarity). Phylogenetic analysis indicated that *A. ramosus*, *A. fucosus* and *A. cerinus* subsp. *nitratus* are the closest relatives to strains 20-5^T and 23-23^T, with similarity values ranging between 95 and 96 %. A phylogenetic tree showing the relationships between members of the genus *Agromyces* and strains 20-5^T and 23-23^T is shown in Fig. 1.

A broad range of physiological characteristics also distinguished strains 20-5^T and 23-23^T from each other, including differences in the decomposition of gelatin and urea, reduction of nitrate, acid production from numerous different carbon sources, enzymic activities tested by the API ZYM galleries and sensitivity to some antibiotics (Table 1).

Differences in the compositions of menaquinones, whole cell sugars, polar lipids, acyl type and cell wall amino acids are shown in Table 2. The predominant fatty acids of strains 20-5^T and 23-23^T were iso-$C_{15:0}$ (6·0 and 12·8 %, respectively), anteiso-$C_{15:0}$ (52·0 and 37·8 %), iso-$C_{16:0}$ (12·9 and 13·0 %), iso-$C_{17:0}$ (2·2 and 3·4 %) and anteiso-$C_{17:0}$ (24·8 and 31·9 %).

The DNA G+C content underlined the distinct taxonomic position of the two strains within the genus *Agromyces*. The DNA G+C contents of strains 20-5^T and 23-23^T were 72·3 and 65·3 mol%, respectively. DNA–DNA relatedness studies showed significant differences between strains 20-5^T and 23-23^T (9 °C; corresponding to approximately 45 % relatedness) and between these two strains and the most

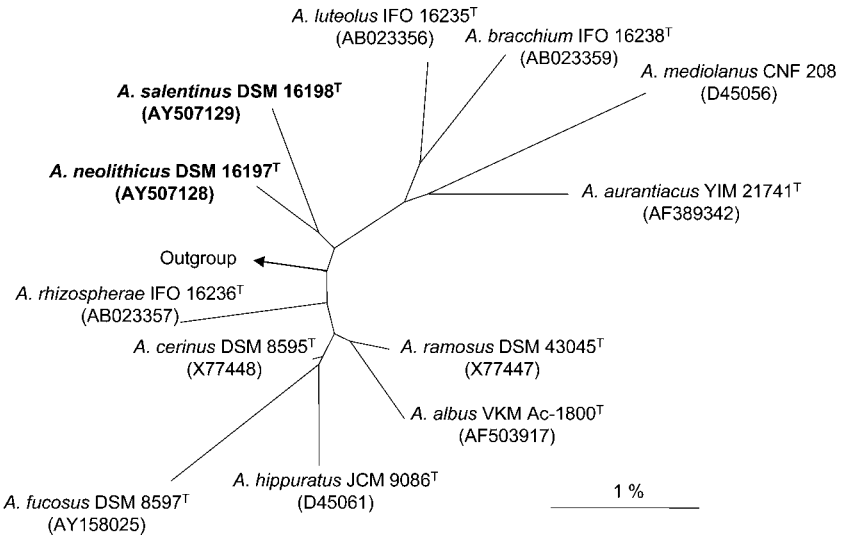

Fig. 1. Phylogenetic tree showing the relationships between species of the genus *Agromyces* and the two studied isolates (*Agromyces salentinus* 20-5^T and *Agromyces neolithicus* 23-23^T). Sequences used in this analysis were from *A. fucosus*, *A. hippuratus*, *A. cerinus*, *A. ramosus*, *A. rhizospherae*, *A. albus*, *A. aurantiacus*, *A. luteolus*, *A. mediolanus*, *A. bracchium*, *A. salentinus* and *A. neolithicus*.

Table 1. Characteristics that can be used to differentiate strains 20-5^T and 23-23^T from their closest relatives within the genus *Agromyces*

Taxa: 1, strain 20-5^T; 2, strain 23-23^T; 3, *A. fucosus* IMET 11529^T; 4, *A. ramosus* IMET 11027^T; 5, *A. cerinus* subsp. *nitratus* IMET 11532^T; 6, *A. cerinus* subsp. *cerinus* IMET 11525^T. Tested strains are all negative for hydrolysis of adenine and Tween 80 and positive for hydrolysis of aesculin and starch. Benzoate and DL-tartrate are not utilized by any of these strains. All strains are positive for the production of hydrogen sulfide. Voges–Proskauer, methyl red and indole tests gave negative results for all strains. All strains are susceptible to chloramphenicol (30 μg), imipenem (10 μg), ofloxacin (10 μg), oxytetracycline (30 μg), rifampicin (30 μg), tetracycline (30 μg), vancomycin (30 μg) and ovobiocin (5 μg) and sensitive to lincomycin (2 μg). All strains have alkaline phosphatase activity; none of them has lipase (C14) activity. −, Negative; +, positive; (+) weakly positive; +/−, variable; (+/−), weak and variable; ND, not determined.

Characteristic	1	2	3	4*	5	6*
G+C content (mol%)	72·3	65·3	70·6	68·9	70·9	70·5
Decomposition/hydrolysis of:						
Casein	+	+	+	−	+	−
Gelatin	−	+	+	−	−	−
Hippurate	+	+	+	ND	+	−
Hypoxanthine	−	−	+	−	+	(+)
Tyrosine	+	+	+	−	+	+
Urea	+	−	−	−	−	−
Xanthine	−	−	+	−	−	−
Acid production from:						
L-Arabinose	+	+	+	+	−	−
β-Gentibiose	(+)	−	−	ND	−	ND
Inulin	+	−	(+)	−	−	−
Lactose	−	−	−	−	−	+
Maltose	+	+	+	−	+	+
Mannitol	−	+	−	(+)	−	−
Melibiose	−	+	−	−†	−	−
N-Acetylglucosamine	−	(+)	+	ND	+	ND
Raffinose	(+)	+	+	+	−	−
Rhamnose	+	−	+	+	+	+
Ribose	+	−	−	−	−	−
Salicin	+	−	+	−	(+)	+
Sucrose	+	(+/−)	+	−†	−	ND
Trehalose	+	−	(+)	−	−	−
D-Turanose	+	−	−	+/−†	−	ND
D-Xylose	+	−	−	−	−	−
Utilization of:						
Acetate	+	−	+	+	+	+
Aconitate	−	−	−	−	−	+
Citrate	−	−	−	−	−	+
Malate	−	−	−	+	−	+
Succinate	−	−	−	+	−	−
Nitrate reduction	−	+	−	+/−	+	−
Catalase reaction	+	+	+	−	+	+
Oxidase test	+/−	+/−	+	−	+/−	+
Microaerobic growth	(+)	+	+	+	+	ND
Antibiotic susceptibility:						
Ampicillin (10 μg)	+	+	−	+	−	+
Ciprofloxacin (5 μg)	+	+	+	+	−	(+)
Kanamycin (30 μg)	+	−	+	+	+	+
Methicillin (5 μg)	+	+	−	ND	−	ND
Norfloxacin (10 μg)	+	+	−	−	−	−
Penicillin G (10 μg)	+	+	−	+	−	−
Polymyxin B (300 μg)	+	+	−	+	+	(+)

Table 1. cont.

Characteristic	1	2	3	4*	5	6*
Streptomycin (10 μg)	+	−	+	+	+	+
Sulfonamide (200 μg)	+	−	−	−	−	−
Nalidixic acid (30 μg)	+	−	−	−	−	−
Enzymic activity:						
α-Chymotrypsin	−	+	−	−	−	−
α-Galactosidase	−	+	−	−	+	+
β-Galactosidase	+	−	+	−	+	+
β-Glucuronidase	−	+	−	−	−	−
β-Glucosidase	+	−	+	+	+	+
α-Mannosidase	−	+	−	−	−	−

*Data from Groth *et al.* (1996), except where marked.
†Data from Gledhill & Casida (1969).

closely related *Agromyces* species, *A. ramosus* (14 and 14 °C for strains 20-5^T and 23-23^T, respectively; corresponding to approximately 20 % relatedness), *A. cerinus* subsp. *nitratus* (16 and 11 °C; 10 and 35 % relatedness, respectively), *A. cerinus* subsp. *cerinus* (15 and 11 °C; 15 and 35 % relatedness, respectively) and *A. fucosus* (9 and 8 °C; corresponding to 45 and 50 % relatedness, respectively). Thus, DNA–DNA relatedness results showed differences below the species threshold (5 °C; Stackebrandt & Goebel, 1994) between strains 20-5^T and 23-23^T and their closest relatives within the genus *Agromyces*.

Based on the results of the polyphasic approach presented in this study, it is proposed that strains 20-5^T and 23-23^T represent two novel species of the genus *Agromyces*, *Agromyces salentinus* sp. nov. and *Agromyces neolithicus* sp. nov., respectively.

Description of *Agromyces salentinus* sp. nov.

Agromyces salentinus (sa.len.ti′nus. N.L. masc. adj. *salentinus* referring to Salentine Peninsula, the location of Grotta dei Cervi, the area from which the organism was isolated).

Cells form branching hyphae (width 0·5–0·7 μm) that break up into irregular diphtheroid and rod-like non-motile fragments. Gram-positive, aerobic and microaerophilic, growing between 10 and 37 °C (optimal growth at 20–28 °C). Colonies are circular, convex, smooth and yellow. Colony diameter is about 1 mm. Phenotypic characteristics, including antibiotic susceptibility, are reported in Table 1. In addition, acid is also produced from starch, amygdalin, D-arabinose, arbutin, cellobiose, aesculin, fructose, L-fucose, galactose, glucose, glycerol, glycogen and mannose. Grows in up to 4 % NaCl. Predominant menaquinones are MK-12 and MK-11. Amino acid composition of the cell wall includes diaminobutyric acid, glutamic acid, glycine and alanine. Whole cell sugars are rhamnose, glucose, galactose, arabinose and ribose. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, an unknown glycolipid and four unknown phospholipids. Acyl type is acetyl. Predominant fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$.

Table 2. Chemotaxonomic characteristics of strains 20-5^T and 23-23^T

For both strains, the acyl type was acetyl. Components are given in order of abundance (most abundant first).

Characteristic	Strain 20-5^T	Strain 23-23^T
Whole-cell sugars*	Rha, Glu, Gal, Ara, Rib	Glu, Gal, Man
Cell-wall amino acids†	DAB, Glu, Gly, Ala	DAB, Gly, Glu, Ala
Major menaquinones	MK-12, MK-11, MK-10, MK-13	MK-13, MK-12
Polar lipids‡	DPG, PG, GL, 4PL	DPG, PG, 2PL, GL

*Ara, Arabinose; Gal, galactose; Glu, glucose; Man, mannose; Rha, rhamnose; Rib, ribose.
†Ala, Alanine; DAB, diaminobutyric acid; Glu, glutamic acid; Gly, glycine.
‡DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; GL, unknown glycolipid; PL, unknown phospholipid.

Type strain is 20-5^T (=HKI 0320^T=DSM 16198^T=NCIMB 13990^T). The G+C content of the type strain is 72·3 mol%.

Description of *Agromyces neolithicus* sp. nov.

Agromyces neolithicus (ne.o.li′thi.cus. N.L. masc. adj. *neolithicus* referring to the origin of the neolithic paintings in Grotta dei Cervi, the source of the soil from which the organism was isolated).

Cells form branching hyphae (width 0·3–0·5 μm) that break up into irregular diphtheroid and rod-like non-motile fragments. Gram-positive, aerobic and microaerophilic, growing between 15 and 37 °C (optimal growth at 28 °C). Colonies are circular, convex, smooth and beige. Colony diameter is about 1 mm. Phenotypic characteristics, including antibiotic susceptibility, are reported in Table 1. In addition, acid is also produced from starch, D-arabinose, arbutin, cellobiose, aesculin, fructose, L-fucose, galactose, glucose, glycerol, glycogen and mannose. No growth occurs in 4 % NaCl. Predominant menaquinones are MK-13 and MK-12. Amino acid composition of the cell wall includes diaminobutyric acid, glutamic acid, glycine and alanine. Whole cell sugars are glucose, galactose and mannose. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, two unknown phospholipids and an unknown glycolipid. Predominant fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$.

Type strain is 23-23^T (=HKI 0321^T=DSM 16197^T=NCIMB 13989^T). The G+C content of the type strain is 65·3 mol%.

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