Taxonomic evaluation of species in the *Streptomyces hirsutus* clade using multi-locus sequence analysis and proposals to reclassify several species in this clade

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Previous phylogenetic analysis of species of the genus *Streptomyces* based on 16S rRNA gene sequences resulted in a statistically well-supported clade (100 % bootstrap value) containing eight species that exhibited very similar gross morphology in producing open looped (*Retinaculum–Apertum*) to spiral (*Spira*) chains of spiny- to hairy-surfaced, dark green spores on their aerial mycelium. The type strains of the species in this clade, specifically *Streptomyces bambergiensis*, *Streptomyces cyanoalbus*, *Streptomyces emeiensis*, *Streptomyces hirsutus*, *Streptomyces prasinopilosus* and *Streptomyces prasinus*, were subjected to multi-locus sequence analysis (MLSA) utilizing partial sequences of the housekeeping genes *atpD*, *gyrB*, *recA*, *rpoB* and *trpB* to clarify their taxonomic status. The type strains of several recently described species with similar gross morphology, including *Streptomyces chlorus*, *Streptomyces herbaceus*, *Streptomyces incanus*, *Streptomyces pratensis* and *Streptomyces viridis*, were also studied along with six unidentified green-spored *Streptomyces* strains from the ARS Culture Collection. The MLSAs suggest that three of the species under study (*S. bambergiensis*, *S. cyanoalbus* and *S. emeiensis*) represent synonyms of other previously described species (*S. prasinus*, *S. hirsutus* and *S. prasinopilosus*, respectively). These relationships were confirmed through determination of *in silico* DNA–DNA hybridization estimates based on draft genome sequences. The five recently described species appear to be phylogenetically distinct but the unidentified strains from the ARS Culture Collection could be identified as representatives of *S. hirsutus*, *S. prasinopilosus* or *S. prasinus*.

Phylogenetic analysis of species of the genus *Streptomyces* based on 16S rRNA gene sequences (Labeda et al., 2012) resulted in a well-supported clade, designated as Clade 6, that had 100 % bootstrap support and was also conserved in maximum-parsimony and maximum-likelihood trees. This clade contained the species *Streptomyces bambergiensis*, *Streptomyces cyanoalbus*, *Streptomyces emeiensis*, *Streptomyces hirsutus*, *Streptomyces prasinopilosus* and *Streptomyces prasinus* and these all produce open looped (*Retinaculum–Apertum*) to spiral (*Spira*) chains of spiny- to hairy-surfaced, dark green spores on their aerial mycelium. Most of these species were observed to belong to Cluster 37 in the numerical taxonomic study of Williams et al. (1983) and were also reported as being closely related by Kämper (2012). Multi-locus sequence analysis (MLSA) has been demonstrated as a valuable tool for assessing species assignments in the genus *Streptomyces* (Guo et al., 2008; Rong et al., 2009; Rong & Huang, 2010, 2012; Labeda, 2011; Labeda et al., 2014) and therefore a study was undertaken to clarify the taxonomic relationships among this group of green-spored species. The type strains of several recently described species having similar morphology, including *Streptomyces chlorus* KACC 20902, *S. herbaceus* NRRL B-59128, *Streptomyces incanus*...
NRRL B-59129\textsuperscript{T}, *Streptomyces pratensis* NRRL B-59131\textsuperscript{T} and *Streptomyces viridis* KACC 21003\textsuperscript{T}, were also selected for study along with six unidentified *Streptomyces* strains from the ARS Culture Collection that also exhibited similar gross colonial morphology (i.e. produced spiral chains of green-colored, spiny- to hairy-surfaced spores).

The strains used in the study were obtained from the ARS Culture Collection, Peoria, IL, USA, or the Korean Agricultural Culture Collection, Suwon, Republic of Korea, and are listed in Table 1. Strains were cultivated on yeast extract-malt extract agar (YM) ISP-2 medium (Shirling & Gottlieb, 1966) at 28 °C.

Genomic DNA was isolated from each strain using Ultra-Clean Microbial DNA isolation kits (MoBio Labs) following the instructions of the manufacturer. Partial sequences of the housekeeping genes *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *rpoB* (RNA polymerase beta subunit), *recA* (recombinase A) and *trpB* (tryptophan synthetase, beta subunit) were amplified and sequenced using the primers and protocols described by Labeda et al. (2014). Amplified products were purified using ExoSAP-IT reagent (Affymetrix) and sequenced using BigDye 3.1 on an ABI model 3730 sequencer in the NCAUR core sequencing facility. Raw contigs for each locus were assembled and corrected from the traces using Sequencher version 5.2 (Gene Codes).

The gene sequences for the five housekeeping loci for each strain were deposited in GenBank (see Table 1) and were also organized using Bacterial Isolate Genomic Sequence (BIGSdb) version 1.10.1 (Jolley & Maiden, 2010) on the ARS Microbial Genomic Sequence Database server (http://199.133.98.43). The sequences for the alleles of each locus for these strains and related strains obtained from GenBank or available locally were aligned by BIGSdb using MUSCLE (Edgar, 2004) during the sequence export process into XMFA format and then the aligned loci were concatenated head to tail in-frame prior to export in FASTA format, providing a dataset of 167 strains and 2593 positions. The sequence alignment was analysed in jModelTest 2, version 2.1.7 (Darriba et al., 2012; Guindon & Gascuel, 2003), to determine the optimal model for phylogenetic analysis. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The phylogenetic tree was inferred by using the maximum-likelihood method based on the General Time Reversible model (Nei & Kumar, 2000). The initial tree for the heuristic search was obtained by applying the BioNJ method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites [four categories (+G, parameter = 0.7568)], which had been determined to be the optimal model for these data in jModelTest2. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 47.4079 % of sites). The analysis involved 167 nucleotide sequences including all codon positions for a total of 2593 positions in the final dataset. The phylogenetic relationships of the strains were also inferred using the Tamura & Nei, (1993) evolutionary distance method with the neighbour-joining model of Saitou & Nei (1987) and maximum-parsimony in MEGA 6.0. All analyses were subjected to 500 bootstrap replications.

**Table 1. *Streptomyces* strains used and gene sequences deposited for the present study**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Genome sequence</th>
<th>atpD</th>
<th>gyrB</th>
<th>recA</th>
<th>rpoB</th>
<th>trpB</th>
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<td>KJ767205</td>
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<td>KJ767220</td>
<td>KJ767227</td>
<td>KJ767230</td>
<td></td>
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<td>KJ767214</td>
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<td>KP890260</td>
<td>KP890263</td>
<td>KP890266</td>
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</table>
replications (Felsenstein, 1985). MLSA evolutionary distances were determined using MEGA 6.0 by calculated the Kimura two-parameter distance (Kimura, 1980) as specified by Rong & Huang (2012) and strain pairs having a distance of less than 0.007 were considered conspecific based on the guideline empirically determined by Rong & Huang (2012) that this distance equates to 70% DNA–DNA relatedness.

The draft genome sequences of six strains, *S. bambergiensis* NRRL B-12521\(^T\), *S. cyanoalbus* NRRL B-3040\(^T\), *S. emeiensis* NRRL B-24621\(^T\), *S. hirsutus* NRRL B-2713\(^T\), *S. prasinopilosus* NRRL B-2711\(^T\) and *S. prasinus* NRRL B-2712\(^T\), were also determined in the course of this study. Libraries were prepared with genomic DNA isolated as described above using a Nextera XT DNA library preparation kit (Illumina) following the manufacturer’s instructions. The library preparations were sequenced with a MiSeq Desktop Sequencer (Illumina) and the resulting short sequence reads were trimmed for quality and removal of adapter sequences, and subsequently de novo assembled with CLC bio Genomics Workbench version 8.0.1 (CLC bio). The draft genome sequences have been deposited in the NCBI Whole Genome Shotgun database with accession numbers LIPR00000000, LIPS00000000, LIQM00000000, LIQT00000000, LIRG00000000 and LIRH00000000, respectively. Other genome sequences utilized in the study were obtained from GenBank.

In silico calculation of estimated genomic DNA–DNA hybridization (DDH) values between the genome sequences of these six type strains was calculated using formula 2 of GGDC 2.0 (Auch et al., 2010a, b; Meier-Kolthoff et al., 2013) at http://ggdc.dsmz.de.

**Results and discussion**

The phylogenetic relationship among the green-spored *Streptomyces* strains under investigation on the basis of the partial sequences of five housekeeping gene loci can be seen in Fig. 1. The larger phylogenetic tree encompassing all 167 strains studied can be seen in Fig. S1 (available in the online Supplementary Material). The green-spored strains form three distinct multi-strain clusters containing closely related strains as well as five single-member nodes representing the most recently described species *S. chlorus* KACC 20902\(^T\), *S. herbasceus* NRRL B-59128\(^T\), *S. incanus* NRRL B-59129\(^T\), *S. pratens* NRRL B-59131\(^T\) and *S. viridis* KACC 21003\(^T\) is > 0.007, as can be seen in Table 2, confirming their status as distinct species on the basis of the guidelines proposed by Rong and Huang (2012).

The phylogenetic position of *S. emeiensis* NRRL B-24621\(^T\) in Fig. 1 suggests that it represents a later synonym of *S. prasinopilosus* NRRL B-2711\(^T\), with the strains containing the identical allele of the *rpoB* locus and exhibiting only 7 bp differences over the entire 2484 bp alignment, predominantly in the *recA* locus. The synonymy of these species is supported further by the MLSA distance of 0.003 observed between these type strains (Table 2).

The MLSA distance between the three multi-strain clades and each of the single-member nodes containing *S. chlorus* KACC 20902\(^T\), *S. herbasceus* NRRL B-59128\(^T\), *S. incanus* NRRL B-59129\(^T\), *S. pratens* NRRL B-59131\(^T\) and *S. viridis* KACC 21003\(^T\) is > 0.007, as can be seen in Table 2, confirming their status as distinct species on the basis of the guidelines proposed by Rong and Huang (2012).

The in silico genomic DDH values determined on the GGDC server at http://ggdc.dsmz.de between the type strains in the *S. hirsutus*, *S. prasinopilosus* and *S. prasinus* clades, as shown in Table 3, confirm the species synonymies proposed based on the phylogenetic relationships observed in Fig. 1 and the MLSA distances calculated from the partial sequences of the five housekeeping genes (Table 2). *S. hirsutus* NRRL B-2713\(^T\) and *S. cyanoalbus* NRRL B-3040\(^T\) have an estimated DDH relatedness of 81.2 %, *S. prasinopilosus* NRRL B-2711\(^T\) and *S. emeiensis* NRRL B-24621\(^T\) have an estimated DDH relatedness of 92.1 %, and *S. prasinus* NRRL B-2712\(^T\) and *S. bambergiensis* NRRL B-12521\(^T\) have an estimated DDH relatedness of 82.1 % to each other while exhibiting less than an estimated DDH relatedness of 57 % to the other four strains for which draft genomes had been determined.

Unidentified *Streptomyces* strains from the ARS Culture collection that produced green, hairy- to spiny-surfaced spores in spiral chains were easily identified to the species level based on the sequences of their five housekeeping loci, particularly because these data had been validated based on the in silico DDH results from the draft genome sequences of the six type strains discussed above. Based on the phylogenetic position observed in Fig. 1 as well as an MLSA distance of 0.001 from the type strain of *S. hirsutus* NRRL B-2713\(^T\) (Table 2), *Streptomyces* sp. NRRL S-1728 is identified as a strain of *S. hirsutus*. Likewise, strains NRRL F-3135, NRRL
S-1099, NRRL S-1729 and NRRL S-1731 are identified as strains of *S. prasinus* based on their phylogenetic position in Fig. 1 and an observed MLSA distance < 0.004 to *S. prasinus* NRRL B-2712\(^T\) (Table 2). Strain NRRL F-4655 is identified as a strain of *S. prasinopilosus* based on the phylogenetic position in Fig. 1 and MLSA distance of 0.001 to *S. prasinopilosus* NRRL B-2711\(^T\) (Table 2).

**Taxonomic proposals**

Most of the species in the present study were originally described in the 1950s and subsequently studied in the International *Streptomyces* Project. The primary reliance on morphological properties and minimal physiological characteristics for species discrimination at that time did not facilitate recognition that strains might belong to the same genomic species. As the cost and complexity of preparing genome sequences continue to decline, it should be reasonable to require that a draft genome sequence accompany all future proposals for novel species within the genus *Streptomyces*. On the basis of MLSA and *in silico* genomic DDH as well as cultural and morphological properties, however, six of the species in the *S. hirsutus* clade could be reduced to three species. According to Rule 38 of the Bacteriological Code (Lapage et al., 1992), the oldest legitimate species name has priority over the names of other species, and should be retained for the merged species. The emended species descriptions follow.

**Emended description of *S. hirsutus* Ettlinger, Corbas & Hüttner 1958\(^{AL}\)**

*Streptomyces hirsutus* (hir.su’tus. L. masc. adj. *hirsutus* shaggy, bristly, with stiff hairs).

Has the following later heterotypic synonym: *S. cyanoalbus* (Krasil’nikov & Agre 1960) Pridham 1970\(^{AL}\).

Flexuous chains of spores forming open spirals to hooks and loops are formed (*Retinaculapietrita* or *Spirales*). Well-developed spirals of one or more turns are rare. Mature spore chains generally have 10–30 spores per chain. Spore surface ornamentation is spiny to hairy. This morphology is observed on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine agar. The colour of the aerial spore mass is grey-green (*Green colour series*) on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine agar. The substrate mycelium is...
<table>
<thead>
<tr>
<th>Strain</th>
<th>MLSA (Kimura 2-parameter) distance</th>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>3</td>
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<tr>
<td>4</td>
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</tr>
<tr>
<td>5</td>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
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<td>20</td>
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Greyish yellow to greyish yellow-green on these media. Substrate mycelium pigment is not a pH indicator. Melanoid pigments are not produced on peptone-yeast extract-iron agar or tyrosine medium. Soluble pigments are not produced. L-Arabinose, D-fructose, D-glucose, i-inositol, D-mannitol, rhamnose and D-xylose are utilized for growth. Utilization of raffinose for growth is variable.

The type strain is NRRL B-2713T (=ATCC 19773T =CBS 511.68T =DSM 40095T =ETH 1666T =NBRC 12786T =ISP 5095T =JCM 4191T =JCM 4587T =RIA 1053T).

Draft genome sequence accession number is LIQT00000000.

**Emended description of *S. prasinopilosus* Ettlinger, Corbas & Hütter 1958**

*S. prasinopilosus* (pra.si.no.pi.lo’sus. L. adj. prasi-nus green; L. adj. pilo.sus hairy; N.L. masc. adj. prasinopilosus green and hairy).

Has the following later heterotypic synonym: *S. emeiensis* Sun, Huang, Zhang & Liu 2007, 1637.

Flexuous chains of spores forming open spirals to hooks and loops are formed (*Retinaculiaperti* or *Spirales*). Spirals are poorly developed and show only up to three turns; loops and hooks are of small diameter on short spore chains, and thus are not typical of well-developed *Retinaculiaperti* spore chains. Mature spore chains are moderately short with three to 10 or sometimes more than 10 spores per chain. This morphology is observed on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine agar. The aerial spore mass is grey-green to green (Green colour series) on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine agar. Spore surface ornamentation is spiny to hairy. Substrate mycelium is yellowish to greyish yellow-green. Substrate mycelium pigment is not a pH indicator. Melanoid pigments are not produced on peptone-yeast extract-iron agar or tyrosine medium. Soluble pigments are not produced. L-Arabinose, D-fructose, D-glucose, i-inositol, D-mannitol and rhamnose are utilized for growth. Utilization of raffinose and D-xylose for growth is variable.

The type strain is NRRL B-2711T (=ATCC 19799T =CBS 551.68T =DSM 40098T =ETH 13765T =NBRC 12809T =ISP 5098T =JCM 4207T =JCM 4404T =NCIB 9842T =RIA 1078T).

Draft genome sequence accession number is LIQR0000000.

**Emended description of *S. prasinus* Ettlinger, Corbas & Hütter 1958**

*S. prasinus* (pra.si.nus. L. masc. adj. prasinus green).

Has the following later heterotypic synonym: *S. bambergiensis* Wallhauer, Nesemann, Prave & Steigler 1966.

Flexuous chains of spores forming open spirals to hooks and loops are formed (*Retinaculiaperti* or *Spirales*). Spirals are poorly developed and show only up to three turns; loops and hooks are of small diameter on short spore chains, and thus are not typical of well-developed *Retinaculiaperti* spore chains. Mature spore chains are moderately short with three to 10 or sometimes more than 10 spores per chain. This morphology is observed on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine agar. The aerial spore mass is grey-green to green (Green colour series) on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine agar. Spore surface ornamentation is spiny to hairy. Substrate mycelium is yellowish to greyish yellow-green. Substrate mycelium pigment is not a pH indicator. Melanoid pigments are not produced on peptone-yeast extract-iron agar or tyrosine medium. Soluble pigments are not produced. L-Arabinose, D-fructose, D-glucose, i-inositol, D-mannitol and rhamnose are utilized for growth. Utilization of L-arabinose for growth is variable. Growth is not observed on raffinose.

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**Table 3.** Comparison of DDH estimate values (lower left triangle) determined from draft genome sequences using formula 2 of GGDC 2.0 (Auch, et al., 2010a, b; Meier-Kolthoff et al., 2013) versus MLSA distances (Rong & Huang, 2012) calculated from the five housekeeping gene alignment for the same strain pairs (upper right triangle)

<table>
<thead>
<tr>
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<th>NRRL B-12521T</th>
<th>NRRL B-2712T</th>
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<th>NRRL B-24621T</th>
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<td><em>S. prasinopilosus</em> NRRL B-2711T</td>
<td>56.6</td>
<td>56.4</td>
<td>–</td>
<td>0.003</td>
<td>0.039</td>
<td>0.039</td>
<td>0.059</td>
</tr>
<tr>
<td><em>S. emeiensis</em> NRRL B-24621T</td>
<td>56.4</td>
<td>56.3</td>
<td>92.1</td>
<td>–</td>
<td>0.040</td>
<td>0.040</td>
<td>0.061</td>
</tr>
<tr>
<td><em>S. caioalbus</em> NRRL B-3040T</td>
<td>50.2</td>
<td>50.4</td>
<td>49.2</td>
<td>49.3</td>
<td>–</td>
<td>0.002</td>
<td>0.053</td>
</tr>
<tr>
<td><em>S. hirsutus</em> NRRL B-2713T</td>
<td>51.0</td>
<td>51.0</td>
<td>49.3</td>
<td>49.2</td>
<td>81.2</td>
<td>–</td>
<td>0.052</td>
</tr>
<tr>
<td><em>S. ghanaensis</em> ATCC 14672T</td>
<td>32.4</td>
<td>32.5</td>
<td>32.9</td>
<td>33.0</td>
<td>32.4</td>
<td>32.7</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 3.** Comparison of DDH estimate values (lower left triangle) determined from draft genome sequences using formula 2 of GGDC 2.0 (Auch, et al., 2010a, b; Meier-Kolthoff et al., 2013) versus MLSA distances (Rong & Huang, 2012) calculated from the five housekeeping gene alignment for the same strain pairs (upper right triangle)
The type strain is NRRL B-2712T (=ATCC 1980T =CBS 552.68T =DSM 40099T =ETH 13815T =NBRC 12810T =ISP 5099T =JCM 4192T =JCM 4603T =RITA 1079T).

Draft genome sequence accession number is LIRH0000000.

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References


