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Molecular Phylogenetic Classification of Streptomycetes Isolated from the Rhizosphere of Tropical Legume (*Paraserianthes falcataria*) (L.) Nielsen

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Intrageneric diversity of 556 streptomycetes isolated from the rhizosphere of tropical legume was determined by using molecular taxonomic method based on 16S rDNA. A total of 46 isolates were taken to represent 37 colour groups of the isolates. 16S rDNA were amplified and subsequently sequenced and the sequences data were aligned with streptomycete sequences retrieved from the ribosomal data base project (RDP) data. Phylogenetic trees were generated by using the PHYLIP software package and the matrix of nucleotide similarity and nucleotide difference were generated by using PHYDIT software. The results confirmed and extended the value of 16S rDNA sequencing in streptomycete systematic. The 16S rDNA sequence data showed that most of the tested colour group representatives formed new centers of taxonomic variation within the genus *Streptomyces*. The generic assignment of these organisms was underpinned by 16S rDNA sequence data which also suggested that most of the strains represented new centers of taxonomic variation. The taxonomic data indicate that diverse populations of streptomycetes are associated with the roots of tropical legume (*P. falcataria*). Therefore, the combination of selective isolation and molecular taxonomic procedures used in this study provide a powerful way of uncovering new centers of taxonomic variation within the genus *Streptomyces*.

Key words: molecular phylogenetic, classification, streptomycetes, rhizosphere, *Paraserianthes falcatharia*

INTRODUCTION

Relatively few attempts have been made to determine the taxonomic integrity of streptomycete clusters defined in the extensive numerical phenetic surveys (Williams *et al*. 1983) using molecular systematic methods. This is surprising considering the ecological and commercial importance of streptomycetes but probably reflects the difficulty of examining a representative sample of strains from a genus which contains almost 600 validly described species (Bacterial Nomenclature, DSMZ GmbH, 2000) and many putatively novel species. Most of the molecular taxonomic investigations which have been carried out on a few representatives of a relatively small number of the numerically defined taxa circumscribed by Williams *et al*. (1983). However, nucleic acid sequencing studies have been used to determine the homogeneity of defined streptomycete species groups and to generate phylogenetic classifications of representative strains. 16S rRNA cataloguing experiments (Stackebrandt *et al.* 1983) demonstrated a close relationship between representatives of the genera *Chainia*, *Elytrosporangium*, *Kitasatoa*, *Microellobosporia*, *Streptoverticillium*, and the genus *Streptomyces*.

It is becoming clear that 16S rRNA sequences should form part of the minimal description of streptomycete species (Chun *et al.* 1997; Kim *et al.* 1998; Kim *et al.* 1999, 2000). However, several descriptions of new species of *Streptomyces* have been based on phenotypic properties, namely cultural characteristics, spore chain morphology as well as spore chain ornamentation (Esnard *et al.* 1995; Li *et al.* 1999) or on limited DNA relatedness data (Goyer *et al.* 1996; Labeda *et al.* 1997).

Stackebrandt *et al.* (1991) analysed partial sequences of 16S and 23S rRNA isolated from members of several *Streptomyces* species in a search for oligonucleotide sequences that could be used to assign unknown streptomycetes to the genus, species-groups and individual *Streptomyces* species. 16S rRNA nucleotide 929 of *Streptomyces ambofaciens* (numbering system; Pernodet *et al.* 1989) was found to be unique to *Streptomyces* strains. A genus specific probe (5'- GCGTCGAATTAAGCCACA -3') was generated incorporating this nucleotide position and its flanking region (Stackerbrandt *et al*. 1991).

The primary aim of the present study was to unravel streptomycete diversity isolated from the rhizosphere of tropical legume (*P. falcatharia*) by application of molecular taxonomy, especially 16S rDNA sequencing analysis. This tree is widely cultivated across the Indonesian archipelago and is grown as a profitable source of timber and paper pulp (Atmosuseno 1997), but it is known to be prone to attack by root-infecting fungi belonging to genera such as *Fusarium*, *Pythium*, and *Rhizoctonia* (Santoso 1992; Atmosuseno 1997). Thus, streptomycete associated with the rhizosphere of the tree could be studied and screened for the development of microbiological agent to suppress the growth of root infecting fungi. The study of the streptomycete diversity could be performed by molecular phylogenetic classification method. Phylogeny tree constructed from the 16S rDNA sequences was then used to evaluate the taxonomic status of the streptomycete isolates. To this end, the isolates were found to form five separated center of taxonomic, which strongly indicated that almost all of the isolates belong to novel species within the realm of the member of the genus *Streptomyces*.

MATERIALS AND METHODS

Test Strains and Reagents. The representative strains of the 37 multi-member colour groups (Sembiring unpublished data) were the subject of 16S rDNA sequencing experiments (Table 1). All of the strains were maintained on modified Bennett's agar (Jones 1949) at 4 °C and as glycerol stock cultures at -70 °C. Molecular biology grade reagents and enzymes were obtained from commercial suppliers (Boehringer Mannheim Biochemica 1997; Sigma Chemical Company 1997; Gibco BRL Products 1998; Promega 1998). Stock solutions were prepared according to Sambrook *et al*. (1989).

Table 1. Cultural, morphological, and chemical characteristics of representatives of the multi-membered colour groups examined in the 16S rDNA sequencing and ribotyping experiments

Colour group	Aerial mycelium colour	Reverse mycelium colour	Soluble pigment colour	Number of strains in colour group	Representatives of the colour group	Spore chain morphology	Spore surface ornamentation	Isomer of diaminopimelic acid
1.1	Gray-brown	Brown	Yellow	19	A1P1	Spirals	Warty	LL
1.2	Gray-brown	Brown	Yellow	11	C1P2	Spirals	Smooth	LL
1.3	Gray-brown	Brown	Yellow	5	B10P3	Spirals	Smooth	LL
2.1	Green-white	Green-black	Green	38	B24P1	Flexuous	Warty	LL
2.2	Green-white	Green-black	Green	27	D4R2	Flexuous	Warty	LL
3.1	Gray-white	Yellow-gray	Yellow	40	A4R2	Spirals	Rugose	LL
3.2	Gray-white	Yellow-gray	Yellow	19	A33R1	Spirals	Rugose	LL
3.3	Gray-white	Yellow-gray	Yellow	35	B13P3	Spirals	Rugose	LL
4.1	White	White	None	42	A13P1	Fragments	Smooth	*meso*
4.2	White-yellow	White-yellow	None	21	D2P2	-	-	*meso*
4.3	White-orange	White-orange	None	6	C28P3	Fragments	Smooth	*meso*
5	Blue	Blue	None	6	A23P1	Spirals	Spiny	LL
6	Brown-gray	Yellow	None	2	A29P1	Spirals	Smooth	LL
7.1	Gray-light brown to dark brown	Gray-yellow	None	14	B20P1	Flexuous	Smooth	LL
7.2	Gray-light brown to dark brown	Gray-yellow	None	27	C25P3	Spirals	Smooth	LL
7.3	Gray-light brown to dark brown	Gray-yellow	None	9	A13P3	Spirals	Smooth	LL
8	Gray-white-yellow-green	Brown-yellow	Yellow	2	B2P1	Spirals	Smooth	LL
9	Gray-brown	Brown	None	3	A21P1	Spirals	Smooth	LL
10	Gray-brown	Yellow	Yellow	2	A46R1	Flexuous	Smooth	LL
11	Gray	Gray-yellow	None	2	B13P1	Flexuous	Smooth	LL
12	Gray-yellow	Yellow-brown	None	16	B12P1	Open loops	Smooth	LL
13	Yellowish pink-white	Brown-yellow-black	None	11	D19P2	Straight	Smooth	LL
14	White	Violet-orange	Violet	3	B11R1	Fragments	Smooth	*meso*
15	Gray-brown	Brown—yellow	Yellow	4	A30P1	Straight	Smooth	LL
16	White-yellow	Yellow-brown	Yellow	18	A33P1	Straight	Warty	LL
17	Gray-white	White-yellow	None	2	A22P1	Flexuous	Smooth	LL
18	White-yellow	Brown-yellow	None	4	A50P1	Open loops	Ridged	*meso*
19	Gray-black	Gray-yellow	None	7	A36P1	Spirals	Rugose	LL
20	Gray	Gray-yellow	None	8	A27P1	Flexuous	Smooth	LL
21	Gray-white	Gray-yellow	Yellow	2	A13P2	Flexuous	Smooth	LL
22	Gray-white	Brown-black	Yellow	2	C7P2	Open loops	Smooth	LL
23	White-gray	White-gray-yellow-black	None	2	A1R2	Spirals	Smooth	LL
24	Gray-white	Gray-orange	Orange	2	A18P2	Spirals	Knobby	LL
25	Blue-gray-white	Gray-yellow	None	4	A5P1	Spirals	Spiny	LL
26	White-violet	Yellow-brown	Violet	2	C32P3	Flexuous	Knobby	*meso*
27	Gray-yellow	White-gray-yellow	Yellow	2	C13R3	Spirals	Rugose	LL
28	Orange	Orange-yellow	None	6	A14R2	Straight	Knobby	LL
29	Gray	Brown	Yellowish brown	2	A6P3	Flexuous	Knobby	LL
30								
31	Greenish gray	Greenish black	Green	4	B25R3	Open loops	Smooth	LL
32	Gray-white-yellow	Yellow-gray	Yellow	3	B17R3	-	-	LL
33	Red-white-orange-pink	Red-white-orange-pink	None	4	A37P3	-	-	LL
34	White-orange-pink	Red-orange	None	2	A22P2	Biverticillate	Twisted	LL
35	Pinkish white	Pinkish white-yellow	None	4	A14P3	Biverticillate	Twisted	LL
36	Greenish gray	Gray-brown-yellow-black	None	2	D12R1	Spirals	Smooth	LL
37	Pinkish orange	Pinkish orange	None	2	D9P2	Fragments	Smooth	*meso*
	Black-brown	Black-brown	None	3	C5P3	Vesicular	Smooth	*meso*

DNA Extraction and Purification. Initially, genomic DNA from the test strains was prepared using the guanidine thiocyanate extraction method of Pitcher *et al*. (1989).

Small Scale Preparation of Genomic DNA. Representative strains from the multi-member colour groups were grown on non-sporulating agar (Sanglier *et al.* 1992) for two to three weeks at 25 °C. Microbial biomass was scrapped from the plates and used for DNA extraction.

Sequencing of 16S rDNA. 16S rDNA preparation from the test strains were amplified by PCR using conserved primers (Lane 1991). The PCR products were separated by electrophoresis and further purified by using DNA purification kits; the purified PCR products were sequenced by using an automatic sequencing procedure.

Oligonucleotide Primers used in PCR Amplification and Sequencing of 16S rDNA. Oligonucleotides primers (Lane 1991) used in the PCR amplification of 16S rDNA were (27f): AGAGTTTGATCCTGGCTCAG (8-27) and (1525r): AAGGA GGTGWTCCARCC (1544-1525).

PCR Amplification of 16S rDNA. Polymerase chain reaction amplifications of the 16S rDNA preparations were carried out in a Perkin Elmer DNA Thermal Cycler 480 using 0.5 ml PCR microfuge tubes. *Taq* DNA polymerase, $MgCl_2$ solution, and *Taq* buffer were purchased from Biotaq™ DNA Polymerase (Bioline) and deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) obtained from Boehringer Mannheim GmbH, Germany at a concentration of 100 mM. The working stock solution of the dNTPs was made by mixing 25 µl of each dNTP stock to obtain a 100 µl mixture of dNTPs; the final concentration of each dNTP was 25 mM. The total 100 µl of PCR reaction mixture containing 2.5 µM of both Primers, 25 mM of dNTPs, 10 x of *Taq* buffer, 2.5 mM of $MgCl_2$, from 2 to 20 ng/ml of DNA template, and 0.0025 U/µl of Taq Polymerase. The PCR amplification was done by one cycle of pre-denaturation of mixture (96 °C; 5 minutes), then followed by 35 cycles of amplification with denaturation (95 °C; 1 minute), annealing (55 °C; 1 minute), and extension (72 °C; 1 minute), ended by final extension (72 °C; 10 minutes), and finally cooling to 4 °C. Electrophoresis was run at 100 V for 1 hour and the size of the amplified 16S rDNA fragments identified by comparison with a molecular size marker (Gene Ruler™ 100 bp DNA Ladder Plus, MBI Fermentas) at the position of 1.5 kb.

Purification of PCR-Amplified 16S rDNA. The PCR-amplified 16S rDNA was separated by preparative agarose electrophoresis (Sambrook *et al.* 1989. Prior to sequencing, the amplicons were purified by using Nucleospin Extract kit (Biogene Limited, Macherey-Nagel GmbH & Co. KG, 1998).

Sequencing of Purified 16S rDNA. The purified 16S rDNA fragments were sequenced by using an ABI PRISM™ 377 DNA Sequencer (PE Applied Biosystems). The sequencing of the complete length of the 16S rDNA molecule was carried out by using the five different oligonucleotide primers (Chun 1995), separately, namely (MG1): AGAGTTTGATCCTGG CTCAG (8 27); (MG3): CTACGGGRSGCAGCAG (352-357); (MG4): AATTCCTGGTGTAGCGGT (675-692); (MG5): AAAC TCAAAGGAATTGACGG (907-926); (MG6): GACGTCAA GTCATCATGCC (1190 -1208).

Analysis of 16S rDNA Sequence Data. The 16S rDNA nucleotide sequences data obtained using the Automatic Sequencer was transferred into AL16S software (Chun 1995 1999). Entries were identified by the strain name and the primers used to generate the sequences. Completely aligned sequences of the 16S rDNA extracted from each of the test strains were used to generate phylogeny trees using the PHYLIP package (Felsenstein 1993).

Alignment of 16S rDNA Sequences. Partial 16S rDNA nucleotide sequences were automatically aligned with corresponding available streptomycete sequences retrieved from the ribosomal database project (RDP) (Maidak *et al.* 1997) and EMBL/GeneBank databases (Benson *et al*. 1998; Appendix D) by using (CLUSTAL X (Thompson *et al.* 1997). PHYDIT (Chun 1999).

Construction of Phylogenic Trees. Evolutionary trees were inferred by using the neighbour-joining (Saitou & Nei 1987), maximum-parsimony (Kluge & Farris 1969) and least-squares algorithms (Fitch & Margoliash 1967). Evolutionary distance matrices for the neighbour-joining and least-squares methods were generated as described by Jukes and Cantor (1969). All of the phylogenic trees were generated by using the PHYLIP software package (Felsenstein 1993) or TREECON (Van De Peer & De Wachter 1994). The resultant unrooted tree topology was evaluated by bootstrap analysis (Felsenstein 1985) of the neighbour-joining method data based on 1000 resampling using the SEQBOOT and CONSENSE programs in the PHYLIP package (Felsenstein 1993). The root position of the unrooted tree based on the neighbour-joining method was estimated by using *Nocardiopsis alborubidus* DSM 40465^T (X97882) as the outgroup strain. A phylogenetic tree based on the partial nucleotide sequences was constructed by using the neighbour-joining algorithm (Saitou & Nei 1987).

RESULTS

16S rDNA Sequencing. Almost complete 16S rDNA nucleotide sequences were obtained for 32 isolates which represented 26 out of the 37 multi-member colour groups. Considerable difficulties were experienced in trying to sequence the 16S rDNA of strains D4R2 (colour group 2.2), A13P1, D2P2, and C28P3 (colour group 4), A23P1 (colour group 5), B11R1 (colour group 14), A22P1 (colour group 17), A50P1 (colour group 18), C7P2 (colour group 22), A1R2 (colour group 23), A5P1 (colour group 25), C32P3 (colour group 26), A14R2 (colour group 28), and B25R3 (colour group 30) hence these organisms were not considered further.

Comparisons of the 16S rDNA nucleotide sequences of the tested strains with the corresponding nucleotide sequences of available representatives of the genus *Streptomyces* clearly showed that 30 out of the 32 strains belong to this genus (Figure 1). It is also apparent from this Figure that several clades, supported by high bootstrap values, can be recognised in the 16S rDNA streptomycete tree. It is also clear that the representatives of the colour

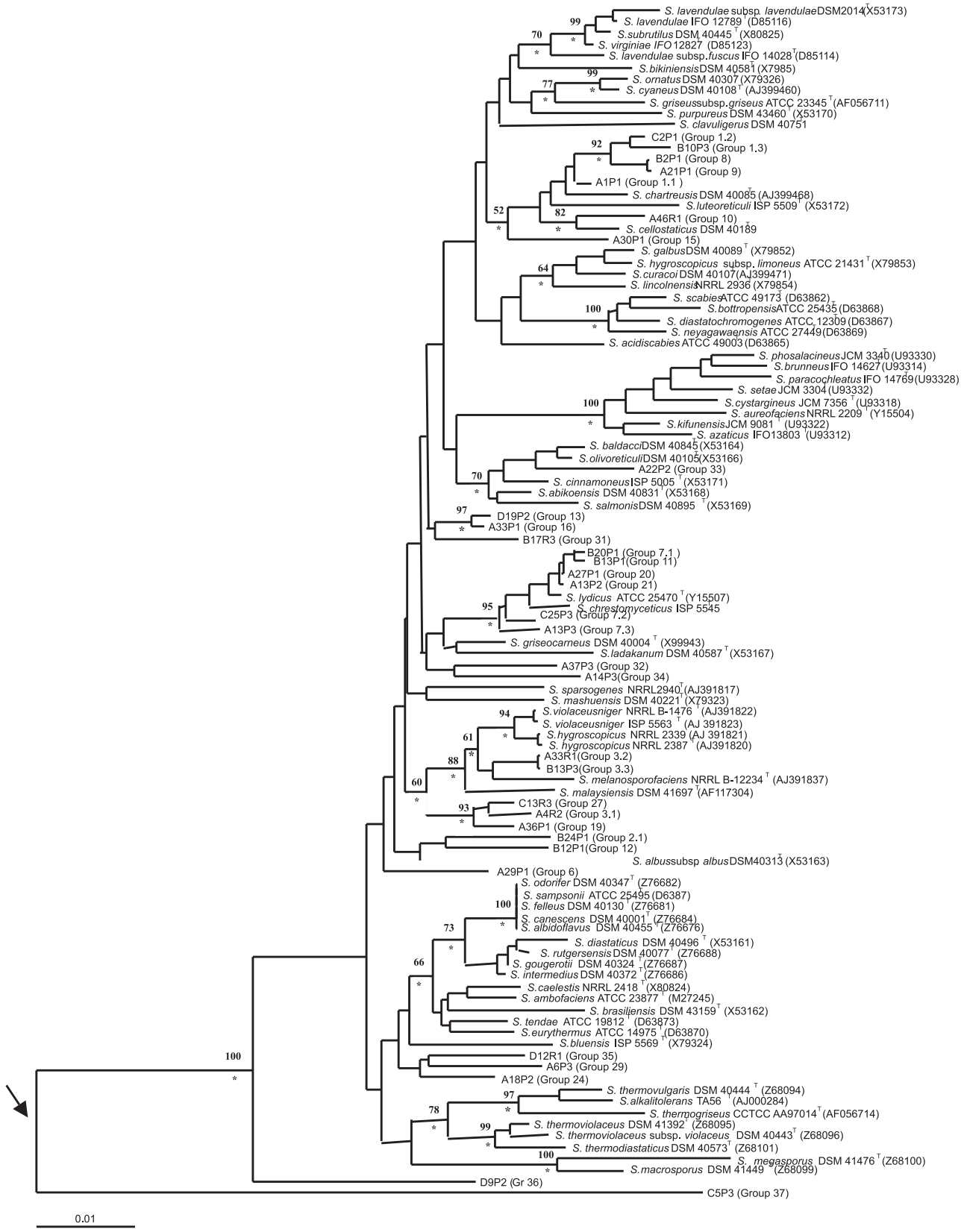

Figure 1. Neighbour-joining tree (Saitou & Nei 1987) based on nearly complete 16S rDNA sequences showing relationships among the representatif isolates of multi-membered colour groups, the member strains of the *Streptomyces hygroscopicus* complex and marker strains of the genus *Streptomyces*. The asterics denote the branches that were also recovered using the least squares (Fitch & Margoliash 1967), maximum likelyhood (Felsenstein 1981) and maximum parsimony (Kluge & Farris 1969) treeing algorithms. The numbers at the nodus indicates the levels of the bootstrap support (%) based on a neighbour-joining analysis of 1,000 resampled data sets. The arrow indicates the estimated root position of the tree. The scale bar indicates one nucleotide substitution per 100 nucleotides in 16S rRNA genes sequences.

groups are quite widely scattered throughout the tree though some of the larger taxa, notably the *S. albidoflavus* clade, did not contain any isolates. Strains C5P3 (colour group 37) and D9P2 (colour group 36) clearly fall outside the zone of evolutionary radiation occupied by members of the genus *Streptomyces* and were not considered further. Some of the isolates were assigned to multi-member clades which were named after the species which had been described first, that is, according to the rule of priority.

It is especially interesting that all of the isolates and marker strains which produced spiral chains of rugose ornamented spores formed a distinct clade with all four treeing algorithms; the taxonomic integrity of this group was also supported by the relatively high bootstrap value of 60%. This taxon, the *S. violaceusniger* clade (Goodfellow *et al.* 2007), encompasses the representatives of colour groups 3, 19, and 27 together with the type strains of *S. hygroscopicus* (Jensen 1931) emended Sembiring *et al*. 2000, *S. malaysiensis* Al-Tai *et al*. 1999, *S. melanosporofaciens* (Arcamone *et al.* 1959) emended Sembiring *et al.* 2000, and *S. violaceusniger* (Waksman & Curtis 1916) emended Sembiring *et al.* 2000. The 16S rDNA nucleotide similarity values and the number of nucleotide differences shown by the members of this clade are given in Table 2. It is interesting that the two *S. hygroscopicus* strains share a 16S rDNA nucleotide similarity value of 99.9%, which corresponds to one nucleotide difference from 1440 sites. Similarly, the representatives of colour groups 3.2 and 3.3 share a 16S rDNA nucleotide similarity value which corresponds to a single nucleotide difference out of 1466 sites. In contrast, the representative of colour group 3.1 was readily distinguished from all of the strains in the *S. violaceusniger* clade, as were the representatives of colour groups 19 and 27 even though these organisms shared a relatively high 16S rDNA similarity value of 99.3% (which is equivalent to 10 nucleotide differences). As expected, the two strains of *S. violaceusniger* had identical 16S rDNA nucleotide sequences.

The representatives of colour groups 7, 11, 20, and 21 together with the type strains of *S. chrestomyceticus* Canevazzi and Scotti 1959 and *S. lydicus* DeBoer *et al*. 1956 formed a well circumscribed clade with all of the treeing algorithms (Figure 1), this taxon was supported by a bootstrap value of 95%. All of the members of this clade shared high 16S rDNA nucleotide similarity values and hence showed relatively few nucleotide differences (Table 3). It was clear from these results that these organisms, with the exception of strain A13P3, are closely related to *S. lydicus*. It was also interesting that the type strains of *S. chrestomyceticus* and *S. lydicus* share a 16S rDNA nucleotide similarity which corresponds to 11 differences at 1454 sites.

A third taxon, the *S. chartreusis* clade, was supported by all of the treeing algorithms and by a bootstrap value of 52%; this clade encompassed representatives of colour groups 1, 8, 9, 10 and 15, and the type strains of *S. cellostaticus* Hamada 1958, *S. chartreusis* Calhoun and Johnson 1956 and *S. luteoreticuli* Katoh and Arai 1957 (Figure 1). It also encompasses a distinct multi-member subclade circumscribed using all four treeing algorithms. This taxon contains the

Table 2. 16S rDNA nucleotide similarity values (%) and the number of nucleotide differences found between the representative strains of colour groups 3, 19, and 27 and marker strains of validly described *Streptomyces* species classified in the *Streptomyces violaceusniger* clade

Strain code	*Streptomyces* sp. A4R2 (group 3.1)	*Streptomyces* sp. A33R1 (group 3.2)	*Streptomyces* sp. B13P3 (group 3.3)	*Streptomyces* sp. A36P1 (group 19)	*Streptomyces* sp. C13R3 (group 27)	*S. malaysiensis* DSM 41697^T	*S. melanosporofaciens* NRRL B-12234^T	*S. hygroscopicus* NRRL 2387^T	*S. hygroscopicus* NRRL 2339	*S. violaceusniger* NRRL B-1476^T	*S. violaceusniger* NRRL-ISP 5563^T
Streptomyces sp. A4R2 (group 3.1)	-	26/1468	25/1466	17/1462	10/1462	26/1434	34/1478	28/1461	27/1458	24/1478	24/1478
Streptomyces sp. A33R1 (group 3.2)	98.2	-	1/1466	36/1460	31/1460	22/1420	19/1465	23/1448	23/1458	16/1467	16/1467
Streptomyces sp. B13P3 (group 3.3)	98.3	99.9	-	37/1459	31/1461	23/1419	19/1463	23/1445	22/1459	15/1466	15/1466
Streptomyces sp. A36P1 (group 19)	98.8	97.5	97.5	-	10/1450	35/1418	39/1459	31/1442	32/1452	32/1460	32/1460
Streptomyces sp. C13R3 (group 27)	99.3	97.9	97.9	99.3	-	28/1414	33/1457	29/1440	30/1459	30/1460	30/1460
S. malaysiensis DSM 41697^T	98.2	98.4	98.4	97.5	98.0	-	28/1433	24/1417	22/1411	26/1431	26/1431
S. melanosporofaciens NRRLB-12234^T	97.7	98.7	98.7	97.3	97.7	98.0	-	23/1462	22/1455	23/1475	23/1475
S. hygroscopicus NRRL 2387^T	98.1	98.4	98.4	97.8	98.0	98.3	98.4	-	1/1440	8/1460	8/1460
S. hygroscopicus NRRL 2339	98.1	98.4	98.5	97.8	97.9	98.4	98.5	99.9	-	7/1460	7/1460
S. violaceusniger NRRL B-1476^T	98.4	99.0	99.0	97.8	97.9	98.2	98.4	99.4	99.5	-	0/1478
S. violaceusniger NRRL-ISP 5563^T	98.4	99.0	99.0	97.8	97.9	98.2	98.4	99.4	99.5	100	-

representatives of colour groups 1.2, 1.3, 8, and 9 and is supported by a bootstrap value of 92%. Strain A1P1, the representative of colour group 1.1, was loosely associated with this subclade. This organism shows 16S rDNA nucleotide similarity values of between 99.1 and 99.6% with the strains in the subclade, these values correspond to between 5 and 10 nucleotide differences (Table 4). It is evident that isolates B2P1 (colour group 8) and A21P1 (colour group 9) are closely related as they share a 16S rDNA nucleotide similarity of 99.9%, which is equivalent to a single nucleotide difference. The representatives of colour groups 1.2 and 1.3 are quite closely related sharing a 16S rDNA nucleotide similarity of 99.4%, which is equivalent to 9 nucleotide differences. The similarity values recorded between isolate A1P1 (colour group 1.1) and the remaining strains need to be interpreted cautiously as they are based on incomplete 16S rDNA nucleotide sequences. Relatively low 16S rDNA similarity values were recorded for the remaining strains assigned to the *S. chartreusis* clade.

Isolate A22P2 (colour group 33) and the type strains of *S. abikoensis* (Umezawa *et al.* 1951) Witt and Stackebrandt 1991^{VP}, *S. baldacci* (Farina & Locci 1966) Witt and Stackebrandt 1991^{VP} *S. cinnamoneus* (Benedict *et al.* 1952) Witt and Stackebrandt 1991^{VP}, *S. olivoreticuli* (Arai *et al.* 1957) Baldacci, Farina and Locci 1966 and *S. salmonis* (Baldacci, Farina & Locci 1966) Locci, Baldacci and Petrolini-Baldan 1969 form a clade that is supported by all of the treeing algorithms and by a bootstrap value of 70%. This organism is sharply separated from all of the other members of the *S. abikoensis* clade showing its highest 16S rDNA nucleotide similarity, 98.7%, with *S. baldacci*; this value corresponds to 14 nucleotide differences from 1089 locations. A relatively close 16S rDNA nucleotide similarity value was shown between the type strains of *S. baldacci* and *S. olivoreticuli*. These organisms have a 16S rDNA nucleotide similarity of 99.6% which equates to 5 differences at 1142 sites (Table 5).

Isolates D19P3 (colour group 13) and A33P1 (colour group 16) form a distinct clade which is supported by a bootstrap value of 97.0%. These organisms share a 16S rDNA nucleotide similarity value of 99.7%, which corresponds to 5 nucleotide differences at 1474 locations (data not shown). The following isolates formed distinct single-member clades: strains B24P1 (colour group 2.1), A29P1 (colour group 6), B12P1 (colour group 12), A18P2 (colour group 24), A6P3 (colour group 29), B17R3 (colour group 31), A37P3 (colour group 32), A14P3 (colour group 34), and D12R1 (colour group 35). All of these isolates formed distinct groups in the phylogenetic tree based

Table 3. 16S rDNA nucleotide similarity values (%) and the number of nucleotide differences between the representative strains of colour grou 7, 11, 20, and 21 and the type strains of the two validly described *Streptomyces* species classified in the *Streptomyces lydicus* clade

Strain code	*Streptomyces* sp. B20P1 (group 7.1)	*Streptomyces* sp. C25P3 (goup 7.2)	*Streptomyces* sp. A13P3 (group 7.3)	*Streptomyces* sp. B13P1 (group 11)	*Streptomyces* sp. A27P1 (group 20)	*Streptomyces* sp. A13P2 (group 21)	*S. chrestomyceticus* ISP 5545^T	*S. lydicus* ATCC 2547^T
Streptomyces sp. B20P1 (group 7.1)	-	4/1147	13/1472	0/1471	2/1467	3/1462	10/1468	2/1449
Streptomyces sp. C25P3 (group 7.2)	99.6	-	2/1146	3/1146	3/1146	3/1145	5/1143	1/1123
Streptomyces sp. A13P3 (group 7.3)	99.1	99.8	-	14/1473	14/1467	14/1462	20/1470	13/1451
Streptomyces sp. B13P1 (group 11)	100.0	99.7	99.0	-	1/1465	2/1461	13/1472	7/1453
Streptomyces sp. A27P1(group 20)	99.9	99.7	99.0	99.0	-	2/1461	11/1463	3/1444
Streptomyces sp. A13P2 (group 21)	99.8	99.7	99.0	99.9	99.9	-	11/1459	5/1440
S.chrestomyceticus ISP 5545^T	99.3	99.6	98.6	99.1	99.2	99.1	-	11/1454
S. lydicus ATCC 2547^T	99.9	99.9	99.1	99.5	99.8	99.6	99.2	-

Table 4. 16S rDNA nucleotide similarity values (%) and the number of nucleotide differences between the representative strains of colour groups 1, 8, 9, 10, and 15 and the type strains of the three validly described *Streptomyces* species classified in the *Streptomyces chartreusis* clade

Strain code	*Streptomyces* sp. A1P1 (group 1.1)	*Streptomyces* sp. C2P1 (group 1.2)	*Streptomyces* sp. B10P3 (group 1.3)	*Streptomyces* sp. B2P1 (group 8)	*Streptomyces* sp. A21P1 (group 9)	*Streptomyces* sp. A46R1 (group 10)	*Streptomyces* sp. A30P1 (group 15)	*S. cellostaticus* DSM 40189^T	*S. chartreusis* DSSM 40085^T	*S. luteoreticuli* ISP 5509^T
Streptomyces sp. A1P1 (group 1.1)	-	6/1132	5/1135	9/1131	10/1132	15/1135	13/1132	9/1131	5/1125	9/784
Streptomyces sp. C2P1 (group 1.2)	99.5	-	9/1471	13/1468	14/1468	33/1471	27/1467	26/1468	23/1460	34/1114
Streptomyces sp. B10P3 (group 1.3)	99.6	99.4	-	13/1480	14/1475	34/1487	28/1471	24/1482	19/1463	31/1129
Streptomyces sp. B2P1 (group 8)	99.2	99.1	99.1	-	1/1474	36/1480	31/1469	29/1480	26/1463	35/1126
Streptomyces sp. A21P1 (group 9)	99.1	99.0	99.0	99.9	-	36/1475	31/1469	29/1474	27/1462	35/1120
Streptomyces sp. A46R1 (group 10)	98.7	97.8	97.7	97.6	97.6	-	35/1470	17/1482	27/1463	26/1129
Streptomyces sp. A30P1 (group 15)	98.8	98.2	98.1	97.9	97.9	97.6	-	33/1469	31/1464	33/1120
S. cellostaticus DSM 40189^T	99.2	98.2	98.4	98.0	98.0	98.8	97.7	-	20/1463	29/1129
S. chartreusis DSSM 40085^T	99.6	98.4	98.7	98.2	98.1	98.1	97.9	98.6	-	21/1116
S. luteoreticuli ISP 5509^T	98.8	96.9	97.2	96.9	96.9	97.7	97.0	97.4	98.1	-

Table 5. 16S rDNA nucleotide similarity values (%) and the number of nucleotide differences found between strain A22P2, the representative of colour group 33 and the type strains of the validly described *Streptomyces* species classified in the *Streptomyces abikoensis* clade

Strain code	*Streptomyces* sp. A22P2 (group 33)	*S. abikoensis* DSM 40831^T	*S. baldacci* DSM 40845^T	*S. cinnamoneus* ISP 5005^T	*S. olivoreticuli* DSM40105^T	*S. salmonis* DSM 40895^T
Streptomyces sp. A22P2 (group 33)	-	27/1102	14/1089	24/1159	19/1154	29/1075
S. abikoensis DSM 40831^T	97.7	-	16/1134	11/1124	14/1143	13/1101
S. baldacci DSM 40845^T	98.7	98.6	-	11/1116	5/1142	18/1099
S. cinnamoneus ISP 5005^T	97.9	99.0	99.0	-	10/1157	14/1091
S. olivoreticuli DSM40105^T	98.8	98.8	99.6	99.1	-	19/1120
S. salmonis DSM 40895^T	97.3	98.8	98.4	98.7	98.3	-

on a comparison of partial 16S rDNA nucleotide sequences (120 bp; Kataoka *et al*. 1997) with 467 representative *Streptomyces* strains (Ueda *et al*. 1997) based on the variable α region (Stackebrandt *et al*. 1991).

DISCUSSION

16S rDNA sequencing data are being used to establish the boundaries and internal taxonomic structure of the genus *Streptomyces* (Kim *et al*. 1996; Takeuchi *et al*. 1996; Chun *et al*. 1997; Hain *et al*. 1997; Shojaei 1997; Kim *et al*. 1998; Kim *et al*. 1999, 2000). It is evident from such studies that some streptomycetes can be assigned to distinct multi-member phyletic lines, such as the *Streptomyces albidoflavus* and *S. thermodiastaticus* clades, and those members of certain evolutionary lines have the capacity to produce commercially significant bioactive compounds. There is also evidence from ecological surveys (Saddler 1988; Sahin 1995; Kim1999; Atalan *et al*. 2000) and from detailed studies of established species (Labeda 1992, 1993, 1996, 1998; Labeda & Lyons 1991a,b, 1992) that the genus *Streptomyces* is underspeciated.

The results of the present investigation confirm and extend the value of 16S rDNA sequencing in streptomycete systematics. The partial and almost complete 16S rDNA sequence data show that most of the tested colour group representatives form new centers of taxonomic variation within the genus *Streptomyces*. It is especially interesting that some of the isolates belong to well delineated phyletic lines that were designated the *S. abikoensis, S. chartreusis, S.lydicus* and *S. violaceusniger* clades. The increased resolution of the 16S rDNA streptomycete tree suggests that the subgeneric structure of this taxonomically complex group may be clarified as new centers of taxonomic variation are highlighted.

The largest 16S rDNA streptomycete clade encompasses the representatives of colour groups 3, 19, and 27 together with marker strains of *S. hygroscopicus, S. malaysiensis, S. melanosporofaciens,* and *S. violaceusniger*. The members of this taxon, the *S. violaceusniger* clade, are characterised by their ability to produce spiral chains of rugose ornamented spores. The two most closely related species, *S. hygroscopicus* and *S. violaceusniger,* share a 16S rDNA nucleotide similarity of 99.5% (which corresponds to 7 nucleotide differences) and a DNA relatedness value of 39% (Labeda & Lyons 1991a) which is well below the 70% cut-off point recommended by Wayne *et al.* (1987) for the recognition of genomic species. Isolates A36P1 (colour group 19) and C13R3 (colour group 27), the representatives of the two most closely related colour-groups, share a 16S rDNA nucleotide similarity of 99.3%, a value which is equivalent to 10 nucleotide differences. The high 16S rDNA nucleotide similarity found between isolates A33R1 (colour group 3.2) and B13P3 (colour group 3.3), namely, 99.9%, suggests that these strains belong to a single species even though they exhibit distinct ribotype patterns. In contrast, isolate A4R2 (colour group 3.1) can readily be distinguished from all of the other members of the *S. violaceusniger* clade on the basis of the 16S rDNA sequence data. These results suggest that the representatives of colour groups 3, 19, and 27 represent the nuclei of at least three novel species.

The 16S rDNA sequence data are also consistent with the view that the isolates assigned to the three other multi-member clades represent new centers of taxonomic variation within the genus *Streptomyces*. The type strains of four out of the five marker species in the *S. abikoensis* clade, namely *S. abikoensis, S. baldacci, S. cinnamoneus,* and *S. salmonis* belong to well separated genomic species (Labeda 1996; Labeda *et al.* 1997). The *S. baldacci* and *S. cinnamoneus* strains have a 16S rDNA nucleotide similarity of 99.0% (which corresponds to 11 nucleotide differences) and share a DNA relatedness value of 28% (Labeda 1996). Similarly, isolate A22P2 (colour group 33) and the *S. baldacci* strain share a 16S rDNA nucleotide similarity of 98.7%, which is equivalent to 14 nucleotide differences. It seems clear, therefore, that isolate A22P2 merits recognition as a new species provided corresponding phenetic data become available. All of the organisms classified in the *S. abikoensis* clade produce aerial mycelia which are composed of long straight filaments that carry branches arranged in verticils, the latter consists of two to many chains of spherical to ellipsoidal, smooth surfaced spores.

The *S. chartreusis* clade contains the type strains of *S. cellostaticus, S. chartreusis,* and *S. luteoreticuli* and the representatives of colour groups 1, 8, 9, 10, and 15. The two most closely related marker species, *S. cellostaticus* and *S. chartreusis*, share a 16S rDNA nucleotide similarity of 98.6%, which corresponds to 20 nucleotide differences. These strains can readily be distinguished from the type strain of *S. luteoreticuli* as they form spiral chains of spiny spores (Shirling & Gottlieb 1968a,b), as opposed to verticils bearing chains of smooth surfaced spores (Shirling & Gottlieb 1972). Some of the isolates formed spiral chains or produced spiny spores; these organisms shared relatively low 16S rDNA nucleotide similarity values with the marker strains. It seems likely that isolates B2P1 (colour group 8) and A2P1 (colour group 9) belong to a distinct new species as they share a 16S rDNA nucleotide similarity of 99.9% (which corresponds to

single nucleotide difference) and form spiral chains of smooth-surfaced spores; these organisms also gave identical ribotype patterns. In contrast, isolates A46R1 (colour group 10) and A30P1 (colour group 15) were sharply distinguished both from one another and from the remaining members of the clade though they did produce flexuous chains of smooth-surfaced spores. Further comparative studies are needed to determine whether isolates C2P1 (colour group 1.2) and B10P3 (colour group 1.3) belong to the same species as these organisms share a 16S rDNA nucleotide similarity of 99.4% (which is equivalent to 9 nucleotide differences) and give similar ribotype patterns.

The *S. lydicus* clade also encompasses marker strains and isolates which show a range of morphological properties. The type strains of *S. chrestomyceticus* and *S. lydicus*, form smooth-surfaced spores in spiral chains (Shirling & Gottlieb 1969, 1972) and share a 16S rDNA nucleotide similarity of 99.2%, which equates to 11 nucleotide differences. Isolates B20P1 (colour group 7.1), C25P3 (colour group 7.2), A27P1 (colour group 20), and A13P2 (colour group 21) are closely related to both one another and to *S. lydicus* given high 16S rDNA nucleotide similarity values. However, only isolate C25P3 (colour group 7.2) forms spiral chains of smooth-surfaced spores. It seems likely, therefore, that the remaining isolates form new centers of taxonomic variation within the *S. lydicus* clade.

All of the isolates which formed single-member clades in the almost complete 16S rDNA streptomycete tree, namely, strains B24P1 (colour group 2.1), A29P1 (colour group 6), B12P1 (colour group 12), A18P2 (colour group 24), A6P3 (colour group 29), B17R3 (colour group 31), A37P3 (colour group 32), A14P3 (colour group 34), and D12R1 (colour group 35), were sharply separated from one another and from representatives of *Streptomyces* species based on the partial nucleotide differences (120 bp; Kataoka *et al.* 1997) of the 16S rDNA. These data are consistent with the view that these organisms represent new zones of taxonomic variation in the genus *Streptomyces*.

The 16S rDNA nucleotide sequence data are encouraging as they provide further evidence that streptomycete colour groups can be taxonomically predictive (Goodfellow & Haynes 1984; Williams & Vickers 1988; Atalan *et al*. 2000). However, it is evident from the present investigation that individual colour groups may encompass isolates that belong to several closely related species. This phenomenon is particularly apparent with respect to the colour group 3 strains as several members of this taxon form distinct phyletic lines within the *S. violaceusniger* clade.

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