

Classification of *Streptomyces griseus* (Krainsky 1914) Waksman and Henrici 1948 and related species and the transfer of '*Microstreptospora cinerea*' to the genus *Streptomyces* as *Streptomyces yanii* sp. nov.

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A soil actinomycete, strain 80-133^T, with the non-validly published name '*Microstreptospora cinerea*', was the subject of a polyphasic study designed to clarify its taxonomic status. Comparative 16S rRNA gene sequence studies indicated that the organism belonged to the genus *Streptomyces*, a result in line with previous chemotaxonomic and morphological data. The strain belonged to the *Streptomyces griseus* clade, but could be distinguished from representatives of species assigned to this taxon by using DNA–DNA relatedness and phenotypic data. In light of these findings, it is proposed that the organism should be recognized as a novel species of the genus *Streptomyces*. The name proposed for this taxon is *Streptomyces yanii* sp. nov., with isolate 80-133^T (= AS 4.1146^T = JCM 3331^T) as the type strain. It was also shown that representative strains of *Streptomyces argenteolus*, *Streptomyces caviscabies*, *S. griseus* and *Streptomyces setonii* belong to the same genomic species and have key phenotypic properties in common. It is proposed that *S. caviscabies* and *S. setonii* should be considered as later heterotypic synonyms of *S. griseus* and that *S. argenteolus* AS 4.1693^T should also be assigned to this taxon.

It is clear from 16S rRNA gene sequence data that the type strains of *Streptomyces caviscabies* Goyer *et al.* 1996, *Streptomyces griseus* (Krainsky 1914) Waksman and Henrici 1948 and *Streptomyces setonii* (Millard and Burr 1926) Waksman 1953 form a distinct clade in the *Streptomyces* tree together with representative strains of *Streptomyces sanglieri* Manfio *et al.* 2003. *Streptomyces argenteolus* AS 4.1693^T also belongs to the *S. griseus* clade. This organism is considered to be the type strain of *S. argenteolus*, a taxon that is incorrectly attributed to Tresner *et al.* (1961) in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) and in the wider microbiological literature (Shirling & Gottlieb, 1968; Williams *et al.*, 1989; Manfio *et al.*, 2003). This organism was first described in the patent literature (Perlman *et al.*, 1955; Fried *et al.*, 1958). *S. griseus* and its near relatives show close phylogenetic relationships with

several species of *Streptomyces* with validly published names, notably *Streptomyces laceyi* Manfio *et al.* 2003, *Streptomyces peucetius* Grein *et al.* 1963, *Streptomyces purpureus* (Matsumae and Hata 1968) Goodfellow *et al.* 1986b and *Streptomyces venezuelae* Ehrlich *et al.* 1948.

The taxonomy of some members of the *S. griseus* clade has been the source of considerable confusion, as exemplified by the proposal that *S. griseus* NRRL-ISP 5236^T and *S. setonii* NRRL-ISP 5395^T should be recognized as heterotypic synonyms of *Streptomyces anulatus* (Williams *et al.*, 1989) and by the assignment of *S. argenteolus* NRRL-ISP 5226^T to the *Streptomyces antibioticus* and *Streptomyces chromofuscus* species-groups in the respective numerical phenetic surveys of Kämpfer *et al.* (1991) and Williams *et al.* (1983). It is now apparent that additional comparative studies are needed to resolve the finer taxonomic relationships between members of the *S. griseus* clade, especially since representative strains of *S. argenteolus*, *S. caviscabies*, *S. griseus* and *S. setonii* share key morphological features (Shirling & Gottlieb, 1968, 1969; Goyer *et al.*, 1996) and have identical

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Streptomyces yanii* AS 4.1146^T is AB015854.

or nearly identical 16S rRNA gene sequences (Manfio *et al.*, 2003). It is also evident from the present study that the strain previously described as '*Microstreptospora cinerea*' belongs to the *S. griseus* 16S rRNA gene clade.

The monospecific genus '*Microstreptospora*' was proposed by Yan *et al.* (1983, 1987) for an actinomycete, strain 80-133^T, which formed single or short chains of spores on the substrate mycelium, chains of spores on aerial hyphae and had whole-organism hydrolysates rich in LL-diaminopimelic acid. The organism was classified as '*M. cinerea*', but was subsequently considered to be closely related to the genus *Elytrosporangium* (Itoh *et al.*, 1987), a later synonym of the genus *Streptomyces* (Goodfellow *et al.*, 1986a). T. Itoh and colleagues were of the view that strain 80-133^T should be classified as belonging to the genus *Streptomyces* as it had chemical and morphological properties that were typical of members of this taxon. The binomial '*M. cinerea*' is not cited in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) and hence has no formal standing in nomenclature.

The present study was designed to establish the taxonomic position of '*M. cinerea*' strain 80-133^T and to clarify the taxonomy of members of the *S. griseus* clade by using a combination of genotypic and phenotypic data. It is evident from the resultant data that the representatives of *S. argenteolus*, *S. caviscabies* and *S. setonii* should be classified as *S. griseus*, and that strain 80-133^T forms a distinct centre of taxonomic variation in the genus *Streptomyces*, for which the name *Streptomyces yanii* sp. nov. is proposed.

Strain 80-133^T and the other strains included in the DNA–DNA relatedness studies, namely *S. argenteolus* AS 4.1693^T, *S. caviscabies* DSM 41811^T, *S. griseus* AS 4.1419^T and AS 4.1321, *S. laceyi* AS 4.1832^T, *S. sanglieri* AS 4.1831^T, *S. setonii* AS 4.1774^T, *S. peucetius* AS 4.1799^T, *S. purpureus* AS 4.1225^T and *S. venezuelae* AS 4.1307^T, were maintained as suspensions of spores and mycelial fragments in glycerol (20%, v/v) and on Gauze's agar (1 g KNO₃, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl, 10 mg FeSO₄, 20 g starch, 30 g agar, 1 litre distilled water) at room temperature. Biomass for the molecular systematic studies was prepared from 7-day-old cultures grown on Gauze's agar at 30 °C. Genomic DNA was extracted from the biomass and purified following an established procedure (Kim *et al.*, 1998). The undisturbed arrangement of hyphae, notably aerial hyphae, and the spore chain morphology of strains 80-133^T and AS 4.1693^T were observed on Gauze's agar after 7 days, using the coverslip technique of Kawato & Shinobu (1959). All of the strains were examined by using a range of phenotypic tests according to standard procedures (Williams *et al.*, 1983).

Purified genomic DNA from strain 80-133^T was sequenced according to a standard procedure (Kim *et al.*, 1998). The resultant sequence was aligned manually with corresponding almost-complete sequences of representatives of available *Streptomyces* species that that been retrieved from

GenBank/EMBL/DDBJ using the CLUSTAL_X program (version 1.64b; Thompson *et al.*, 1997). The resultant dataset was examined by using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms from the PHYLIP suite of programs (Felsenstein, 1993). Evolution distance matrices for the least-squares and neighbour-joining methods were generated according to Jukes & Cantor (1969). Tree topologies were evaluated by a bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset by using the SEQBOOT and CONSENSE programs from the PHYLIP package (Felsenstein, 1993). A partial nucleotide sequence (120 bp) from the variable γ -region of the 16S rRNA gene of the tested strain was compared with corresponding nucleotide sequences of nearly 500 strains belonging to the genus *Streptomyces* retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>). A phylogenetic tree based on these partial nucleotide sequences was constructed by using the neighbour-joining algorithm (Saitou & Nei, 1987). DNA–DNA relatedness studies were carried out (Table 1) by using the thermal denaturation procedure described by De Ley *et al.* (1970) and a UV-1206 spectrophotometer (Shimadzu) equipped with a TB-85 thermobath.

Comparison of the almost-complete sequence of strain 80-133^T (1463 nt) with corresponding sequences of the reference *Streptomyces* strains showed that the strain formed a phyletic line within the *S. griseus* subclade (Fig. 1). The tested organism was also distinguished from representatives of over 500 members of the genus *Streptomyces* in a comparative study based on the 120 bp γ -region of the 16S rRNA gene (data not shown). It was evident from these studies that the tested strain is a bone fide member of the genus *Streptomyces*, a conclusion that was supported by chemotaxonomic data drawn from previous studies (Yan *et al.*, 1983, 1987; Itoh *et al.*, 1987). The morphological data acquired in the present investigation are in line with those from these earlier studies in showing that strain 80-133^T forms single or short chains of spores on substrate hyphae and aerial hyphae that differentiate into short chains of spores.

Table 1. Percentage DNA–DNA relatedness between representatives of species assigned to the *S. griseus* clade

Strain	1	2	3	4	5	6	7
1. <i>S. yanii</i> 80-133 ^T	100						
2. <i>S. argenteolus</i> AS 4.1693 ^T	13	100					
3. <i>S. caviscabies</i> DSM 41811 ^T	30	85	100				
4. <i>S. griseus</i> AS 4.1419 ^T	31	94	100	100			
5. <i>S. griseus</i> AS 4.1321	16	84	100	100	100		
6. <i>S. sanglieri</i> AS 4.1831 ^T	33	32	45	36	40	100	
7. <i>S. setonii</i> AS 4.1774 ^T	25	84	84	100	99	48	100

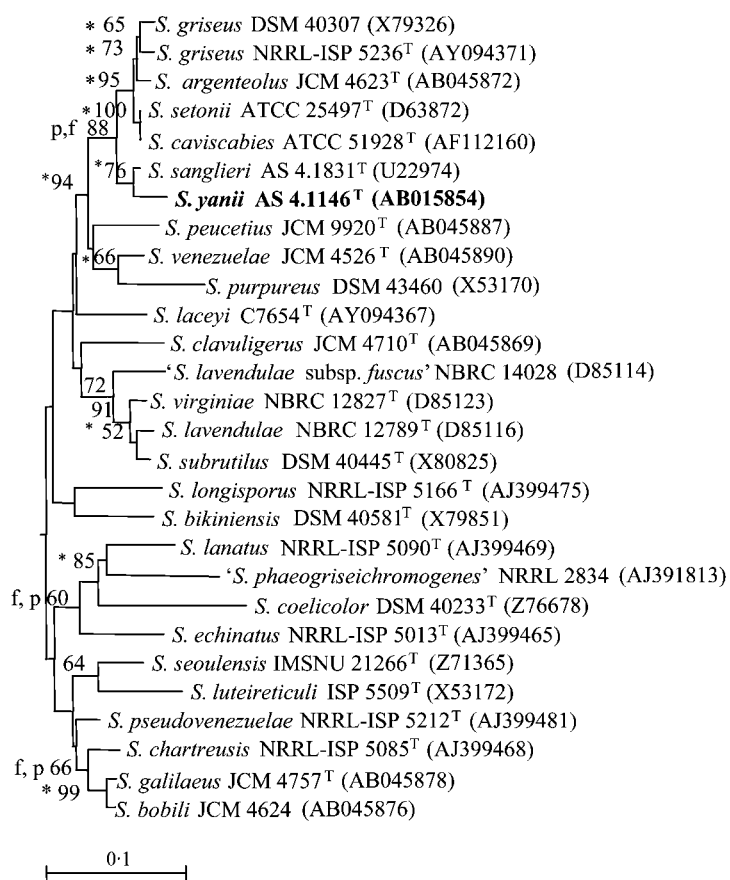


Fig. 1. Neighbour-joining tree showing the position of strain 80-133^T in relation to phylogenetically close organisms in the 16S rRNA *Streptomyces* gene tree. Asterisks indicate branches that were also recovered using the least-squares, maximum-likelihood and maximum-parsimony tree-making algorithms. f and p indicate branches that were also recovered using the least-squares and maximum-parsimony methods, respectively. Numbers at nodes are percentage bootstrap values based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are given. Bar, 0.1 substitutions per site.

It is evident from Fig. 1 that strain 80-133^T lies at the periphery of the *S. griseus* clade together with the type strain of *S. sanglieri*; the relationship between these strains was underpinned by the results from all of the tree-making algorithms and by a bootstrap value of 76% in the neighbour-joining analysis. The taxonomic status of the *S. griseus* clade is supported by the results from two of the tree-making algorithms and by a bootstrap value of 88% in the neighbour-joining analysis. The type strains of *S. peuceitius*, *S. purpureus* and *S. venezuelae* formed a loose phylogenetic group that was closely related to the *S. griseus* clade; members of these taxa formed a distinct group that was supported by a bootstrap value of 94% in the neighbour-joining analysis.

Strain 80-133^T shared relatively high 16S rRNA gene similarity values with other members classified in the *S. griseus* clade, that is, with *S. sanglieri* AS 4.1831^T (99.4%), *S. argenteolus* AS 4.1693^T (99.2%), *S. griseus* AS 4.1419^T (99.2%), *S. caviscabies* DSM 41811^T (99.1%) and *S. setonii* AS 4.1774^T (99.1%), values that correspond to 8, 12, 12, 13 and 13 nucleotide differences, respectively. 16S rRNA gene similarities within this range are consistent with the assignment of closely related streptomycetes to distinct species (Sembiring *et al.*, 2000; Manfio *et al.*, 2003). The 16S rRNA gene data are in line with results from previous studies

which showed that strain 80-133^T had chemical and morphological properties that were typical of members of the genus *Streptomyces* (Itoh *et al.*, 1987; Yan *et al.*, 1987).

DNA–DNA relatedness studies are used routinely to resolve finer taxonomic relationships between closely related actinomycetes (Kim *et al.*, 2003; Groth *et al.*, 2004). The minimum level of DNA relatedness between strains recommended to define genomic species is 70% (Wayne *et al.*, 1987), although comprehensive studies on specific groups of streptomycetes indicate that relatedness values above 80% correspond to species level relatedness within this taxon (Labeda, 1993, 1998; Labeda & Lyons, 1992). It is evident from Table 1 that strain 80-133^T shared relatedness values that were well below these cut-off points with strains of *S. argenteolus*, *S. caviscabies*, *S. griseus* and *S. setonii*; it was also sharply separated from its nearest phylogenetic neighbour, the type strain of *S. sanglieri*. Strain 80-133^T showed uniformly low DNA–DNA relatedness values with the type strains of *S. laceyi* (14%), *S. peuceitius* (14%), *S. purpureus* (11%) and *S. venezuelae* (16%). The DNA–DNA pairing data also provide further evidence that strain AS 4.1321, previously designated '*Streptomyces ornatus*', belongs to the same genomic species as the type strain of *S. griseus* (Okanishi *et al.*, 1972).

Table 2. Phenotypic properties that differentiate strain 80-133^T from representatives of *Streptomyces* species classified in the *S. griseus* clade

Taxa: 1, strain 80-133^T; 2, *S. argenteolus* AS 4.1693^T; 3, *S. caviscabies* DSM 41811^T; 4, *S. griseus* AS 4.1419^T; 5, *S. sanglieri* AS 4.1831^T; 6, *S. setonii* AS 4.1774^T. Spore chain arrangement data for the reference strains were taken from: Shirling & Gottlieb (1968), AS 4.1419^T; Goyer *et al.* (1996), DSM 41811^T; Shirling & Gottlieb (1969), AS 4.1774^T; Manfio *et al.* (2003), AS 4.1831^T. +, Positive/present; -, negative/absent. All of the strains were positive for degradation of adenine, hypoxanthine and xanthine; hydrolysis of aesculin and arbutin; utilization of (+)-D-fructose, (+)-D-galactose, (+)-D-glucose, (+)-D-lactose, (+)-D-mannitol, (+)-D-mannose, methyl α -D-glucoside, (+)-D-raffinose, (+)-D-ribose and (+)-D-xylose (1%, w/v) and acetate, formate, malonate and propionate (0.1%, w/v); and growth in the presence of 7% NaCl. All of the strains were negative for growth at 45 °C, and production of melanin pigments on either peptone yeast extract iron agar or tyrosine agar.

Characteristic	1	2	3	4	5	6
Morphology and pigmentation on oatmeal agar:						
Aerial spore mass colour	Brown	Yellowish grey	Grey	Yellowish grey	Grey	Yellowish grey
Substrate mycelium colour	Grey-black	Yellow-brown	Yellow-brown	Yellowish grey-orange	Reddish orange	Yellowish grey
Diffusible pigment	-	-	-	-	Yellow-brown	-
Spore arrangement	Short to slightly curved	Long, straight to flexuous	Long, straight to flexuous	Long, straight to flexuous	Long, spiral and open loops	Long, straight to flexuous
Spores on substrate mycelium	+	-	-	-	-	-
Degradation of:						
Elastin	-	+	+	+	-	+
Tween 20	-	+	+	+	+	+
Tween 80	-	+	+	+	+	-
Growth in the presence of sodium azide (0.01%, w/v)	-	-	-	-	+	-
Utilization of citrate	-	+	+	+	+	+

It is apparent from Table 2 that strain 80-133^T can be distinguished from representatives of species classified in the *S. griseus* clade by using a combination of phenotypic properties, notably morphological features. Since '*M. cinerea*' 80-133^T can be distinguished from all of these organisms by using a combination of genotypic and phenotypic data, it is proposed that it should be classified as a novel species of the genus *Streptomyces*. The name proposed for this new taxon is *Streptomyces yanii* sp. nov.

The results of the present study provide further evidence that the genus *Streptomyces* as currently constituted is overspeciated (Hatano *et al.*, 2003; Lanoot *et al.*, 2002, 2004, 2005). It is apparent from Table 1 that the type strains of *S. argenteolus*, *S. caviscabies* Goyer *et al.* 1996, *S. griseus* (Krainsky 1914) Waksman and Henrici 1948 and *S. setonii* (Millard and Burr 1926) Waksman 1953 share DNA-DNA relatedness values between 84 and 100%, results which provide further evidence for the recognition of the 80% cut-off for species level relatedness within the genus *Streptomyces* (Labeda, 1993, 1998; Labeda & Lyons, 1992). Members of these taxa also have key phenotypic properties in common, notably spore chain morphology (Table 2). It is therefore proposed that *S. argenteolus*, *S. caviscabies* and *S. setonii* are heterotypic synonyms of *S. griseus*, the earliest described of these species with validly published names. An emended description of *S. griseus* is given below.

Description of *Streptomyces yanii* sp. nov.

Streptomyces yanii (ya'ni.i. N.L. gen. n. *yanii* of Yan, named in honour of Dr Xunchu Yan, a Chinese microbiologist).

The description is based on data taken from this and earlier studies (Yan *et al.*, 1983, 1987; Itoh *et al.*, 1987). Aerobic, Gram-positive, non-acid-alcohol-fast actinomycete that forms an extensively branched substrate mycelium and aerial hyphae. The substrate mycelium carries abundant spherical, smooth-surfaced spores (about 2.5 μ m in diameter) singly or in short chains of 2 to 4 spores on short sporophores. Short chains of ellipsoidal smooth-surfaced spores (0.8 \times 2.0 μ m in diameter) are formed on aerial hyphae. The latter are usually formed only on inorganic media; an abundant grey aerial spore mass is apparent on oatmeal and Krass's No. 1 agars. Forms an abundant grey to black substrate mycelium on Czapek's, Krass's and oatmeal agars. Gelatin and starch are degraded, but not cellulose or tyrosine. Nitrate is reduced. H₂S is not formed. Milk is coagulated but not peptonized. Additional phenotypic properties are shown in Table 2. Good growth occurs at 18–35 °C and pH 6–9. Whole-organism hydrolysates contain major proportions of LL-diaminopimelic acid, glycine, galactose and mannose, and a trace of ribose. The muramic acid of the peptidoglycan is N-acetylated. The predominant isoprenologues are tetra- and

hexa-hydrogenated menaquinones with nine isoprene units, and the major phospholipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. The G+C content of the DNA is 69.4 mol%. The sole strain was isolated from a mud sample collected from a sewage ditch in Zhanjiang City, Guangdong Province, China.

The type strain is 80-133^T (= AS 4.1146^T = JCM 3331^T).

Emended description of *Streptomyces griseus* (Krainsky 1914) Waksman and Henrici 1948, 948^{AL}

Streptomyces griseus (gri'se.us. M.L. masc. adj. *griseus* grey).

The description is based on data taken from this and earlier studies (Shirling & Gottlieb, 1968, 1969). Spore chains are *rectiflexibiles*; the spore surface is smooth. Aerial spore mass colour places the organism in the grey colour series; the reverse side of colonies is grey–yellow. Melanin pigment is not formed nor are soluble pigments produced. Additional phenotypic properties are shown in Table 2. The G+C content of the DNA is 71–72 mol%. The type strain is ATCC 23921^T (= AS 4.1419^T = DSM 40236^T = NRRL-ISP 5236^T = JCM 4047^T = NCIMB 13023^T). Heterotypic synonyms: *Streptomyces argenteolus*, *Streptomyces caviscabies* Goyer *et al.* 1996 and *Streptomyces setonii* (Millard and Burr 1926) Waksman 1953.

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