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ORIGINAL ARTICLE

Diversity of members of the *Streptomyces violaceusniger* 16S rRNA gene clade in the legumes rhizosphere in Turkey

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Abstract Large numbers of putatively novel streptomycetes were isolated from rhizosphere soils of *Albizia distachya*, *Colutea arborescens*, *Gleditsia triancanthos*, *Medicago arborea*, *Robinia pseudoacacia*, *Sophora japonica*, *Spartium junceum*, *Tipuana tipu* and *Wisteria sinensis*. Representative isolates were determined to 6 multi-membered and 11 single-membered colour groups based on their ability to form pigments on oatmeal and peptone yeast extract iron agars. The largest colour groups, which encompassed 40 isolates with morphological properties typical of members of the *Streptomyces violaceusniger* 16S rRNA gene clade, were tested for a characteristic PCR amplification product with taxon-specific primers. In spite of highest 16S rRNA gene nucleotide similarity among the isolated strains belonging to the *S. violaceusniger* 16S rRNA gene clade, it is evident from the phenotypic, molecular and chemical results obtained that many new species will emerge.

Keywords Biodiversity · *Streptomyces violaceusniger* clade · Polyphasic taxonomy

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Introduction

The actinomycetes, especially *Streptomyces* spp. are well-known saprophytic organisms that decompose organic matter, particularly biopolymers such as lignocellulose, starch and chitin, in soil (Crawford et al. 1993; Strap and Crawford 2006). The taxon contains aerobic, Gram-positive actinomycetes with DNA rich in G + C (Williams et al. 1989; Manfio et al. 1995). They produce extensive branching substrate and aerial mycelia that typically differentiate into chains of spores, and contain LL-diaminopimelic acid but no characteristic sugars in the cell wall (Lechevalier and Lechevalier 1970). The filamentous of growth of *Streptomyces* gives them a competitive advantage in colonising not only solid substrates such as decomposing plant residues, but also the rhizosphere (Strap and Crawford 2006). Plant rhizospheres remain an insufficiently explored reservoir of novel microorganisms producing bioactive metabolites. The soil of the rhizosphere zone is influenced directly by plant roots, and is a biologically complex and distinct microhabitat within the terrestrial ecosystem (Zaehner and Fiedler 1995; Paterson et al. 1995). The rhizosphere represents a substantial biological niche that supports a quantity of diverse saprophytic microorganisms due to the a high input of organic material provided from plant roots and root exudates (Merckx et al. 1987). Rhizospheres are also an environment in which complex interactions abound between beneficial and deleterious microorganisms and their plant hosts (Loper 1998).

Specific rhizosphere-colonising *Streptomyces*, such as members of the *Streptomyces violaceusniger* 16S rRNA

gene clade, are known to grow in close association with plant root systems, where their presence and activity benefit plant growth and protect plant roots against attack by fungal pathogens (Doumbou et al. 2002; Tokala et al. 2002; Coombs and Franco 2003; Conn and Franco 2004; Goodfellow et al. 2007). In addition, streptomycetes synthesis an array of biodegradative enzymes, such as chitinases (Blaak et al. 1993; Gupta et al. 1995; Mahadevan and Crawford 1997), glucanases (Harchand and Singh 1997; Thomas and Crawford 1998; Trejo-Estrada et al. 1998a), peroxidases (Ramachandra et al. 1988; Tuncer et al. 2004, 2009), and other metabolites possibly involved in mycoparasitic activity.

Taxonomic relationships within the genus *Streptomyces* have been clarified by the application of genotypic and phenotypic methods (Goodfellow et al. 1992; Manfio et al. 1995; Anderson and Wellington 2001; Kumar and Goodfellow 2008). It is now apparent that many streptomycete type strains can be assigned to distinct multimembered species groups, as exemplified by species classified in the *Streptomyces albidoflavus* (Lanoot et al. 2005), *Streptomyces griseus* (Liu et al. 2005), *Streptomyces violaceoruber* (Duangmal et al. 2005), *Streptomyces violaceusniger* (Sembiring et al. 2000) and *Streptomyces yeochonensis* (Xu et al. 2006) 16S rRNA gene clades.

Members of the *S. violaceusniger* 16S rRNA gene clade form a grey aerial spore mass and greyish-yellow substrate mycelium on oatmeal agar, produce aerial hyphae that differentiate into spiral chains of rugose ornamented spores (Sembiring et al. 2000; Ward and Goodfellow 2004; Goodfellow et al. 2007; Kumar and Goodfellow 2008), and give a characteristic amplification product with taxon-specific primers (Kumar et al. 2007). Strains belonging to the *S. violaceusniger* 16S rRNA gene clade have been isolated from non-rhizosphere (Al-Tai et al. 1999; Saintpierre et al. 2003; Hayakawa et al. 2004) and rhizosphere (Sembiring et al. 2000) soil from various sites around the world. PCR amplification of DNA extracted from marine and terrestrial samples using *S. violaceusniger*-clade-specific primers provides further evidence for the widespread distribution of novel members of the clade in natural habitats (Kumar et al. 2007).

The primary aim of the present study was to determine the nature and extent of the taxonomic diversity of members of the *S. violaceusniger* clade associated with the rhizosphere of natural or planted legumes in Turkey, i.e. *Albizia distachya* (Vent.) Macb., *Colutea arborescens* L., *Gleditsia triancanthos* L., *Medicago arborea* L., *Robinia pseudoacacia*, *Sophora japonica* L., *Spartium junceum* L., *Tipuana tipu* (Benth.) Kuntze and *Wisteria sinensis* Sweet.

Materials and methods

Selective isolation

Rhizosphere samples were collected from and around the root systems of legumes in Izmir and Samsun, Turkey. One gram of soil sample was suspended in 9 ml sterile one-quarter strength Ringer's solution (Oxoid, Basingstoke, UK) and shaken for 30 min on a tumble shaker (Luckham, Burgess Hill, UK) at speed setting 8 at room temperature. This 10^{-1} dilution was then treated in a pre-warmed water bath at 60°C for 15 min. Aliquots (0.1 ml) of 10^{-2} to 10^{-4} tenfold serial dilutions were spread over the surface of dried starch casein agar (Küster and Williams 1964) plates supplemented with filter sterilised cycloheximide (50 μg ml^{-1}), nystatin (50 μg ml^{-1}) and rifampicin (0.5 μg ml^{-1}) and incubated at 28°C for 14 days.

A total of 305 representatives of isolates putatively assigned to the genus *Streptomyces* on the basis of colony morphology—notably aerial spore mass colour, substrate mycelial pigmentation and the colour of any diffusible pigments—were sub-cultured on glucose yeast malt extract agar, incubated at 28°C for 14 days, and checked for purity by microscopic examination of Gram-stained smears. The isolates were maintained as suspensions of spores and mycelial fragments in glycerol (20%, v/v) at −20°C.

Colour grouping, maintenance and morphological characteristic of strains

Representative isolates were inoculated onto oatmeal (ISP3; Küster 1959) and peptone-yeast extract-iron agar plates (ISP6; Shirling and Gottlieb 1966), which were incubated at 28°C for 14 and 4 days, respectively. The oatmeal agar plates were examined for aerial spore mass colour, substrate mycelium pigmentation; colour of any diffusible pigments was recorded using the National Bureau of Standards (NBS) Colour Name Charts (Kelly 1958). The peptone yeast extract-iron agar plates were used to determine whether the isolates produced melanin pigments. The isolates were assigned to 6 multi- (5–40 isolates) and 11 single-membered colour groups. The largest colour group, which contained 40 isolates considered to have colonial properties consistent with their assignment to the *S. violaceusniger* 16S rRNA gene clade (Sembiring et al. 2000), was subjected to a polyphasic approach (Table 1).

Spore-chain morphology was determined by light and scanning electron microscopy (SEM) of 14-day-old

Table 1 Source of isolates identified as members of the *Streptomyces violaceusniger* 16S rRNA gene clade using molecular techniques

	Laboratory number	Source of strains
Streptomyces sp.	M5038, M5047, M5055, M5077, M5090	*Albizia distachya*, Izmir, Turkey
Streptomyces sp.	M4031, M4032, M4033, M4039, M4041, M4042, M4050	*Colutea arborescens*, Izmir, Turkey
Streptomyces sp.	M1331, M1345, M1351, M1362, M1375, M1399	*Gleditsia triancanthos*, Samsun, Turkey
Streptomyces sp.	M1001, M1075, M1082	*Medicago arborea*, Izmir, Turkey
Streptomyces sp.	M1499, M1400, M1444, M1455, M1456, M1470, M1473, M1481, M1491	*Robinia pseudoacacia*, Samsun, Turkey
Streptomyces sp.	M2002, M2062, M2048	*Tipuana tipu*, Izmir, Turkey
Streptomyces sp.	M1241, M1243, M1244, M1247, M1256, M1286, M1287	*Wisteria sinensis*, Samsun, Turkey

cultures grown at 28°C on inorganic salts-starch agar (ISP 4; Shirling and Gottlieb 1966). Spore chain morphology was observed using a Nikon Optiphot binocular light microscope fitted with long working distance objectives; spore-chains were assigned to the morphological categories proposed by Pridham et al. (1958). Spore surface ornamentation was determined on SEM preparations using a Cambridge Stereoscan 240 instrument; ornaments were assigned to the categories of Tresner et al. (1961).

Chemotaxonomy

The isomeric form of diaminopimelic acid (A_{2pm}) was determined by thin-layer chromatography of whole-organism hydrolysates on cellulose acetate sheets following the procedure described by Staneck and Roberts (1974), with a modified solvent system, namely methanol:H_2O:HCl:10 N pyridine (85:15:5:10, v/v). Fatty acids were extracted, methylated and analysed by GC using the standard MIDI (Microbial Identification; Microbial ID, Newark, DE) system (Sasser 1990; Kämpfer and Kroppenstedt 1996).

DNA preparation, amplification and sequencing of 16S rRNA genes

Extraction of genomic DNA and PCR-amplification of 16S rRNA genes from the 40 strains were carried out as described by Pitcher et al. (1989), using the modifications of Sembiring et al. (2000). The amplified fragments were purified with QIAquick purification kits (Qiagen, Valencia, CA) and sequenced directly using ABI PRISM BigDye Terminator Cycle Sequencing kits (Applied Biosystems, Foster City, CA) and previously described oligonucleotide primers (Lane 1991; Chun and Goodfellow 1995). Sequencing gel electrophoresis was performed and nucleotide sequences were obtained automatically using an Applied Biosystem DNA sequencer (model 377) and software provided by the manufacturer.

PCR amplification using taxon-specific primers

Representatives of the resulting colour groups, which were considered to have colonial properties consistent with their assignment to the *S. violaceusniger* clade (Sembiring et al. 2000; Goodfellow et al. 2007) in that they formed a gray-to-blackish aerial spore mass on oatmeal agar, did not produce melanin pigments on peptone-yeast extract-iron agar, and produced spiral chains of rugose ornamented spores, were tested for a characteristic PCR amplification product with taxon-specific primers (Goodfellow et al. 2007; Kumar et al. 2007).

Phylogenetic analysis

The resultant 16S rRNA gene sequences were aligned manually using the PHYDIT program (Chun 1995) against the corresponding streptomycete sequences retrieved from the GenBank, EMBL and DDBJ databases. An unrooted phylogenetic tree was inferred using the neighbour-joining (Saitou and Nei 1987) tree-making algorithm from the PHYLIP suite of programs (Felsenstein 1993). An evolutionary distance matrix was generated for the neighbour-

joining as described by Jukes and Cantor (1969). The resultant tree topology was evaluated by a bootstrap analysis (Felsenstein 1985) of the neighbour-joining dataset, based on 1000 bootstrap resampling, using the SEQBOOT and CONSENSE programs from the PHYLIP package (Felsenstein 1993).

Phenotypic characterisation

Forty isolated strains were examined for 49 unit characters using the media and methods described by Williams et al. (1983).

Results and discussion

Selective isolation, enumeration and colour grouping

Isolates presumptively assigned to the genus *Streptomyces* were distinguished from other bacterial colonies growing on the starch casein agar plates by their characteristic colonial and pigmentation properties. Large numbers of the target organisms were isolated from the heat-treated suspension of rhizosphere soil following incubation on selective isolation media. Representatives of presumptive streptomycetes growing on starch-casein agar plates were assigned to 6 multi-membered (5–40 isolates) and 11 single-membered colour groups (data not shown). The largest colour group encompassed 40 isolates that formed a grayish aerial spore mass, a grayish-yellow reverse colour and yellow diffusible pigment on oatmeal agar, but did not produce melanin pigments on peptone-yeast extract-iron agar. These organisms formed an extensively branched substrate mycelium, and aerial hyphae that differentiate into rugose-ornamented spores in spiral spore chains; apart from the production of the yellow diffusible pigment, these properties are consistent with the largest colour group in the *S. violaceusniger* 16S rRNA gene clade (Sembiring et al. 2000; Goodfellow et al. 2007). Members of the largest colour group were tested with taxon-specific primers described by Kumar et al. (2007) and gave a PCR amplification product characteristic of members of the *S. violaceusniger* clade (data not shown). These data show that the selected 40 strains are authentic members of the *S. violaceusniger* 16S rRNA gene clade (Goodfellow et al. 2007; Kumar et al. 2007).

Most actinomycetes in soil belong to the genus *Streptomyces*, and there are several reports of the presence of large populations of *Streptomyces* in the rhizosphere of legumes, which are common in plant root systems (Watson and Williams 1974; Upton 1994; Atalan et al. 2000; Sembiring et al. 2000; Suzuki et al. 2000), producing biologically active compounds such as antifungal and antibacterial agents. They also produce plant-growth-promoting substances that have been improved for agricultural use (Ilic et al. 2007). Plant root exudates increase microbial colonisation and activity in the rhizosphere substantially. However, the composition and quantity of root exudates varies depending on the plant species in terms of components such as ions, free oxygen and water, enzymes, mucilage, and a range of primary and secondary metabolites (Uren 2000). It is interesting that we found no members of the *Streptomyces violaceusniger* 16S rRNA gene clade among the strains that were isolated from *Sophora japonica* and *Spartium junceum* rhizosphere soils.

It has already been pointed out that members of the *S. violaceusniger* 16S rRNA gene clade are the source of many useful products, and they have also been considered as useful biological control agents (Chamberlain and Crawford 1999; Getha and Vikineswary 2002; Trejo-Estrada et al. 1998a, b). However, despite the industrial and ecological importance of members of the *S. violaceusniger* clade, little is known about their taxonomic diversity, geographical distribution or ecological role in their natural habitats. It is interesting that all 40 isolates inhibited the growth of *Aspergillus flavus* NRLL 1957, *Aspergillus niger* (isolated strain), *Aspergillus parasiticus* NRRL 465 and *Candida albicans* ATCC 10231 (data not shown). Further studies are needed to establish the roles that the constituent members of this clade play in soil ecosystems, notably their interactions with fungal pathogens in root systems.

Chemical, cultural and morphological properties

All isolates contained LL-diaminopimelic acid and were rich in iso- and anteiso-branched fatty acid. The chain lengths of the major fatty acid components were between 14 and 17 carbon atoms, with 12-methyltetradecanoic (anteiso-C15:0), 14-methylpentadecanoic (iso-C16:0), hexadecanoic (C16:0) and 14-methylhexadecanoic (anteiso-C17:0) fatty acids as the predominant components (Table 2). Forty representative isolates belong to *S. violaceusniger* clade, formed rugose ornamented spores in spiral spore chains, as exemplified in Fig. 1.

Phenotypic characterization

The isolated strains were examined for a range of phenotypic features, including enzyme activities, degrada-

Table 2 Percentage fatty acid composition of isolated strains, including some related type strains of the *S. violaceusniger* clade

Strain number	*i*-14:0	*i*-15:0	*ai*-15:0	15:0	16:0	*i*-16:1-H	*i*-16:0	16:1 CIS 9	16:0 9- Methyl	*ai*-17:1 C	*i*-17:0	*ai*-17:0
M1345	2.14	25.54	19.95	1.22	9.10	1.40	11.55	5.68	4.72	1.74	6.15	8.20
M1399	4.14	27.80	16.34	1.43	10.11	2..46	15.73	5.66	4.80	1.70	7.61	6.98
M1499	3.80	26.20	10.62	2.36	3.78	5.24	16.80	6.12	9.90	1.74	6.50	4.30
M1400	4.28	28.17	8.62	1.19	4.60	4.63	18.40	5.60	10.23	2..48	6.10	4..64
M5090	2.40	42.70	10.80	1.10	7.20	0.90	6.30	5.10	7.10	1.12	9.15	3.00
M1001	3.80	37.85	11.53	2.0	9.80	1.0	10.10	4.70	6.80	0.80	5.90	3.14
M2002	11.40	13.21	4.87	1.0	1.65	12.85	37.68	4.33	2.84	1.26	2.14	1.78
M1491	12.33	11.88	5.63	0.83	1.41	13.65	40.24	2.77	3.03	1.58	1.94	2.80
M5038	3.80	24.88	14.95	1.36	5.38	4.20	17.57	5.95	5.62	1.85	5.28	6.59
M5047	5.86	30.81	15.57	1.18	5.90	2.90	14.91	4.35	4.78	1.19	5.10	4.70
M1351	2.60	35.29	18.73	1.30	7.68	0.86	7.14	6.20	5.10	1.21	5.74	4.75
M1444	3.20	25.85	17.11	0.80	8.91	1.93	16.10	4.44	4.34	1.39	6.80	7.45
M1331	8.52	13.01	11.71	0.82	2.97	9.26	35.23	2.53	2.78	2.13	3.28	5.78
M1481	6.55	16.33	13.01	0.83	4.93	7.39	29.10	5.11	3.85	1.92	3.30	5.57
M5077	5.42	7.13	11.52	0.49	4.12	10.10	39.56	3.02	2.27	2.63	2.28	6.38
M1256	13.26	11.04	8.57	0.99	4.93	8.90	37.99	2.57	3.64	-	4.95	3.15
M1247	3.25	32.25	19.71	1.92	10.47	1.02	7.47	6.54	3.38	1.05	4.73	4.30
M4033	22.48	14.32	7.09	1.48	0.43	10.41	27.62	-	4.47	2.20	1.77	2.00
M1243	7.60	17.00	15.14	1.71	7.62	4.35	25.52	5.11	3.47	1.44	5.04	6.04
M1470	15.03	9.50	10.14	0.85	1.67	10.85	39.23	2.25	1.92	1.36	1.65	2.55
M1286	21.33	7.85	7.51	-	4.02	6.91	37.89	2.12	6.89	-	3.22	2.25
M1241	15.13	16.66	10.55	0.75	2.05	9.37	28.19	5.47	3.43	1.18	2.16	3.36
M4052	6.17	16.24	14.46	0.91	5.74	1.82	15.90	3.46	14.08	5.90	9.57	5.74
M5055	7.06	23.49	13.60	-	6.34	3.79	23.82	3.60	4.46	1.49	6.94	5.40
M4042	10.61	11.05	13.12	0.98	1.89	10.31	35.98	2.69	2.43	2.13	2.13	4.86
M4032	25.46	12.99	8.61	3.20	1.22	7.03	26.09	2.65	2.54	0.99	1.41	2.20
M4039	16.50	6.69	9.64	0.48	1.23	11.89	43.78	1.28	1.43	1.82	1.00	3.50
M1082	3.19	31.42	15.78	0.57	5.71	1.89	12.10	5.23	6.78	1.38	8.21	5.30
M4031	6.77	17.99	18.81	1.56	2.82	6.48	22.64	5.36	4.05	2.46	3.20	7.10
M1075	15.01	13.77	16.81	0.87	2.94	7.06	28.59	3.15	2.50	1.68	2.30	3.56
M1244	5.43	16.56	11.01	0.86	2.48	9.22	33.94	3.33	4.87	2.47	3.11	3.57
M1362	11.55	10.77	10.34	1.51	1.13	17.30	31.91	3.68	2.64	2.09	1.16	1.72
M1456	10.53	16.03	10.06	-	1.36	11.64	32.43	3.63	4.69	2.01	3.06	2.68
M1375	6.40	18.22	18.61	1.30	2.75	6.40	23.14	5.20	4.35	2.16	3.40	6.76
M1473	12.47	14.46	9.18	0.50	1.64	11.82	33.39	3.43	4.02	1.94	2.79	2.44

Table 2 (continued)

Strain number	*i*-14:0	*i*-15:0	*ai*-15:0	15:0	16:0	*i*-16:1-H	*i*-16:0	16:1 CIS 9	16:0 9- Methyl	*ai*-17:1 C	*i*-17:0	*ai*-17:0
M1455	8.45	14.81	10.69	0.65	-	10.85	30.93	4.86	4.17	2.11	2.87	2.65
M2048	9.05	15.21	10.39	0.52	-	11.25	31.19	4.81	4.10	2.01	2.78	2.85
M2062	9.64	14.69	12.73	0.82	2.08	11.03	31.66	2.46	3.54	2.59	2.65	3.43
M4041	8.88	10.90	11.42	0.90	2.89	7.77	31.84	3.26	7.00	2.04	3.06	4.03
M1287	15.10	16.78	11.05	0.65	2.25	9.45	27.67	5.60	3.55	1.40	2.0	3.20
DSM 41759 *Streptomyces indonesiensis*	2.96	35.17	12.12	5.59	11.27	0.62	5.80	5.35	4.39	0.80	5.74	3.35
DSM 41761 *Streptomyces asiaticus*	3.57	36.13	7.94	2.21	7.45	2.52	12.72	4.84	6.74	0.80	7.57	3.22
DSM 41766 *Streptomyces yogyakartensis*	3.88	28.17	8.62	1.19	3.78	4.63	17.61	5.71	9.50	1.82	6.29	3.90
DSM 41760 *Streptomyces rhizosphaericus*	2.63	33.46	9.64	4.36	14.21	0.55	6.90	5.86	5.09	0.69	7.59	3.52
DSM 41769 *Streptomyces cangkringensis*	2.37	35.90	10.65	1.96	9.49	1.18	8.21	5.53	6.36	0.98	9.12	4.40
NRRL B-1865 *Streptomyces griseiniger*	7.98	26.84	3.32	1.15	1.55	6.96	26.47	1.97	9.42	1.08	8.38	2.00
NRRL B-1477 *Streptomyces hygroscopicus.*	3.23	27.62	25.13	1.15	9.81	0.63	7.40	5.96	2.93	0.93	6.57	5.00
NRRL B-3602 *Streptomyces geldanamycininus*	3.72	38.53	11.47	1.89	8.75	0.96	9.94	5.65	5.80	0.74	6.38	3.13
DSM41764 *Streptomyces javensis*	2.22	42.70	10.87	1.04	7.16	0.84	6.24	4.85	7.08	0.89	9.12	2.72
NRRL B-1478 *Streptomyces demainii*	4.45	27.58	22.73	3.25	7.20	1.21	10.70	4.09	2.99	1.01	4.77	4.79
DSM40563 *Streptomyces violaceusniger*	7.44	22.73	4.91	0.36	2.42	9.24	30.24	2.42	6.55	1.42	7.36	1.87

i-14:0 12-Methyltridecanoic acid, *i-15:0* 13-methyltetradecanoic acid, *ai-15:0* 12-methyltetradecanoic acid, *15:0* pentadecanoic acid, *16:0* hexadecanoic acid, *i-16:0* 14-methylpentadecanoic acid, *16:1* cis-9 hexadecanoic acid (palmitoleic acid), *i-17:0* 15-methylhexadecanoic acid, *ai-17:0* 14-methylhexadecanoic acid, *17:0* methylheptadecanoic acid

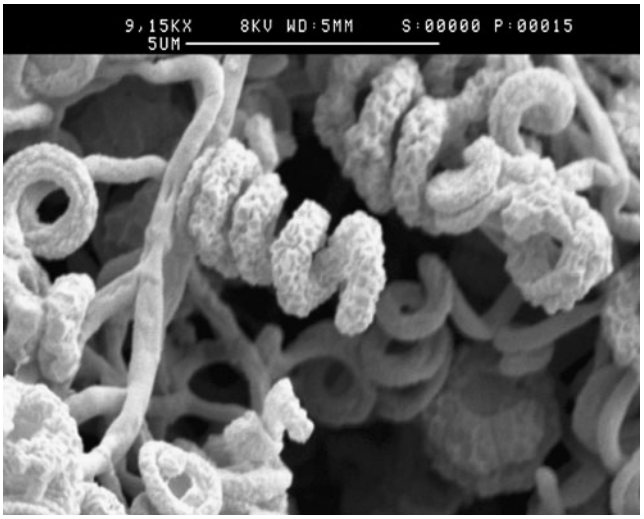

Fig. 1 Scanning electron micrograph (SEM) showing spiral chains of rugose ornamented spores of strain M1082 grow on inorganic salts-starch agar (ISP medium no.4) at 28°C for 14 days

tion, growth on sole carbon and nitrogen sources, growth at different pH and temperatures and antibiotic resistances. The phenotypic profiles of the isolated strains are shown in Tables 3 and 4.

Phylogenetic characterization

Comparison of the almost complete 16S rRNA nucleotide (nt) gene sequences obtained for the isolated strains with the corresponding sequences of the *Streptomyces violaceusniger* 16S rRNA gene clade showed that 35 out of the 40 strains (exceptions being strains M1082, M1001, M1400, M1499 and M5090) were assigned according to morphological properties and diagnostic PCR amplification with clade specific primers to the *S. violaceusniger* 16S rRNA gene clade. The others formed a closely related but distinct group with the species *S. malaysiensis* (Fig. 2); 15 out of the 40 strains (M1243, M1244, M1247, M1256, M1331, M1456, M1470, M1473, M2062, M4039, M4041, M4052, M5047, M5055 and M5077) showed 100% 16S rRNA gene nt similarities. The corresponding 16S rRNA gene sequence similarities for these 15 strains and their nearest neighbours, namely *Streptomyces malaysiensis* DSM 41697^T (AF117304), were 99.4% (9 nt differences at 1439 sites). Similarly, strains M1345, M1351 and M5038 presented 100% 16S rRNA gene nucleotide (nt) similarities, and the corresponding 16S rRNA gene sequence similarities for these three strains and their nearest neighbour type strain, namely *S. malaysiensis* DSM 41697^T (AF117304), were 99.4% (10 nt differences at 1439 sites). Nevertheless, two strains, namely M1399 and M2002, are most closely associated and share highest 16S rRNA gene similarities with the species *S. malaysiensis* (99.9 and 99.1%; values, which correspond to 1 and 13 nt differences at 1439 sites, respectively). The 16S rRNA similarity values and the associated differences found between the remaining 17 isolates and the most closely related marker strain, *S. malaysiensis*, ranged from 99.8 to 99.0% (1 to 14 nt differences at 1439 sites). It can be seen from the phylogenetic tree (Fig. 2) that isolates M1082 and M1001 belong to, or are most closely associated with, the type strain *S. antimycoticus* NBRC12839^T. Strain M1001 and *S. antimycoticus* share 16S rRNA gene similarities of 100% over 1446 locations, while M1082 and *S. antimycoticus* shared 16S rRNA gene similarities of 99.86%, a value that corresponds to 2 nt differences in 1446 positions. The two strains M1400 and M1499 belong to the type strains of *S. violaceusniger* NRRL B-1476^T, with 100% 16S rRNA gene similarities at 1449 positions. The remaining strain M5090 is also closely related to *S. violaceusniger* NRRL B-1476^T. These organisms share a 16S rRNA gene similarity of 99.1%, a value that corresponds to 13 nt differences at 1449 locations, and can be separated readily using a range of phenotypic properties (Tables 3, 4).

Media low in organic nutrients, such as starch-casein agar, were found to be good for isolating large numbers of *Streptomyces* from rhizosphere soils, confirming the findings of others workers (e.g., Sembiring et al. 2000). The fact that rhizosphere-associated soils yielded almost twice as many actinomycetes as non-rhizosphere-associated soil show that members of the *Streptomyces violaceusniger* 16S rRNA gene clade are especially abundant in the rhizosphere (Crawford et al. 1993; Sembiring et al. 2000; Goodfellow et al. 2007).

The 40 isolates belonging to the largest colour group formed rugose-ornamented spores in spiral spore chains on inorganic salts starch agar—properties also shown by the members of the *S. violaceusniger* clade. This assignment was confirmed both by PCR products generated with clade-specific primers and by the results of 16S rRNA sequence analysis, which showed that all 40 representative isolates belong to a phylogenetic group with members of the *S. violaceusniger* clade. It is apparent from Fig. 2 that isolates M1082, M1001, M1400, M1499 and M5090 formed a subclade together with *S. malaysiensis* DSM 41697^T, separate from other type strains of the *S. violaceusniger* 16S rRNA gene clade. However, further comparative studies are needed to resolve the complex taxonomic relationship between strains assigned to the *S. violaceusniger* clade. The isolation of members of the *S. violaceusniger* 16S rRNA gene clade from the rhizospheres of legume plants suggests that a specific root-colonizing relationship between *Streptomyces* sp. and legumes could exist. These rhizosphere-colonizing streptomycetes show huge potential as a potential source of novel antibacterial, antifungal and plant growth-regulatory metabolites.

Table 3 Phenotypic properties that separate members of the *Streptomyces violaceusniger* 16 S rRNA gene clade

Phenotipic Tests	*S. asiaticus* DSM 41761 T	*S. cangkringensis* DSM 41769 T	*S. hygroscopicus* NRRL 2387 T	*S. hygroscopicus* NRRL 2339 T	*S. hygroscopicus* NRRL B-1477	*S. geldanamycinus* NRRL B-3602 T	*S. indonesiensis* DSM 41759 T	*S. javensis* DSM 41764 T	*S. malaysiensis* DSM 41697 T	*S. melanosporofaciens* NRRL B-12234 T	*S. sporoclivatus* NRRL B-24330 T	*S. rhizosphaericus* DSM 41760 T
Enzyme activity												
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	-
Nitrate reduction	-	-	+	+	+	-	+	+	+	-	-	-
Urea hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Degradation tests (%, w/v)												
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+
Casein (1)	+	+	+	+	+	+	+	+	+	+	+	-
Elastin	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+
Guanine (0.05)	-	+	-	-	-	-	-	+	-	-	-	+
Hypoxanthine (0.4)	+	+	+	+	+	+	-	+	+	+	+	-
RNA	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80 (l%v/v)	+	+	+	+	+	+	+	+	+	+	+	+
Xanthine	-	-	-	-	-	-	-	-	-	-	-	-
Xylan	-	-	-	-	-	-	-	-	-	-	-	-
Growth on sole C sources at %1 w/v												
Adonitol	+	-	-	-	-	+	+	+	+	+	+	+
Aesculin	-	-	-	-	-	-	-	-	-	-	-	-
L(+)Arabinose	+	+	-	-	-	+	+	+	+	+	+	+
Arbutin	-	-	-	-	-	-	-	-	-	-	-	-
D(+)Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+
Dextrin	+	+	+	+	+	+	+	+	+	+	+	+
D(-) Fructose	+	+	+	+	+	+	+	+	-	+	+	+
α-Lactose	+	+	-	-	-	+	+	-	+	+	+	+
D-Mannitol (% 1)	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose (% 1)	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	-	-	-	-	-	-	-	+	-	-	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+
L-Valine (% 0.1)	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+
Growth on sole N sources at %1 w/v												
L(-) Alanine	+	-	-	+	+	-	-	+	+	-	-	-
L(-)Histidine	+	+	+	+	+	+	+	+	+	+	+	+
L(-)Leucine	+	+	-	-	-	-	+	+	+	-	-	-
Dl(-)Methionine	-	-	-	-	-	-	+	-	-	-	-	-
L(-)Serine	-	+	+	+	+	-	+	+	+	-	-	-
Growth at												
Sodium chloride (% 4)	+	+	+	+	+	+	+	+	+	+	+	+

Sodium chloride (% 7)	+	+	+	+	+	+	+	+	+	+	+	+
pH 4.0	-	-	-	-	+	-	-	-	+	-	-	-
pH 5.0	-	+	+	+	-	-	-	+	-	-	-	-
pH 9.0	-	+	-	-	-	-	+	-	+	-	-	+
pH 10	+	+	-	-	-	-	+	-	+	-	-	+
4°C	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotics (μg/ml)												
Ampicillin (32)	+	+	+	+	+	+	+	+	+	+	+	+
Gentamycin sulphate (8)	-	-	-	-	-	-	-	-	-	-	-	-
Gentamycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin sulphate (16)	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-
Penicillin (10)	+	+	+	+	+	+	+	+	+	+	+	+
Penicillin (20)	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin sulphate (8)	-	-	-	-	-	-	-	-	-	-	-	-
Streptomycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-

Data for type strains were taken from Goodfellow et al. 2007

Table 3 (continued)

Phenotipic Tests	*S. violaceusniger* ISP 5563	*S. violaceusniger* NRRL B-1476	*S. albiflaviniger* NRRL B-1356	*S. demainii* NRRL B-1478 ^{T}	*S. griseiniger* NRRL B-1865	*S. yatensis* DSM 41771 ^{T}	*S. yogyakartensis* DSM 41766 ^{T}	M4032	M1075	M1244	M4041	M1345
Enzyme activity												
Aesculin hydrolysis	+	+	-	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	-	+	+	-	-	+	+	+	+	-
Urea hydrolysis	+	+	+	+	+	+	+	+	-	-	+	-
Degradation tests (%, w/v)												
Arbutin	+	+	+	+	+	+	+	-	+	+	+	+
Casein (1)	+	+	-	+	+	+	+	+	+	+	+	+
Elastin	+	+	+	+	+	+	+	-	-	-	+	-
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+
Guanine (0.05)	+	-	-	+	-	-	-	-	-	-	-	-
Hypoxanthine (0.4)	+	+	+	+	+	+	+	+	+	-	-	-
RNA	+	+	+	+	+	+	+	-	-	-	-	-
Starch	+	+	+	+	+	+	+	-	+	+	+	+
Tween 80 (l%v/v)	+	+	+	+	-	+	-	+	+	+	+	+
Xanthine	-	-	-	-	-	-	-	-	-	-	-	-
Xylan	-	-	-	-	-	-	-	+	+	+	+	+
Growth on sole C sources at %1 w/v												
Adonitol	-	-	-	+	+	-	+	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	+	+	+	+	+
L(+)Arabinose	+	+	+	+	-	+	+	+	+	+	-	+
Arbutin	-	-	-	-	-	-	-	-	+	+	+	+
D(+)Cellobiose	+	+	+	+	-	+	+	-	+	+	-	+
Dextrin	+	+	+	+	-	+	+	-	-	-	-	-
D(-) Fructose	+	+	+	-	+	+	+	+	+	+	+	+
α-Lactose	+	+	-	+	+	+	+	+	+	+	+	+
D-Mannitol (% 1)	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose (% 1)	+	+	+	+	+	+	+	-	-	-	-	+
Salicin	-	-	-	+	-	+	+	+	+	+	+	-
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+
L-Valine (% 0.1)	+	+	+	+	+	+	+	+	+	+	+	-
D-Xylose	+	+	+	+	+	+	+	-	-	+	-	-
Growth on sole N sources at %1 w/v												
L(-) Alanine	+	+	-	+	+	+	+	+	+	+	+	+
L(-)Histidine	+	+	+	-	+	+	+	+	+	-	+	-
L(-)Leucine	+	+	-	-	+	+	+	+	+	+	+	+
Dl(-)Methionine	-	-	-	-	-	-	-	-	+	+	+	+
L(-)Serine	+	+	+	+	-	+	+	+	+	+	+	+
Growth at												
Sodium chloride (% 4)	+	+	+	+	+	+	+	+	-	-	-	+
Sodium chloride (% 7)	+	+	+	+	+	+	+	-	-	-	-	-

pH 4.0	-	-	-	+	-	-	-	-	-	-	-	-
pH 5.0	-	-	+	-	-	-	-	+	+	+	+	+
pH 9.0	-	-	-	-	-	+	-	+	+	+	+	+
pH 10	+	-	-	+	-	+	-	-	-	-	-	+
4°C	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotics (μg/ml)												
Ampicillin (32)	+	+	+	+	+	+	+	+	+	+	+	+
Gentamycin sulphate (8)	-	-	-	-	-	-	-	-	-	-	-	-
Gentamycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin sulphate (16)	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-
Penicillin (10)	+	+	+	+	+	+	+	+	+	+	+	+
Penicillin (20)	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin sulphate (8)	-	-	-	-	-	-	-	-	+	-	+	-
Streptomycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	+	-

Table 4 Phenotypic properties that separate members of the *Streptomyces violaceusniger* 16S rRNA gene clade

Phenotipic Tests	M5038	M4052	M2062	M4039	M1243	M2048	M4031	M1491	M1241	M5047	M1286	M5077	M1375	M1473	M1082	M5055	M1455	M1362
Enzyme activity																		
Aesculin hydrolysis	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urea hydrolysis	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Degradation tests (%, w/v)																		
Arbutin	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+
Casein (1)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Elastin	-	+	-	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+
Guanine (0.05)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hypoxanthine (0.4)	-	-	-	+	-	+	-	+	-	+	-	+	+	-	-	-	-	-
RNA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80 (l% v/v)	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Xanthine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylan	+	+	-	+	-	-	+	+	+	+	+	-	-	+	-	-	+	-
Growth on sole C sources at %1 w/v																		
Adonitol	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
L(+)Arabinose	+	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+
D(+)Cellobiose	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+
Dextrin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D(-) Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+
D-Mannitol (% 1)	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose (% 1)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Valine (%0.1)	+	+	+	-	-	+	+	+	+	-	+	+	+	-	-	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on sole N sources at %1 w/v																		
L(-) Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
L(-)Histidine	+	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+
L(-)Leucine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dl(-)Methionine	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
L(-)Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at																		
Sodium chloride (% 4)	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Sodium chloride (% 7)	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
pH 4.0	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-
pH 5.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.0	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 10	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
4°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotics (μg/ml)																		
Ampicillin (32)	+	+	+	-	-	+	-	-	-	+	+	-	+	+	+	+	-	-
Gentamycin sulphate (8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gentamycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin sulphate (16)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillin (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillin (20)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin sulphate (8)	-	-	+	-	-	+	+	+	+	+	-	-	-	+	-	+	-	-
Streptomycin sulphate (32)	-	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	-	-

Phenotipic Tests	M4033	M4042	M1247	M1456	M1331	M1256	M1351	M1470	M1481	M1287	M1444	M1399	M1499	M1400	M5090	M1001	M2002
Enzyme activity																	
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+
Urea hydrolysis	+	+	-	+	-	+	+	+	+	-	+	+	+	+	+	+	+
Degradation tests (%, w/v)																	
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein (1)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Elastin	-	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Guanine (0.05)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Hypoxanthine (0.4)	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
RNA	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80 (l% v/v)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
Xanthine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylan	+	-	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-
Growth on sole C sources at %1 w/v																	
Adonitol	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-
Aesculin	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
L(+)Arabinose	-	-	-	+	-	+	+	+	-	-	+	+	+	+	+	+	-
Arbutin	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
D(+)Cellobiose	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Dextrin	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-
D(-) Fructose	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+
α-Lactose	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+	-
D-Mannitol (% 1)	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose (% 1)	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Salicin	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Valine (%0.1)	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	+	-	+	-	-	-	+	+	+	+	+	+
Growth on sole N sources at %1 w/v																	
L(-) Alanine	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+
L(-)Histidine	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	-
L(-)Leucine	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+
Dl(-)Methionine	+	+	-	+	-	+	+	+	-	-	-	-	-	-	+	-	+
L(-)Serine	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+
Growth at																	
Sodium chloride (% 4)	+	-	-	-	+	-	-	-	+	-	-	+	+	+	+	+	+
Sodium chloride (% 7)	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-
pH 4.0	-	-	-	-	-	+	-	+	-	-	+	+	-	-	-	-	-
pH 5.0	+	+	+	+	-	+	+	+	+	+	+	-	-	-	+	-	+
pH 9.0	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+
pH 10	-	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-
4°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Antibiotics (μg/ml)																	
Ampicillin (32)	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Gentamycin sulphate (8)	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+
Gentamycin sulphate (32)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin sulphate (16)	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Neomycin sulphate (32)	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Penicillin (10)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillin (20)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptomycin sulphate (8)	-	-	-	-	+	+	-	+	+	+	+	-	-	-	-	-	+
Streptomycin sulphate (32)	-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-	+

Data for type strains were taken from Goodfellow et al. 2007

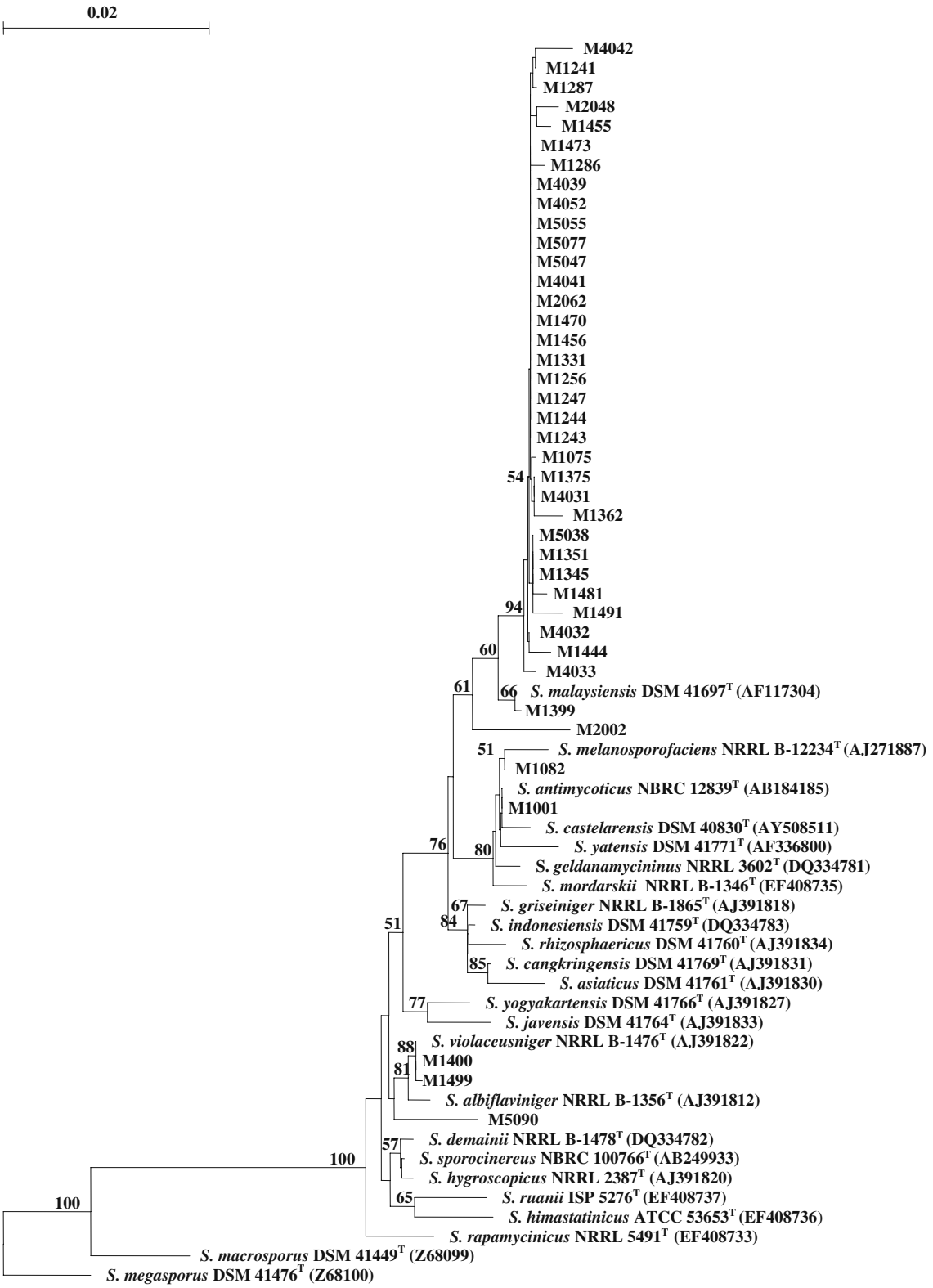

Fig. 2 Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships among new isolates and the type strains of the *Streptomyces violaceusniger* 16S *rRNA* gene clade. The numbers at the nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50% are cited. *Bar* 0.02 substitutions per site

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