Taxonomic evaluation of *Streptomyces albus* and related species using multilocus sequence analysis and proposals to emend the description of *Streptomyces albus* and describe *Streptomyces pathocidini* sp. nov.

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In phylogenetic analyses of the genus *Streptomyces* using 16S rRNA gene sequences, *Streptomyces albus* subsp. *albus* NRRL B-1811^T forms a cluster with five other species having identical or nearly identical 16S rRNA gene sequences. Moreover, the morphological and physiological characteristics of these other species, including *Streptomyces almquistii* NRRL B-1685^T, *Streptomyces flocculus* NRRL B-2465^T, *Streptomyces gibsonii* NRRL B-1335^T and *Streptomyces rangoonensis* NRRL B-12378^T are quite similar. This cluster is of particular taxonomic interest because *Streptomyces albus* is the type species of the genus *Streptomyces*. The related strains were subjected to multilocus sequence analysis (MLSA) utilizing partial sequences of the housekeeping genes *atpD*, *gyrB*, *recA*, *rpoB* and *trpB* and confirmation of previously reported phenotypic characteristics. The five strains formed a coherent cluster supported by a 100 % bootstrap value in phylogenetic trees generated from sequence alignments prepared by concatenating the sequences of the housekeeping genes, and identical tree topology was observed using various different tree-making algorithms. Moreover, all but one strain, *S. flocculus* NRRL B-2465^T, exhibited identical sequences for all of the five housekeeping gene loci sequenced, but NRRL B-2465^T still exhibited an MLSA evolutionary distance of 0.005 from the other strains, a value that is lower than the 0.007 MLSA evolutionary distance threshold proposed for species-level relatedness. These data support a proposal to reclassify *S. almquistii*, *S. flocculus*, *S. gibsonii* and *S. rangoonensis* as later heterotypic synonyms of *S. albus* with NRRL B-1811^T as the type strain. The MLSA sequence database also demonstrated utility for quickly and conclusively confirming that numerous strains within the ARS Culture Collection had been previously misidentified as subspecies of *S. albus* and that *Streptomyces albus* subsp. *pathocidicus* should be redescribed as a novel species, *Streptomyces pathocidini* sp. nov., with the type strain NRRL B-24287^T.

The characterization and systematics of the species of the genus *Streptomyces* has evolved from a largely morphology-based classification system, with attempts to fine-tune these methods through cooperative projects such as the International Streptomyces Project. This project, however, did not attempt to identify species that were likely to be synonymous. The application of numerical taxonomy to classification of members of the genus *Streptomyces* (Williams *et al.*, 1983) attempted to bring some order to the confused state of taxonomy of the genus through the evaluation of significantly more phenotypic traits than the classical morphology-based classification, and although it succeeded in grouping phenotypically related strains for further investigation, this approach still was not a useful solution to the problem. The introduction of classification schemes based on molecular biological tools provided meaningful insights into species relationships in the genus *Streptomyces*. Whole-genomic DNA relatedness determinations among species of the genus *Streptomyces* (Labeda &

Abbreviation: MLSA, multi-locus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the sequences of five housekeeping genes from 11 strains of the genus *Streptomyces* examined in this study are listed in Table 2.

A supplementary figure and two supplementary tables are available with the online version of this paper.

Table 1. Strains used in the present study and their provenance

Species	Strain	Provenance
Streptomyces albus subsp. *albus*	NRRL B-1811^T	ATCC 3004 ← Waksman 3004
S. albus subsp. *albus*	NRRL B-2208^T	Waksman 3004
'*Streptomyces albus* subsp. *cobaltofaciens*'	NRRL B-3902	Lederle Laboratories AC-630
'*Streptomyces albus* subsp. *cretosus*'	NRRL B-1812	ATCC 3005 ← CBS 137.21
'*Streptomyces albus* subsp. *ochroleucus*'	NRRL B-1813	ATCC 3006
Streptomyces albus subsp. *pathocidicus*	NRRL B-24287^T	DSM 40799 ← Lederle Laboratories BK-513
Streptomyces almquistii	NRRL B-1685^T	Waksman Collection ←ATCC 618
Streptomyces flocculus	NRRL B-2465^T	Waksman 3863 ← ETH 24454← Nicot 373
Streptomyces gibsonii	NRRL B-1335^T	ATCC 6852 ←NCTC 4575
Streptomyces rangoonensis	NRRL B-12378^T	ISP 5452←ATCC 6860
Streptomyces willmorei	NRRL B-1332^T	ATCC 6867← NCTC 1856 ← J. Willmore

Lyons, 1991a, b; Labeda, 1992, 1993, 1998) provided evidence that the numerical taxonomic schemes were not always valid, but the onerous nature of these determinations precluded their widespread usage for routine identification of novel species. A comprehensive phylogenetic analysis of all species within the family *Streptomycetaceae* with validly published names based on the sequence of the 16S rRNA gene has been published (Labeda *et al.*, 2012) and although it was not possible to assess the evolutionary relationships among all species within the genus due to the highly conserved nature of the gene sequenced, the nearest neighbouring taxa to an individual species could be easily determined. The use of multi-locus sequence analysis (MLSA) in systematics of the genus *Streptomyces* has been pioneered by the studies at the Institute of Microbiology, Chinese Academy of Sciences by Dr Y. Huang and her colleagues (Guo *et al.*, 2008; Rong *et al.*, 2009; Rong & Huang, 2010, 2012) and has also been applied to the study of phytopathogenic species of the genus *Streptomyces* by Labeda (2011) as well as homologous recombination in species of the genus *Streptomyces* by Doroghazi & Buckley (2010). This technique was found to be extremely valuable in determining species-level relationships because of the increased phylogenetic signal available in even partial sequences of single-copy housekeeping-protein-coding genes.

It has been observed in the phylogenetic study of the family *Streptomycetaceae* based on sequences of the 16S rRNA gene (Labeda *et al.*, 2012) that several species, including *Streptomyces almquistii*, *Streptomyces flocculus*, *Streptomyces gibsonii* and *Streptomyces rangoonensis*, are very closely related phylogenetically to *Streptomyces albus* subsp. *albus*, the type species of the genus. The 16S rRNA gene sequence similarity between *S. albus* subsp. *albus* NRRL B-1811^T and *S. almquistii* NBRC 13015^T, *S. gibsonii* LMG 19912^T and *S. rangoonensis* NBRC 13078^T is 99.93 % and the similarity between *S. flocculus* NRRL B-2465^T and the rest of the clade members is 99.73 % based on searches in EzTaxon-e (Kim *et al.*, 2012). Kämpfer (2012) made a similar observation in the description of these species in his recent chapter in Bergey's Manual of Systematic Bacteriology. In the present investigation, an MLSA study was carried out utilizing the type strains of the species within the *S. albus* subsp. *albus* 16S rRNA gene clade [i.e. Clade 126 of Labeda *et al.* (2012)] along with the type strain of *Streptomyces albus* subsp. *pathocidicus* NRRL B-24287^T and other strains designated as subspecies of *S. albus* within the ARS Culture Collection in order to clarify their taxonomic status.

Table 2. Gene sequences of species of the genus *Streptomyces* deposited for the present study

Species	Strain	*atpD*	*gyrB*	*recA*	*rpoB*	*trpB*
S. albus subsp. *albus*	NRRL B-1811^T	JX486035	JX486040	JX486045	JX486050	JX486055
S. albus subsp. *albus*	NRRL B-2208^T	KF528055	KF528056	KF528057	KF528058	KF528059
'*S. albus* subsp. *cobaltofaciens*'	NRRL B-3902	KC965070	KC965078	KC965086	KC965094	KC965102
'*S. albus* subsp. *cretosus*'	NRRL B-1812	KC965066	KC965074	KC965082	KC965090	KC965098
'*S. albus* subsp. *ochroleucus*'	NRRL B-1813	KC965067	KC965075	KC965083	KC965091	KC965099
S. albus subsp. *pathocidicus*	NRRL B-24287^T	KC965072	KC965080	KC965086	KC965096	KC965104
S. almquistii	NRRL B-1685^T	JX486034	JX486039	JX486044	JX486049	JX486054
S. flocculus	NRRL B-2465^T	JX486036	JX486041	JX486046	JX486051	JX486056
S. gibsonii	NRRL B-1335^T	JX486033	JX486038	JX486043	JX486048	JX486053
S. rangoonensis	NRRL B-12378^T	JX486032	JX486037	JX486042	JX486047	JX486052
S. willmorei	NRRL B-1332^T	KC965065	KC965073	KC965081	KC965089	KC965097

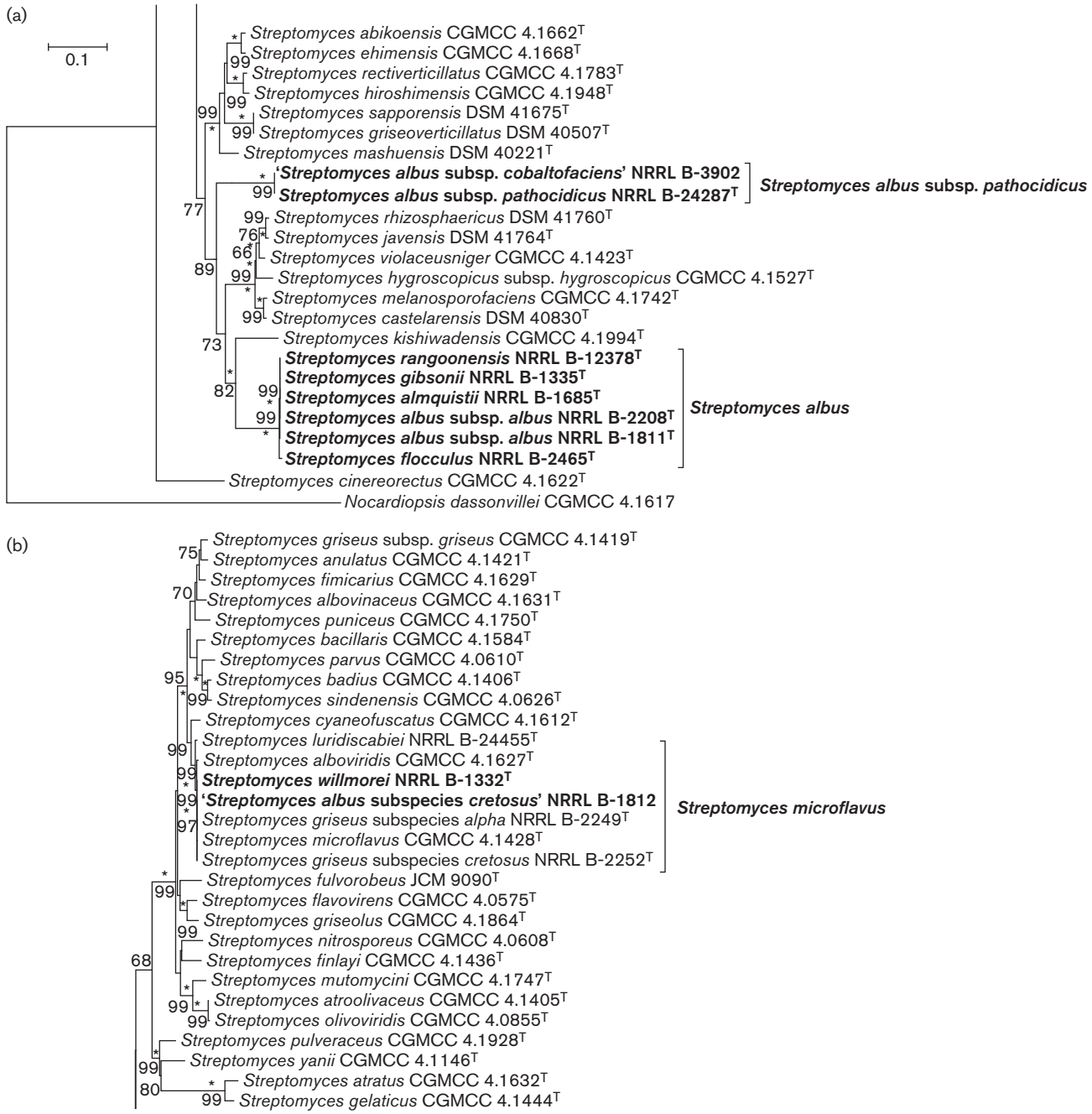
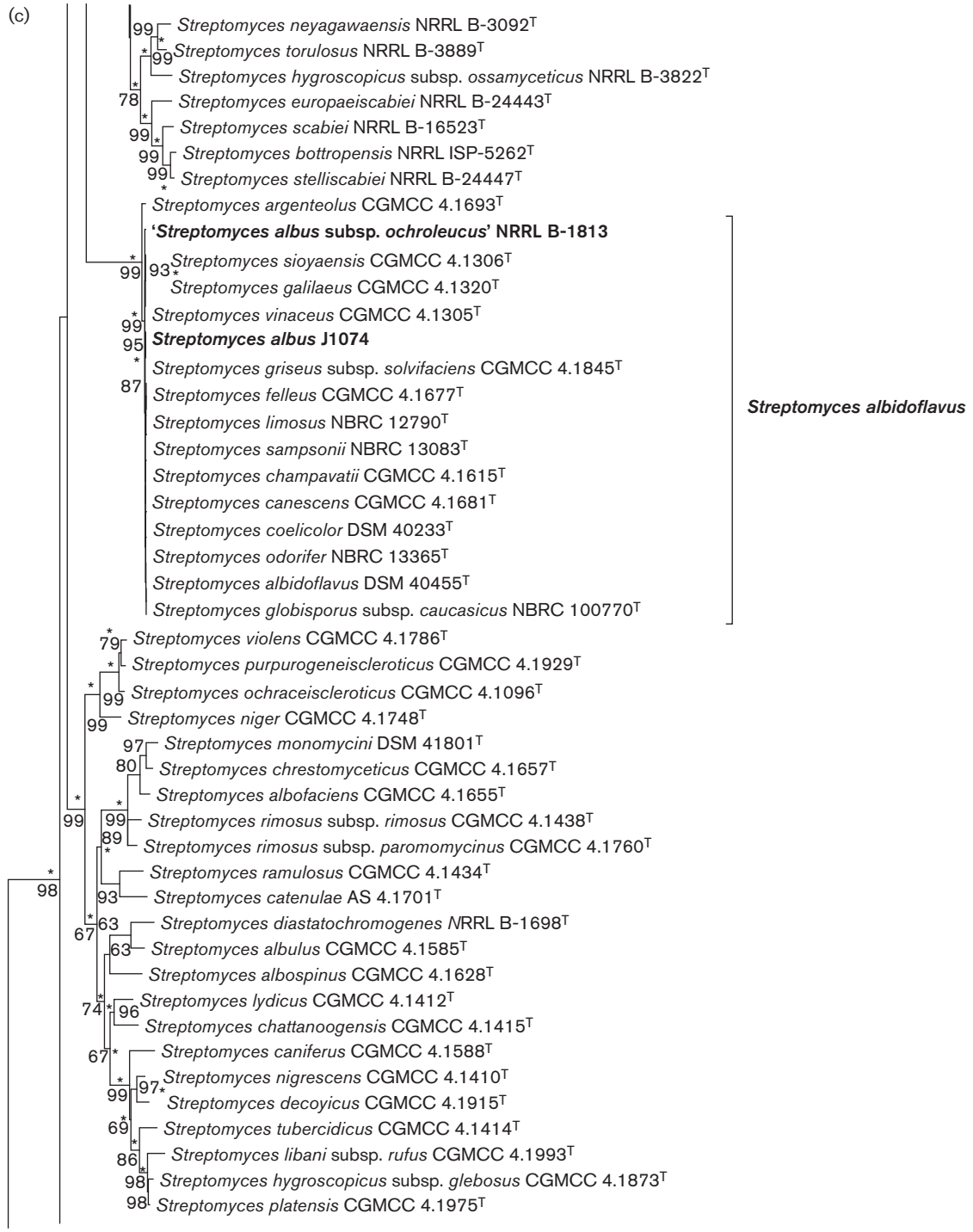


Fig. 1. Subsections of the phylogenetic tree inferred in MEGA 5.1 (Tamura *et al*., 2011) using the maximum-likelihood method based on the general time reversible model (Nei & Kumar, 2000). There were 2488 positions in the final dataset. Trees were also inferred using the evolutionary distance method (Tamura & Nei, 1993) with the neighbour-joining algorithm of Saitou & Nei (1987), and the neighbour-joining and maximum-parsimony models in MEGA 5.1 and branches conserved in all methods are marked with asterisks. Percentages at the nodes represent levels of bootstrap support from 1000 resampled datasets (Felsenstein, 1985) with values less than 60 % not shown. The strains within the *Streptomyces albus* subsp. *albus* clade and all other valid and invalid subspecies of *Streptomyces albus* are indicated with bold node labels. Bar, 0.1 substitutions per site. (a) Subtree showing the phylogenetic positions of species in the *S. albus* subsp. *albus* clade and *Streptomyces albus* subsp. *pathocidicus*. (b) Subtree showing the phylogenetic position of '*Streptomyces albus* subsp. *cretosus*' NRRL B-1812 and *Streptomyces willmorei* NRRL B-1332 confirming their identity as strains of *Streptomyces microflavus*. (c) Subtree showing the phylogenetic position of '*Streptomyces albus* subsp. *ochroleucus*' NRRL B-1813 and *Streptomyces albidoflavus* J1074 and demonstrating their identity as strains of *Streptomyces albidoflavus.*



METHODS

The strains used in the study were obtained from the ARS Culture Collection, Peoria, IL, 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 all strains using UltraClean Microbial DNA isolation kits (MoBio Laboratories) following the instructions of the manufacturer. The 16S rRNA gene of *S. albus* subsp. *albus* NRRL B-1811^T was amplified and sequenced as described by Labeda *et al.* (2012) and partial sequences of the housekeeping genes *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit) and *rpoB* (RNA polymerase beta subunit) were amplified and sequenced using the primers and protocols described previously by Guo *et al.* (2008) and Rong et *al.* (2009). Modified primers shown in Table S1, available in the online Supplementary Material, were designed for the amplification and sequencing of the housekeeping genes *recA* (recombinase A) and *trpB* (tryptophan synthetase, beta subunit) because the previously described primers did not work adequately for the species of the genus *Streptomyces* being studied.

Table 3. MLSA distance values for selected strains in this study

Strains: 1, *Streptomyces microflavus* CGMCC 4.1428^T; 2, '*Streptomyces albus* subsp. *cretosus*' NRRL B-1812; 3, *Streptomyces willmorei* NRRL B-1332^T; 4, *Streptomyces albidoflavus* DSM 40455^T; 5, '*Streptomyces albus* subsp. *ochroleucus*' NRRL B-1813; 6, *Streptomyces albus* J1074; 7, *Streptomyces albus* subsp. *pathocidicus* NRRL B-24287^T; 8, '*Streptomyces albus* subsp. *cobaltofaciens*' NRRL B-3902; 9, *Streptomyces rangoonensis* NRRL B-12378^T; 10, *Streptomyces gibsonii* NRRL B-1335^T; 11, *Streptomyces almquistii* NRRL B-1685^T; 12, *Streptomyces albus* subsp. *albus* NRRL B-1811^T; 13, *Streptomyces albus* subsp. *albus* NRRL B-2208^T; 14, *Streptomyces flocculus* NRRL B-2465^T. Bold type indicates distances below the threshold for classification of strains as representatives of distinct species.

Strain	MLSA distance (Kimura two-parameter)												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1													
2	**000.0**												
3	**000.0**	**000.0**											
4	0.115	0.114	0.114										
5	0.113	0.112	0.112	**0.003**									
6	0.115	0.115	0.115	**0.001**	**0.002**								
7	0.137	0.137	0.137	0.135	0.135	0.136							
8	0.137	0.137	0.137	0.135	0.135	0.136	**0.000**						
9	0.128	0.128	0.128	0.144	0.143	0.145	0.132	0.132					
10	0.128	0.128	0.128	0.144	0.143	0.145	0.132	0.132	**0.000**				
11	0.128	0.128	0.128	0.144	0.143	0.145	0.132	0.132	**0.000**	**0.000**			
12	0.128	0.128	0.128	0.144	0.143	0.145	0.132	0.132	**0.000**	**0.000**	**0.000**		
13	0.128	0.128	0.128	0.144	0.143	0.145	0.132	0.132	**0.000**	**0.000**	**0.000**	**0.000**	
14	0.133	0.132	0.132	0.145	0.144	0.145	0.135	0.135	**0.005**	**0.005**	**0.005**	**0.005**	**0.005**

PCR conditions were those described by Guo *et al.* (2008) and Rong *et al.* (2009). Amplified products were purified using ExoSAP-IT (Affymetrix) and sequenced using BigDye 3.1 on an ABI model 3730 sequencer in the National Center for Agricultural Utilization Research (NCAUR) core sequencing facility.

Sequence data for the five housekeeping loci for each strain were deposited in GenBank (Table 2) and the multilocus sequences for these and those in the literature were also organized using version 1.5.1 of the Bacterial Isolate Genomic Sequence Database (BIGSdb) software package (Jolley & Maiden, 2010) that is publicly available on the ARS Microbial Genomic Sequence Database server (http://ars.usda.gov/amgsdb). The sequences for all loci for each strain were concatenated head to tail in-frame and exported in FASTA format, providing a dataset of 153 strains and 2488 positions. Sequences were aligned with MUSCLE (Edgar, 2004) in MEGA 5.1 (Tamura *et al.*, 2011) and phylogenetic relationships were reconstructed using the maximum-likelihood algorithm based on the general time reversible model (Nei & Kumar, 2000), that had been determined to be the optimal model for these data using jmodeltest2 (Darriba *et al.*, 2012; Guindon & Gascuel, 2003). Trees were also inferred using the evolutionary distance method (Tamura & Nei, 1993) with the neighbour-joining algorithm of Saitou & Nei (1987) and maximum-parsimony models in MEGA 5.1. Bootstrap support for all analyses was determined from 1000 resampled datasets (Felsenstein, 1985). *Nocardiopsis dassonvillei* NRRL B-16336 (=CGMCC 4.1617), formerly classified as *Streptomyces flavidofuscus* (Tamura *et al.*, 2008), was used as the outgroup for all phylogenetic analyses. The full phylogenetic tree resulting from these analyses can be seen in Fig. S1. MLSA evolutionary distances were determined using MEGA 5.1 to calculate the Kimura two-parameter distances (Kimura 1980) as shown in Table 2. Strain pairs having less than 0.007 MLSA distance were considered conspecific based on the guideline empirically determined by Rong & Huang (2012) to be the distance that equates to 70 % DNA–DNA homology.

Morphological and physiological properties of the strains in the *S. albus* 16S rRNA phylogenetic clade were confirmed using the methods of the International Streptomyces Project (Shirling & Gottlieb, 1966). In addition, tolerance to NaCl was evaluated using YM agar to which 2 %, 2.5 %, 3 %, 5 % or 7 % (w/v) NaCl was added prior to sterilization. Additional physiological properties were evaluated using ApiCoryne and ApiZym test strips (bioMérieux) following the manufacturer's instructions.

RESULTS AND DISCUSSION

The close phylogenetic relationship of the species *S. albus* subsp. *albus*, *S. almquistii*, *S. flocculus*, *S. gibsonii* and *S. rangoonensis* described in the reports of Labeda *et al.* (2012) and Kämpfer (2012) was confirmed by the results of the phylogenetic analyses based on the 2488 bp alignment of the concatenated partial sequences of the housekeeping genes *atpD*, *gyrB*, *recA*, *rpoB* and *trpB* as can be seen in Fig. 1a. The second isolate of the type strain of *Streptomyces albus* held in the ARS Culture Collection, NRRL B-2208^T, was included in the study to confirm identity with NRRL B-1811^T and it was observed to contain identical alleles for all five housekeeping loci. The other species formed a very tight cluster which is extremely well supported by high bootstrap values and similar tree topology was observed in phylogenetic trees reconstructed using the neighbour joining and maximum-parsimony algorithms (not shown). *S. flocculus* NRRL B-2465^T is slightly distant from the other species in this cluster, whose sequences for all of the housekeeping genes are 100 % similar, but the differences amount to only 13 bp over the total alignment of 2488 bp

(4 bp in *atpD*, 2 bp in *gyrB*, 2 bp in *recA*, 2 bp in *rpoB* and 3 bp in *trpB*). These differences are reflected as an MLSA distance of 0.005 from the other strains in this clade, as can be seen in Table 3, and this value is less than the species level threshold of 0.007 proposed by Rong & Huang (2012). All of these strains were observed to form white spores in spiral chains, did not produce melanin pigments from tyrosine and had physiological properties that were also very similar, as shown in Table S2, supporting the conclusion based on MLSA distances that *S. albus*, *S. almquistii*, *S. flocculus*, *S. gibsonii* and *S. rangoonensis* should be considered as a single species with the name *Streptomyces albus* having priority. The other species names should be classified as later heterotypic synonyms.

The phylogenetically distant position of *S. albus* subsp. *pathocidicus* NRRL B-24287^T relative to the *S. albus* subsp. *albus* clade (see Fig. 1a.) demonstrates that it cannot represent a subspecies of *S. albus*. Moreover, this observation is further supported by the fact that the 16S rRNA sequence similarity of this strain with *S. albus* subsp. *albus* NRRL B-1811^T is 94.3 % (51 mismatches over 1459 bp) and the MLSA distance from the *S. albus* MLSA clade (Table 3) is 0.132, well above the species-definitive MLSA distance of 0.007. During the course of the present investigation it was also observed that the sequences for all five of the housekeeping genes of strain NRRL B-3902, deposited in the ARS Culture Collection in 1970 as the invalid subspecies '*S. albus* subsp. *cobaltofaciens*', were identical to those of NRRL B-24287^T (MLSA distance= 0.000), thus indicating that they represent the same species. It is proposed that the taxon represented by these two strains be classified as the novel species *Streptomyces pathocidini* sp. nov. with NRRL B-24287^T as the type strain.

The growing multigene database for members of the genus *Streptomyces* proved to be a very useful tool for rapid and accurate identification of strains of questionable taxonomic status in the ARS Culture Collection. Two strains whose accession records indicated that they represented subspecies of *S. albus* for which details are unpublished were included in the present study and were conclusively identified (MLSA distance=0.000) as representatives of other species, as can be seen in Figs 1b and 1c. '*S. albus* subsp. *cretosus*' NRRL B-1812 was identified as a strain of *Streptomyces microflavus*, while '*S. albus* subsp. *ochroleucus*' NRRL B-1813 was found to be a strain of *Streptomyces albidoflavus*. The five housekeeping gene sequences for *Streptomyces willmorei* NRRL B-1332^T were also determined in the present study and the MLSA distance of 0.000 from *S. microflavus* CGMCC 4.1428^T that was observed (Table 3) confirmed the proposal by Lanoot *et al.* (2005) that this species should be considered as a later synonym of *S. microflavus*.

S. albus J1074, whose genome had been sequenced by the Broad Institute (GenBank accession number NZ_ABYC00000000), was included in this study and was found to be misidentified. The sequences for the five housekeeping gene loci were extracted from the draft genome sequence using the capabilities of the BIGSdb package for inclusion in the phylogenetic analyses. The phylogenetic position of this strain (Fig. 1c) is within *Streptomyces albidoflavus* and the MLSA distance of 0.001 to the type strain, *S. albidoflavus* DSM 40455^T, confirms its identity as a representative of this species.

The results reported in this study conclusively support a proposal to emend the description of *S. albus* to include *S. almquistii*, *S. gibsonii*, *S. flocculus* and *S. rangoonensis* as later heterotypic synonyms. The data also support the redescription of *Streptomyces albus* subsp. *pathocidicus* as a novel species for which the name *Streptomyces pathocidini* is proposed. The formal descriptions of *Streptomyces albus* and *Streptomyces pathocidini* sp. nov. follow:

Emended description of *Streptomyces albus* (Rossi Doria 1891) Waksman and Henrici 1943, 339^{AL}

Later heterotypic synonyms: *Streptomyces almquistii* (Duché 1934) Pridham *et al.* 1958^{AL}, *Streptomyces flocculus* (Duché 1934) Waksman and Henrici 1948^{AL}, *Streptomyces gibsonii* (Erikson 1935) Waksman and Henrici 1948^{AL} and *Streptomyces rangoonensis* corrig. (Erikson 1935) Pridham *et al.* 1958^{AL}.

Morphology is as described by Kämpfer (2012). Growth on D-fructose, D-galactose, D-glucose, i-inositol, D-mannitol, salicin and D-xylose as sole carbon source; variable growth on raffinose; weak to no growth on L-arabinose, rhamnose and sucrose. Good growth in the presence of up to 5 % NaCl and variable growth in the presence of 7 % NaCl. Utilizing apiCoryne and apiZym test strips, the following positive test reactions are observed: pyrolidonyl arylamidase, alkaline phosphatase, N-acetyl-glucosaminidase, esculin hydrolysis, gelatin hydrolysis, leucine arylamidase; the following negative test reactions are observed: nitrate reduction, β-glucuronidase, β-glucosidase, esterase (C4), esterase (C6), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-fucosidase; the following variable test reactions are observed: pyrazymidase (3 of 5 positive), β-galactosidase (4 of 5 positive), urease (4 of 5 positive), valine arylamidase (2 of 5 weakly positive), acid phosphatase (3 of 5 positive), α-mannosidase (1 of 5 positive). Fermentation of glucose, ribose and xylose was weak for all strains using the apiCoryne test strips. The test results for α-glucosidase varied between the apiCoryne test strips where results were variable (3 of 5 positive) and apiZym test strips where all were negative.

Type strain is NRRL B-1811^T (=ATCC 25426^T=ATCC 3004^T=CBS 410.63^T=CBS 924.69^T=BCRC 10802^T=CCUG 33990^T=CECT 3077^T=CGMCC 4.1640^T=CIP 104432^T=DSM 40313^T=HUT 6613^T=IFM 1119^T=IMET 40241^T=IMRU 3004^T=JCM 4450^T=JCM 4177^T=KCTC 1082^T=NBRC 13014^T=NBRC 3710^T=NCIMB 9558^T=NRRL B-2208^T=RIA 1206^T=VKM Ac-35^T). The GenBank/DDBJ/EMBL accession number for the 16S rRNA gene for the type strain is JX486031.

Description of *Streptomyces pathocidini* sp. nov.

Streptomyces pathocidini. (pa.tho.ci.di′ni. N.L. neut n. *pathocidini* referring to the antibiotic pathocidin).

Basonym *Streptomyces albus* subsp. *pathocidicus*.

Phenotypic description is that of Nagatsu *et al.* (1962).

Type strain is NRRL B-24287^T (=ATCC 14510^T=B-28^T=BCRC 12331^T=CCRC 12331^T=CGMCC 4.1633^T=CIP 104431^T=DSM 40799^T=JCM 4166^T=KCTC 9671^T=NBRC 13812^T=VKM Ac-598^T). The GenBank/DDBJ/EMBL accession number for the 16S rRNA gene of the type strain is AB184501.

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