Agromyces italicus sp. nov., *Agromyces humatus* sp. nov. and *Agromyces lapidis* sp. nov., isolated from Roman catacombs

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A polyphasic study was carried out to clarify the taxonomic positions of three Gram-positive isolates from the Catacombs of Domitilla, Rome (Italy). 16S rRNA gene sequence comparisons placed these strains within the genus *Agromyces*. The morphological and chemotaxonomic characteristics of these isolates were consistent with the description of the genus *Agromyces*. The three isolates could be readily distinguished from one another and from representatives of all *Agromyces* species with validly published names by a broad range of phenotypic characteristics and DNA–DNA relatedness studies. Therefore, these isolates are proposed to represent three novel species of the genus *Agromyces*, *Agromyces italicus* sp. nov. (type strain CD1^T = HKI 0325^T = DSM 16388^T = NCIMB 14011^T), *Agromyces humatus* sp. nov. (type strain CD5^T = HKI 0327^T = DSM 16389^T = NCIMB 14012^T) and *Agromyces lapidis* sp. nov. (type strain CD55^T = HKI 0324^T = DSM 16390^T = NCIMB 14013^T).

Strains of the genus *Agromyces* are common inhabitants of different soils ranging from fertile meadows to deserts [*Agromyces ramosus* Gledhill and Casida 1969, *Agromyces cerinus* subsp. *cerinus* Zgurskaya *et al.* 1992, *Agromyces cerinus* subsp. *nitratus* Zgurskaya *et al.* 1992, *Agromyces fucosus* Zgurskaya *et al.* 1992 emend. Ortiz-Martinez *et al.* 2004, *Agromyces hippuratus* (Zgurskaya *et al.* 1992) Ortiz-Martinez *et al.*, 2004, *Agromyces mediolanus* Suzuki *et al.* 1996 and *Agromyces aurantiacus* Li *et al.* 2003]. The rhizosphere of plants harbours a wide diversity of these organisms, as has been shown by Takeuchi & Hatano (2001) with the description of three species, *Agromyces bracchium*, *Agromyces luteolus* and *Agromyces rhizospherae*, from mangrove rhizosphere soil. Dorofeeva *et al.* (2003) isolated *Agromyces albus* from the leaves and inflorescences of members of the Primulaceae, while *Agromyces ulmi* was isolated from the decayed stump of an elm tree, *Ulmus nigra* (Rivas *et al.*, 2004). Recently, two novel species of this genus, *Agromyces salentinus* and *Agromyces neolithicus*, have been isolated from the Grotta dei Cervi, a cave in southern Italy with singular neolithic rock art paintings (Jurado *et al.*, 2005).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CD1^T, CD5^T and CD55^T are AY618215, AY618216 and AY618217, respectively.

Strains CD1^T and CD5^T were obtained from the wall of a tomb located in the Little Apostle cubicle, Domitilla Catacombs, Rome, Italy (Sanchez-Moral *et al.*, 2004). Strain CD55^T was isolated by touching the stone wall of a tomb (first arcosolium on the left side behind the entrance of Domitilla Catacombs) with a sterile cotton swab and suspending the attached bacteria in 1 : 10 diluted organic medium 79 (OM79) (Prauser & Falta, 1968; Jurado *et al.*, 2005). The strains were isolated on nutrient agar (Difco) or, in the case of strain CD55^T, on peptone/yeast extract/brain heart infusion agar (Yokota *et al.*, 1993), using a standard dilution plate procedure.

All the methods used in this study have been recently described by Jurado *et al.* (2005). Range of pH for growth was established using liquid OM79 medium adjusted to initial pH values between 4 and 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. albus* VKM Ac-1800^T, *A. cerinus* subsp. *cerinus* IMET 11525^T, *A. fucosus* IMET 11529^T, *A. mediolanus* VKM Ac-1388^T, *A. neolithicus* DSM 16197^T, *A. ramosus* IMET 11027^T and *A. salentinus* DSM 16198^T. Morphological and physiological traits are summarized in the species descriptions and Table 1.

Differences referring to the compositions of whole-cell sugars, menaquinones and polar lipids are shown in Table 2. Cell-wall amino acids in strains CD1^T, CD5^T and CD55^T

Table 1. Characteristics that differentiate strains CD1^T, CD5^T and CD55^T and their closest relatives within the genus *Agromyces*

Strains: 1, *A. italicus* sp. nov. CD1^T; 2, *A. humatus* sp. nov. CD5^T; 3, *A. lapidis* sp. nov. CD55^T; 4, *A. albus* VKM Ac-1800^T; 5, *A. cerinus* subsp. *cerinus* IMET 11525^T; 6, *A. fucosus* IMET 11529^T; 7, *A. mediolanus* VKM Ac-1388^T; 8, *A. neolithicus* DSM 16197^T; 9, *A. ramosus* IMET 11027^T; 10, *A. salentinus* DSM 16198^T. Data for *A. cerinus* subsp. *cerinus* and *A. ramosus* were taken from Groth *et al.* (1996). −, Negative; +, positive; (+) weakly positive; V, variable; ND, not determined. Strains CD1^T, CD5^T and CD55^T share the following properties. They produce acid from starch, arbutin, D-fructose, glycogen, maltose, D-mannose, but not from adonitol, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, gluconate, 2-ketogluconate, 5-ketogluconate, β-gentiobiose, inositol, D-lyxose, melibiose, melezitose, sorbitol, L-sorbose; D-tagatose, L-xylose, methyl β-xyloside or xylitol. They produce alkaline phosphatase, esterase (C1), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase, but not α-galactosidase, *N*-acetyl-β-glucosaminidase, lipase (C14), trypsin or α-fucosidase. They are sensitive to chloramphenicol (30 μg), imipenem (10 μg), ofloxacin (10 μg), oxytetracycline hydrochloride (30 μg), rifampicin (5 μg) and vancomycin hydrochloride (30 μg), but not to kanamycin (30 μg), lincomycin (2 μg), norfloxacin (10 μg) or sulfonamide (200 μg). They hydrolyse aesculin and starch, but not adenine or Tween 80. Voges–Proskauer, methyl red and indole tests give negative results. They are positive for H_2S production.

Characteristic	1	2	3	4	5	6	7	8	9	10
DNA G+C content (mol%)	70·8	70·6	70·4	69·0	70·5	70·6	72·3	65·3	68·9	72·3
Growth at 10 °C	(+)	−	(+)	(+)	(+)	+	(+)	−	+	+
Growth in 4·0 % NaCl (w/v)	+	−	+	(+)	−	+	+	−	(+)	(+)
Microaerophilic growth	+	−	+	−	−	+	+	+	+	(+)
Decomposition or hydrolysis of:										
Casein	+	+	+	+	−	+	+	+	−	+
Gelatin	+	+	V	+	−	+	−	+	−	−
Hippurate	+	+	+	+	−	+	+	+	+	+
Hypoxanthine	+	(+)	−	−	(+)	+	+	−	−	−
Tyrosine	+	+	+	−	+	+	+	+	−	+
Urea	−	−	−	+	−	−	−	−	−	+
Xanthine	+	−	−	−	−	+	+	−	−	−
Biochemical tests										
Nitrate reduction	+	+	+	−	−	−	−	+	V	−
Catalase reaction	+	+	+	+	+	+	+	+	−	+
Oxidase test	+	−	V	+	+	+	V	V	−	V
Acid production from (API 50 CH B/E):										
Amygdalin	(+)	−	+	−	+	+	+	−	+	(+)
D-Arabinose	+	−	+	+*	+	+	(+)*	+	+	+
L-Arabinose	+	+	−	+	−	+	−	+	+	+
Cellobiose	+	V	+	−	+	+	+	+	−	+
L-Fucose	+	−	+	−	(+)	+	−	+	+	+
Galactose	+	+	+	(+)	+	+	+	+	−	+
D-Glucose	+	+	+	(+)	+	+	+	+	−	+
Glycerol	+	+	+	−	+	+	+	+	+	+
Inulin	−	+	+	−	−	(+)	−	−	+	+
Lactose	(+)	−	−	−	+	−	+	−	−	−
Methyl α-D-mannoside	−	−	+	−	−	+	−	−	−	−
Methyl α-D-glucoside	−	−	−	−	−	+	−	−	−	−
Mannitol	−	+	−	+	−	−	−	+	(+)	−
N-Acetylglucosamine	−	V	+	−	−	+	−	(+)*	+	−
D-Raffinose	−	+	(+)	+*	−	+	−	+	+	(+)
Rhamnose	−	+	−	+	+	+*	+	−	+	+
Ribose	−	−	−	+*	−	−	+	−	−	+
Salicin	+	−	+	−	+	+	(+)	−	−	+
Sucrose	−	+	+	(+)	−	+	+	(V)	+	+
Trehalose	−	−	−	−	−	(+)*	+	−	−	+
D-Turanose	−	−	−	−	−	−	(+)	−	−	+
D-Xylose	+	−	−	+*	−	−	−	−	−	+
Utilization of:										
Aconitate	−	−	−	−	+	−	−	−	−	−

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10
Citrate	−	−	−	−	+	−	−	−	−	−
Malate	−	+	−	+	+	−	−	−	+	−
Succinate	−	+	−	+	−	−	−	−	+	−
Enzyme assays (API ZYM):										
α-Chymotrypsin	−	−	+	−	−	−	−	+	−	−
α-Galactosidase	−	−	−	−	+	−	−	+	−	−
β-Galactosidase	+	+	+	+	+	+	+	−	−	+
β-Glucuronidase	−	+	−	−	−	−	−	+	−	−
N-Acetyl-β-glucosaminidase	−	−	−	+	−	+	+	+	−	+
α-Mannosidase	−	+	−	−	−	−	−	+	−	−
Antibiotic susceptibility:										
Ampicillin (10 μg)	+	+	+	+	+	−	+	+	+	+
Ciprofloxacin (5 μg)	+	+	+	+	V	+	−	+	+	+
Kanamycin (30 μg)	−	−	−	+	+	+	−	−	+	+
Methicillin (5 μg)	+	+	+	−	ND	−	−	+	ND	+
Nalidixic acid (30 μg)	−	−	+	+	−	−	−	−	−	+
Norfloxacin (10 μg)	−	−	−	−	−	−	−	+	−	+
Novobiocin (5 μg)	+	+	+	+	+	+	−	+	−	+
Penicillin G (10 IU)	+	−	+	−	−	−	−	+	+	+
Polymyxin B (300 IU)	−	+	−	+	V	−	−	+	+	+
Streptomycin sulfate (10 μg)	−	+	−	+	+	+	−	−	+	+
Sulfonamide (200 μg)	−	−	−	−	−	−	−	−	−	+

*Delayed response.

were diaminobutyric acid, glycine, glutamic acid and alanine. Acyl type for these three strains was acetyl. The predominant fatty acids of strains CD1^T, CD5^T and CD55^T were anteiso-$C_{15:0}$ (50·9, 41·5 and 48·1 %, respectively), anteiso-$C_{17:0}$ (15·3, 34·0 and 13·0 %), iso-$C_{16:0}$ (14·7, 15·5 and 16·5 %), iso-$C_{15:0}$ (14·2, 5·4 and 10·5 %) and $C_{16:0}$ (1·7, 1·3 and 5·9 %).

Sequence comparisons of 16S rRNA genes from strains CD1^T, CD5^T and CD55^T showed a close phylogenetic relationship to *A. ramosus*, *A. cerinus* subsp. *cerinus*, *A. salentinus*, *A. neolithicus*, *A. albus*, *A. mediolanus* and *A. fucosus* with percentages of similarity ranging from 94 to 98 %. Strains CD1^T and CD55^T showed high similarity (97 %). The recently described species *A. salentinus* (Jurado *et al.*, 2005) was highly related to strains CD1^T and CD55^T with 97 % similarity. *A. neolithicus* was the closest relative to strain CD5^T (98 %). A phylogenetic tree generated by the neighbour-joining method showing the relationships between members of the genus *Agromyces* and the three novel isolates, CD1^T, CD5^T and CD55^T, is shown in Fig. 1.

The DNA G+C contents of strains CD1^T, CD5^T and CD55^T were 70·8, 70·6 and 70·4 mol%, respectively. DNA–DNA relatedness studies showed significant differences between the three isolates as well as with their closest phylogenetic neighbours. In all cases, differences in melting temperatures were $>7·7\,^{\circ}C$ (roughly $<51\,\%$ DNA–DNA relatedness;

Table 2. Chemotaxonomic characteristics of strains CD1^T, CD5^T and CD55^T

Components are listed in decreasing order of abundance.

Characteristic	Strain CD1^T	Strain CD5^T	Strain CD55^T
Whole-cell sugars*	Gal, Rib, Glc, Man	Glc, Gal, Rha, Man	Glc, Gal, Man, Rib
Major menaquinones	12, 13	13, 12	12, 13
Polar lipids†	DPG, PG, 2 PL, GL	DPG, PG, PL, 2 GL	DPG, PG, PL, 2 GL

*Gal, Galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose.

†DPG, Diphosphatidylglycerol; GL, unknown glycolipid; PG, phosphatidylglycerol; PL, unknown phospholipid.

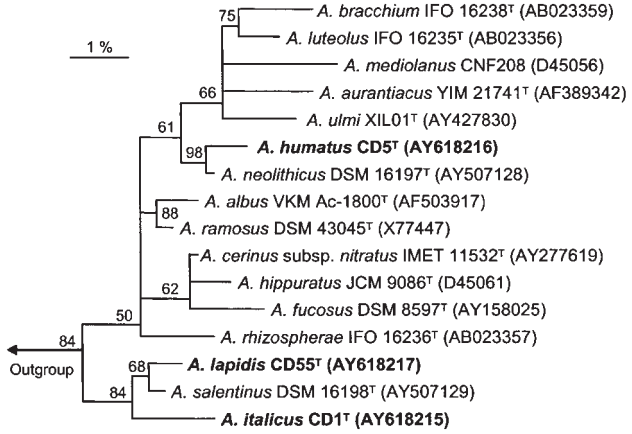

Fig. 1. Neighbour-joining phylogenetic tree showing the relationships between species of the genus *Agromyces* and isolates CD1^T, CD5^T and CD55^T based on 16S rRNA gene sequences. Bar, 1 % sequence divergence. The outgroup (not shown) was *Corynebacterium* sp. QSSC3-5 (AF170740).

Rosselló-Mora & Amann, 2001), which is well above the 5 °C cut-off point recommended for the delineation of species (Stackebrandt & Goebel, 1994).

The genotypic and phenotypic characteristics of strains CD1^T, CD5^T and CD55^T are consistent with their classification in the genus *Agromyces* (Gledhill & Casida, 1969; Zgurskaya *et al.*, 1992). The isolates can be readily distinguished from representatives of *Agromyces* species with validly published names, and from one another, by a broad range of phenotypic properties (Table 1) and composition of whole-cell sugars (Table 2). DNA–DNA relatedness data support the above distinctions between strains CD1^T, CD5^T and CD55^T and their closest relatives within the genus *Agromyces*. Based on the results of this polyphasic approach, we propose that the three studied isolates are classified within novel species of the genus *Agromyces*, *Agromyces italicus* sp. nov. (strain CD1^T), *Agromyces humatus* sp. nov. (strain CD5^T) and *Agromyces lapidis* sp. nov. (strain CD55^T).

Description of *Agromyces italicus* sp. nov.

Agromyces italicus (i.ta′li.cus. L. masc. adj. *italicus* from Italy, the origin of the type strain).

Gram-positive, aerobic to microaerophilic actinomycete that forms branching hyphae (width 0·4–0·6 μm) which break up into irregular diphtheroid and rod-like, non-motile fragments. Colonies are circular, convex, smooth and cream. Colony diameter is about 1 mm. Growth occurs between 10 and 37 °C (optimal growth at 28 °C) and at pH 5–9·5. NaCl is tolerated up to 4 % (w/v), but not at 6 % (w/v). Phenotypic characteristics including antibiotic susceptibility and enzymic activities are reported in Table 1. Chemotaxonomic characteristics are reported in Table 2. Cell-wall amino acids are diaminobutyric acid, glycine, glutamic acid and alanine. Acyl type is acetyl. Predominant fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$; mycolic acids are absent. G+C content is 70·8 mol%.

The type strain is CD1^T (=HKI 0325^T=DSM 16388^T=NCIMB 14011^T), isolated from the wall of a tomb located in the Little Apostle cubicle, Domitilla Catacombs, Rome, Italy.

Description of *Agromyces humatus* sp. nov.

Agromyces humatus (hu.ma′tus. L. masc. part. adj. *humatus* buried).

Gram-positive, aerobic actinomycete that forms branching hyphae (width 0·3–0·5 μm) which break up into irregular diphtheroid and rod-like, non-motile fragments. Colonies are circular, convex, smooth and yellow. Colony diameter is about 1 mm. Growth occurs between 15 and 37 °C (optimal growth at 28 °C) and at pH 5–9·5. NaCl is tolerated up to 2 % (w/v), but not at 4 % (w/v). Phenotypic characteristics including antibiotic susceptibility and enzymic activities are reported in Table 1. Chemotaxonomic characteristics are reported in Table 2. Cell-wall amino acids are diaminobutyric acid, glycine, glutamic acid and alanine. Acyl type is acetyl. Predominant fatty acids are anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$; mycolic acids are absent. G+C content is 70·6 mol%.

The type strain is CD5^T (=HKI 0327^T=DSM 16389^T=NCIMB 14012^T), isolated from the wall of a tomb located in the Little Apostle cubicle, Domitilla Catacombs, Rome, Italy.

Description of *Agromyces lapidis* sp. nov.

Agromyces lapidis (la.pi′dis. L. gen. n. *lapidis* of a stone).

Gram-positive, aerobic to microaerophilic actinomycete that forms branching hyphae (width 0·4–0·6 μm) which break up into irregular diphtheroid and rod-like, non-motile fragments. Colonies are circular, convex, smooth and yellow. Colony diameter is about 1 mm. Growth occurs between 10 and 37 °C (optimal growth at 28 °C) and at pH 5–9·5. NaCl is tolerated up to 4 % (w/v), but not at 6 % (w/v). Phenotypic characteristics including antibiotic susceptibility and enzymic activities are reported in Table 1. Chemotaxonomic characteristics are reported in Table 2. Cell-wall amino acids are diaminobutyric acid, glycine, glutamic acid and alanine. Acyl type is acetyl. Predominant fatty acids are anteiso-$C_{15:0}$ and iso-$C_{16:0}$; mycolic acids are absent. G+C content is 70·4 mol%.

The type strain is CD55^T (=HKI 0324^T=DSM 16390^T=NCIMB 14013^T), isolated from a carved stone wall of the Domitilla Catacombs, Rome, Italy.

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References

Dorofeeva, L. V., Krausova, V. I., Evtushenko, L. I. & Tiedje, J. M. (2003). *Agromyces albus* sp. nov., isolated from a plant (*Androsace* sp.). *Int J Syst Evol Microbiol* **53**, 1435–1438.

Gledhill, W. E. & Casida, L. E. (1969). Predominant catalase-negative soil bacteria. III. *Agromyces*, gen. nov., microorganisms intermediary to *Actinomyces* and *Nocardia*. *Appl Microbiol* **18**, 340–349.

Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.

Jurado, V., Groth, I., Gonzalez, J. M., Laiz, L. & Saiz-Jimenez, C. (2005). *Agromyces salentinus* sp. nov. and *Agromyces neolithicus* sp. nov. *Int J Syst Evol Microbiol* **55**, 153–157.

Li, W. J., Zhang, L. P., Xu, P., Cui, X. L., Xu, L. H., Zhang, Z., Schumann, P., Stackebrandt, E. & Jiang, C. L. (2003). *Agromyces aurantiacus* sp. nov., isolated from a Chinese primeval forest. *Int J Syst Evol Microbiol* **53**, 303–307.

Ortiz-Martinez, A., Gonzalez, J. M., Evtushenko, L. I., Jurado, V., Laiz, L., Groth, I. & Saiz-Jimenez, C. (2004). Reclassification of *Agromyces fucosus* subsp. *hippuratus* as *Agromyces hippuratus* sp. nov., comb. nov. and emended description of *Agromyces fucosus*. *Int J Syst Evol Microbiol* **54**, 1553–1556.

Prauser, H. & Falta, R. (1968). Phagensensibilität, Zellwand-Zusammensetzung und Taxonomie von Actinomyceten. *Z Allg Mikrobiol* **8**, 39–46 (in German).

Rivas, R., Trujillo, M. E., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2004). *Agromyces ulmi* sp. nov., a xylanolytic bacterium isolated from *Ulmus nigra* in Spain. *Int J Syst Evol Microbiol* **54**, 1987–1990.

Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Sanchez-Moral, S., Luque, L., Cañaveras, J. C., Laiz, L., Jurado, V., Hermosin, B. & Saiz-Jimenez, C. (2004). Bioinduced barium precipitation in St Callixtus and Domitilla catacombs. *Ann Microbiol* **54**, 1–12.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Suzuki, K., Sasaki, J., Uramoto, M., Nakase, T. & Komagata, K. (1996). *Agromyces mediolanus* sp. nov., nom. rev., comb. nov, a species for ''*Corynebacterium mediolanum*'' Mamoli 1939 and for some aniline-assimilating bacteria which contain 2,4-diaminobutyric acid in the cell wall peptidoglycan. *Int J Syst Bacteriol* **46**, 88–93.

Takeuchi, M. & Hatano, K. (2001). *Agromyces luteolus* sp. nov., *Agromyces rhizospherae* sp. nov. and *Agromyces bracchium* sp. nov., from the mangrove rhizosphere. *Int J Syst Evol Microbiol* **51**, 1529–1537.

Yokota, A., Takeuchi, M., Sakane, T. & Weiss, N. (1993). Proposal of six new species of the genus *Aureobacterium* and transfer of *Flavobacterium esteraromaticum* Omelianski to the genus *Aureobacterium* as *Aureobacterium esteraromaticum* comb. nov. *Int J Syst Bacteriol* **43**, 555–564.

Zgurskaya, H. I., Evtushenko, L. I., Akimov, V. N., Voyevoda, H. V., Dobrovolskaya, T. G., Lysak, L. V. & Kalakoutskii, L. V. (1992). Emended description of the genus *Agromyces* and description of *Agromyces cerinus* subsp. *cerinus* sp. nov., subsp. nov., *Agromyces cerinus* subsp. *nitratus* sp. nov., subsp. nov., *Agromyces fucosus* subsp. *fucosus* sp. nov., subsp. nov., and *Agromyces fucosus* subsp. *hippuratus* sp. nov., subsp. nov. *Int J Syst Bacteriol* **42**, 635–641.