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Lechevalieria nigeriaca sp. nov., isolated from arid soil

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A novel actinobacterium, designated strain NJ2035^T, was isolated from soil collected from Abuja, Nigeria and was characterized to determine its taxonomic position. The isolate was found to have chemical and morphological properties associated with members of the genus *Lechevalieria*. Phylogenetic analyses based on almost-complete 16S rRNA gene sequences indicated that the isolate was closely related to members of the genus *Lechevalieria*, and was shown to form a distinct phyletic line in the *Lechevalieria* phylogenetic tree. Strain NJ2035^T was most closely related to *Lechevalieria roselyniae* C81^T, *Lechevalieria atacamensis* C61^T and *Lechevalieria deserti* C68^T (98.5 % 16S rRNA gene sequence similarity). Sequence similarities with other members of the genus *Lechevalieria* were less than 98.2 %. The cell wall of the novel strain contained *meso*-diaminopimelic acid, and galactose, mannose and rhamnose as the diagnostic sugars. The predominant menaquinone was MK-9(H_4). The polar lipids detected were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol. DNA–DNA relatedness and phenotypic data showed that the novel isolate and *L. roselyniae* C81^T, *L. atacamensis* C61^T and *L. deserti* C68^T belong to distinct genomic species. On the basis of data from this taxonomic study using a polyphasic approach, strain NJ2035^T represents a novel species of the genus *Lechevalieria*, for which the name *Lechevalieria nigeriaca* sp. nov. is proposed. The type strain is NJ2035^T (=DSM 45680^T=KCTC 29057^T=NRRL B-24881^T).

The genus *Lechevalieria* was proposed by Labeda *et al.* (2001) for aerobic, Gram-reaction-positive actinobacteria that form an extensively branched vegetative mycelium and scant aerial hyphae on some media. Members of the genus are also characterized by a number of chemical markers, including the presence of *meso*-diaminopimelic acid in the whole-cell hydrolysate peptidoglycan (wall chemotype III *sensu* Lechevalier & Lechevalier, 1970), galactose, mannose and rhamnose as diagnostic sugars, MK-9(H_4) as the predominant menaquinone, phosphatidylethanolamine as the main polar lipid (type II *sensu* Lechevalier *et al.*, 1977), and a fatty acid profile mainly consisting of iso- and anteiso-branched fatty acids. Members of the genus *Lechevalieria* form a distinct branch in the phylogenetic tree based on 16S rRNA gene sequences of the family *Pseudonocardiaceae* (Labeda *et al.*, 2001) and can be distinguished from one another by a combination of phenotypic properties (Zhang *et al.*, 2007; Wang *et al.*, 2007; Okoro *et al.*, 2009, 2010). At the time of writing, the genus *Lechevalieria* contains seven species with validly published names isolated from environmental samples, *Lechevalieria aerocolonigenes* (Labeda, 1986, emended Labeda *et al.* 2001), *Lechevalieria flava* (Gauze *et al.*, 1974, emended Labeda *et al.* 2001), *Lechevalieria fradiae* (Zhang *et al.*, 2007), *Lechevalieria xinjiangensis* (Wang *et al.*, 2007), and *Lechevalieria atacamensis*, *Lechevalieria deserti* and *Lechevalieria roselyniae* (Okoro *et al.*, 2010).

The present investigation was designed to determine the taxonomic position of the isolate NJ2035^T by using a polyphasic approach. Strain NJ2035^{T}was isolated from SM2 agar plates (Tan *et al.*, 2006), supplemented with (+)-D-melezitose (1 %, w/v), cycloheximide (50 μg ml^{-1}), neomycin sulphate (4 μg ml^{-1}) and nystatin (50 μg ml^{-1}), after incubation at 28 °C for 21 days, following inoculation with a suspension of a soil sample collected from Abuja, Nigeria. The organism was maintained on modified Bennett's agar slopes (modified after Jones, 1949) at 4 °C and as suspensions of mycelial fragments in 20 % (v/v) glycerol at

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NJ2035^T is JN989291.

Three supplementary figures and a supplementary table are available with the online version of this paper.

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–20 °C. Biomass for chemotaxonomic and molecular systematic analyses was prepared by growing strain NJ2035^T in ISP 2 broth cultures, at 160 r.p.m. for 10 days at 28 °C; cells were harvested by centrifugation, washed twice in distilled water, recentrifuged and freeze-dried.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out following Chun & Goodfellow (1995). The almost-complete 16S rRNA gene sequence of strain NJ2035^T (1473 bp) was determined using an ABI PRISM 3730 XL automatic sequencer. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim *et al.*, 2012). Multiple alignments with sequences from closely related species was performed by using the program CLUSTAL W in the MEGA5 software package (Tamura *et al.*, 2011). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5 (Tamura *et al.*, 2011). Evolutionary distance matrices were prepared according to Jukes & Cantor (1969). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The almost-complete 16S rRNA gene sequence of strain NJ2035^T (1473 bp) was used for phylogenetic analysis. The phylogenetic tree based on the neighbour-joining algorithm showed that strain NJ2035^T was a member of the genus *Lechevalieria* and formed a distinct branch from other species of the genus *Lechevalieria*, which was also supported by a high bootstrap value (Fig. 1). The other two tree-making algorithms (maximum-likelihood and maximum-parsimony) resulted in trees showing similar topologies (Figs S1 and S2 available in IJSEM Online). Strain NJ2035^T shared 16S rRNA gene sequence similarities of 98.5 % (22 nt differences at 1471 locations), 98.5 % (22 nt differences at 1464 locations) and 98.49 % (22 nt differences at 1453 locations), with its nearest relatives, *L. roselyniae* C81^T, *L. atacamensis* C61^T and *L. deserti* C68^T, respectively. Sequence similarities with all other members of the genus *Lechevalieria* were <98.2 %.

DNA–DNA relatedness values between strain NJ2035^T and related type strains, *L. roselyniae* DSM 45481^T, *L. atacamensis* DSM 45479^T and *L. deserti* DSM 45480^T were determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ; Braunschweig, Germany). DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), incorporating the modifications described by Huß *et al.* (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The DNA–DNA relatedness values between strain NJ2035^T and *L. roselyniae* DSM 45481^T, *L. atacamensis* DSM 45479^T and *L. deserti* DSM 45480^T were 51.5 ± 1.6, 49.0 ± 2.3 and 46.6 ± 0.8 % (values are means of duplicate determinations), respectively. These values are well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of genomic species. DNA–DNA hybridization was not carried out between strain NJ2035^T and other species of the genus *Lechevalieria* because they were positioned in different clusters in the phylogenetic tree and shared relatively low 16S rRNA gene sequence similarities (98.2–97.7 %).

Amino acid and sugar analyses of whole-cell hydrolysates were performed according to the procedures described by Hasegawa *et al.* (1983). Polar lipid and respiratory quinones analyses were carried out by the Identification Service of the DSMZ. Respiratory quinones were extracted from 100 mg freeze-dried cells based on the two-stage method described by Tindall (1990a; 1990b). Respiratory quinones were separated into their different classes (menaquinones and ubiquinones) by thin layer chromatography on silica gel (Macherey-Nagel Art. no. 805 023), using hexane/tert-butylmethylether (9 : 1, v/v) as the solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse-phase column (Macherey-Nagel, 2 mm × 125 mm, 3 μm, RP18) using methanol as the eluant. Respiratory quinones were detected at 269 nm.

Cellular fatty acids were extracted, methylated and separated by gas chromatography using an Agilent Technologies 6890N instrument, fitted with an auto sampler and a 6783 injector, according to the standard protocol of the Sherlock Microbial identification (MIDI) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996), and the fatty acid methyl ester peaks were identified and quantified using TSBA 5.0 software. The DNA G + C content of the isolate was determined following the procedure of Gonzalez & Saiz-Jimenez (2005).

Chemotaxonomic analyses revealed that strain NJ2035^T displayed chemical characteristics that were consistent with those of the genus *Lechevalieria*. The cell-wall diamino acid in the peptidoglycan layer of strain NJ2035^T was *meso*-diaminopimelic acid, and the whole-cell hydrolysates contained galactose, mannose and rhamnose (major components), glucose and small amounts of ribose (cell-wall chemotype III *sensu* Lechevalier & Lechevalier, 1970). The polar lipids of strain NJ2035^T were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, two unidentified phospholipids, one aminophospholipid and three glycolipids (Fig. S3). The predominant menaquinone of strain NJ2035^T was MK-9(H_4) (80.0 %); MK-9(H_2) (11.0 %), MK-11(H_2) (5.0 %) and MK-9(H_6) (1.0 %) were also detected. The major cellular fatty acids were iso-$C_{16:0}$ (36.1 %), anteiso-$C_{15:0}$ (8.7 %), iso-$C_{14:0}$ (8.6 %), iso-$C_{15:0}$ (7.5 %), $C_{16:0}$ (7.2 %)

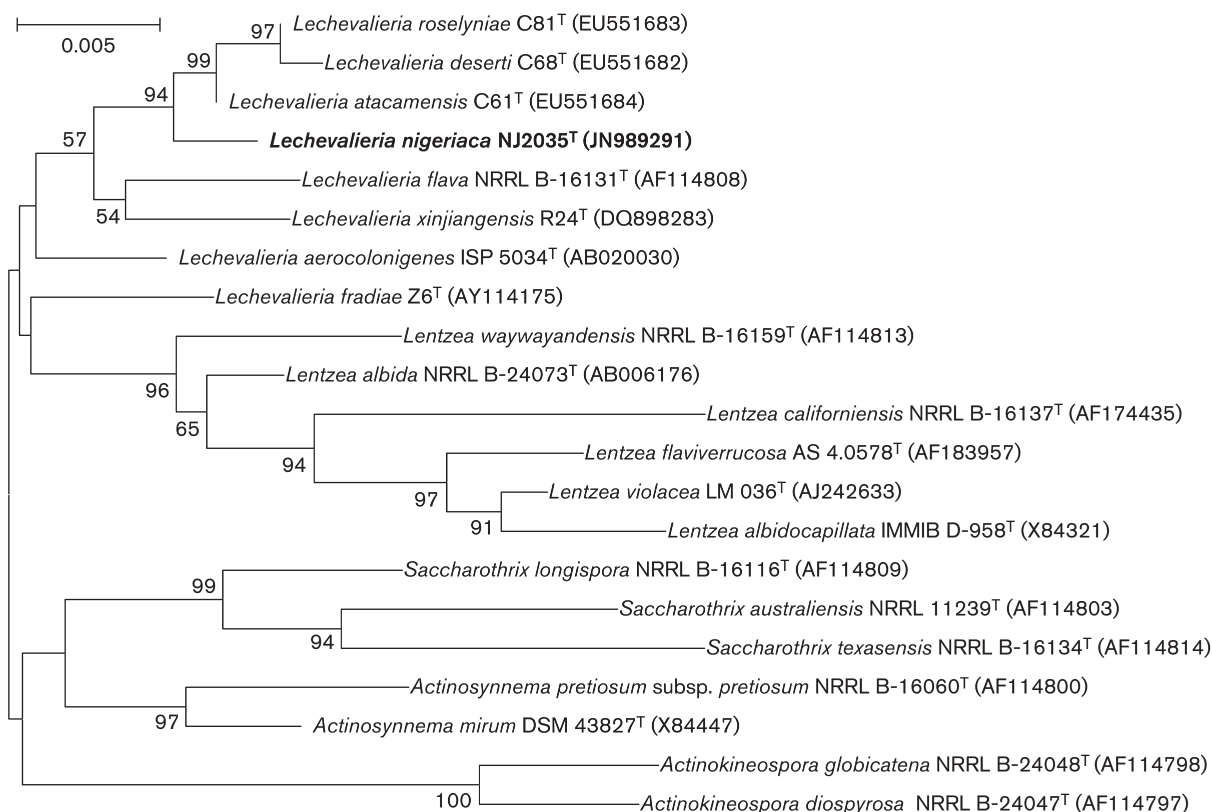

Fig. 1. Neighbour-joining phylogenetic tree (Saitou & Nei, 1987) based on a comparative analysis of 16S rRNA gene sequences, showing the relationships between strain NJ2035^T and closely related species. Numbers at nodes indicate bootstrap values (expressed as percentages of 1000 replications); only values $\geqslant$50 % are shown. GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per site.

and anteiso-$C_{17:0}$ (6.8 %). Comparative cellular fatty acid compositions of strain NJ2035^T and *L. roselyniae* C81^T, *L. atacamensis* C61^T and *L. deserti* C68^T are shown in Table S1. The DNA G+C content of strain NJ2035^T was 68.4 mol%.

Cultural characteristics were investigated on media from the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966), modified Bennett's agar (MBA; Jones, 1949), Czapek's and tryptic soy agar (TSA; Difco). The degree of growth, aerial mycelium and pigmentation were recorded after 14 days of incubation at 28 °C. The National Bureau of Standards (NBS) Colour Name Charts (Kelly, 1964) was used for determining colour designation and names. Colony morphology and micro-morphological properties of strain NJ2035^T were determined by examining gold-coated dehydrated specimens of 14-day-old cultures from ISP 3 medium using a JSM 6060 instrument (JEOL). Growth at different temperatures (4, 10, 20, 28, 30, 37, 45, 50 and 55 °C), at pH 4.0–11.0 (at intervals of 1.0 pH unit), and in the presence of NaCl (0–10 %; w/v) (at intervals of 1.0 pH unit) was determined on ISP 2. Established methods were used to determine whether the strains degraded chitin (Hsu & Lockwood, 1975), RNA (Goodfellow *et al.* 1979) and Tweens 20 and 80 (Nash & Krent, 1991); the remaining degradation tests were examined using methods described by Williams *et al.* (1983). Carbon source utilization was tested using carbon source utilization (ISP 9) medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 % of the tested carbon sources. Nitrogen source utilization was examined using the basal medium recommended by Williams *et al.* (1983) supplemented with a final concentration of 0.1 % of the tested nitrogen sources. The type strains *L. roselyniae* DSM 45481^T, *L. atacamensis* DSM 45479^T and *L. deserti* DSM 45480^T were included for comparison in all tests.

Strain NJ2035^T exhibited very good growth on tested media. The aerial mycelium was not produced on ISP 2, ISP 5, ISP 6, Czapek's or nutrient agar, while grey aerial mycelium was produced on ISP 3, ISP 4 and ISP 7 media, and light yellow aerial mycelium was produced on modified Bennett's agar medium. The strain did not produce melanoid pigments on ISP 6 or ISP 7 media. The aerial mycelium was fragmented into rod-shaped spores (0.7–1.1 μm wide × 0.8–1.7 μm long) (Fig. 2). The detailed results of the morphological and physiological tests are given in Table 1 and the species description.

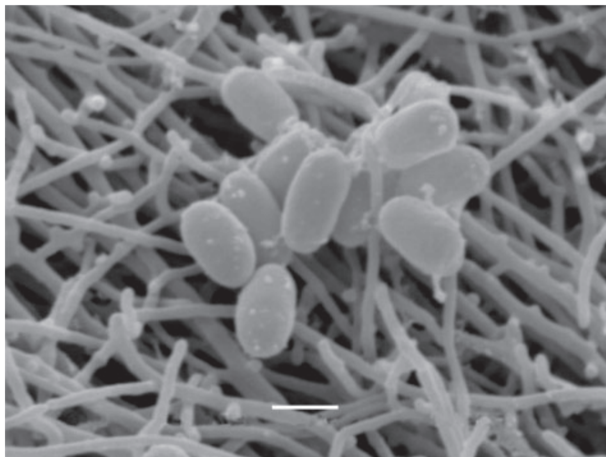

Fig. 2. Scanning electron micrograph of strain NJ2035^T grown on ISP 3 agar at 28 °C for 14 days. Bar, 1 μm.

It is evident from these genotypic and phenotypic data that strain NJ2035^T can be distinguished from its closest phylogenetic neighbours, *L. roselyniae* C81^T, *L. atacamensis* C61^T and *L. deserti* C68^T. We therefore suggest that strain NJ2035^T represents a novel species of the genus *Lechevalieria*, for which the name *Lechevalieria nigeriaca* sp. nov., is proposed.

Description of *Lechevalieria nigeriaca* sp. nov.

Lechevalieria nigeriaca (ni.ge.ri′a.ca. N.L. fem. adj. *nigeriaca* of or belonging to Nigeria).

Aerobic, Gram-reaction-positive, catalase-positive, non-motile actinomycete which forms an extensively branched substrate mycelium that fragments into rod-shaped elements. Growth occurs at 20–45 °C (optimum, 28–30 °C), pH 5.0–10.0 (optimum, pH 7.2) and in the presence of 1.0–5.0 % NaCl. Positive for aesculin hydrolysis, but negative for allantoin, arbutin and urea hydrolysis, and nitrate reduction. Elastin, hypoxanthine and Tweens 20 and 80 are degraded, but not guanine or L-tyrosine. Dextrin, cellobiose, D-galactose, D-mannitol, D-mannose, inulin, lactose, L-arabinose, L-rhamnose, maltose, starch and sucrose are used as sole carbon sources for energy and growth but adonitol, D-sorbitol and melezitose are not. Utilizes α-isoleucine, D-phenylalanine, L-alanine, L-arginine, L-cysteine, L-hydroxyproline, L-methionine, L-phenylalanine, L-valine, L-proline, L-serine and L-threonine as sole nitrogen sources, but not glycine. The predominant menaquinone is MK-9(H_4). The polar lipid profile contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol. The major fatty acids of the type strain are iso-$C_{16:0}$, anteiso-$C_{15:0}$, iso-$C_{14:0}$, iso-$C_{15:0}$, $C_{16:0}$ and anteiso-$C_{17:0}$.

The type strain, NJ2035^T (=DSM 45680^T=KCTC 29057^T=NRRLB-24881^T), was isolated from an arid soil sample collected in Abuja, Nigeria. The DNA G+C content of the type strain is 68.4 mol%.

Table 1. Phenotypic properties of strain NJ2035^T and type strains of closely related species of the genus *Lechevalieria*

Strains: 1, NJ2035^T; 2, *L. roselyniae* DSM 45481^T; 3, *L. atacamensis* DSM 45479^T; 4, *L. deserti* DSM 45480^T. All strains were positive for degradation of elastin (0.3 %) and Tweens 20 (1.0 %) and 80 (1.0 %); utilization of cellobiose, D-galactose, D-mannose, D-mannitol, dextrin, inulin, L-arabinose, L-rhamnose, lactose, maltose, starch and sucrose as sole carbon sources (1.0 %); utilization of L-alanine, L-arginine, L-hydroxyproline, L-proline, L-serine, L-threonine as sole nitrogen sources (0.1 %); and growth at pH 5, 6, 7, 8, 9 and 10, at temperatures of 28, 37 and 45 °C, and in the presence of 1, 2, 3 and 4 % NaCl. All strains were negative for hydrolysis of urea, degradation of guanine (0.05 %), utilization of melezitose as a sole carbon source (1.0 %) and glycine as a sole nitrogen source (0.1 %), and growth at pH 4 and 11, at temperatures of 4 and 50 °C, and in the presence of ≥6.0 % NaCl. All data were obtained in this study. +, Positive; −, negative.

Characteristic	1	2	3	4
Hydrolysis of:				
Aesculin	+	−	+	+
Arbutin	−	+	−	+
Allantoin	−	−	−	+
Nitrate reduction	−	+	−	−
Degradation of:				
L-Tyrosine (0.5 %)	−	+	+	+
Hypoxanthine (0.4 %)	+	−	−	−
Carbon source utilization (1.0 %)				
Adonitol	−	+	−	−
D-Sorbitol	−	−	+	−
Nitrogen source utilization (0.1 %)				
α-Isoleucine	+	−	−	−
D-Phenylalanine	+	−	−	−
L-Cysteine	+	+	−	+
L-Methionine	+	−	−	−
L-Phenylalanine	+	−	−	−
L-Valine	+	−	−	+
Growth at/with:				
10 °C	−	+	+	+
5 % NaCl	+	−	−	+

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