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ORIGINAL PAPER

Actinomadura physcomitrii sp. nov., a novel actinomycete isolated from moss [*Physcomitrium sphaericum* (Ludw) Fuernr]

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Abstract A novel endophytic actinomycete, designated strain LD22^T, was isolated from moss [*Physcomitrium sphaericum* (Ludw) Fuernr] collected from Yunnan Province, Southwest China. A polyphasic taxonomic study was carried out to establish the status of this strain. Morphological and chemotaxonomic characteristics of strain LD22^T confirmed the affiliation of the isolate to the genus *Actinomadura*. The diamino acid present in the cell wall is *meso*-diaminopimelic acid. Glucose, madurose, galactose and ribose occur in whole cell hydrolysates. The menaquinones were identified as MK-9(H_4), MK-9(H_8), MK-9(H_6) and MK-9(H_2). The polar lipid profile was found to contain diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside and an unidentified phospholipid. The major fatty acids were found to be $C_{16:0}$, 10-methyl $C_{18:0}$ and $C_{18:1}$ ω9c. The DNA G + C content of the draft genome sequence, consisting of 10.0 Mbp, was 72.5%. Phylogenetic analysis of 16S rRNA gene sequences showed that strain LD22^T belongs to the genus *Actinomadura* with the highest sequence similarity to *Actinomadura montaniterrae* CYP1-1B^T (99.2%). However, phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate formed a phyletic line with *Actinomadura rudentiformis* HMC1^T (98.6%). The low level of DNA–DNA relatedness and some different phenotypic characteristics allowed the strain to be distinguished from the above-mentioned two strains. Therefore, it is concluded that strain that strain LD22^T represents a novel species of the genus of *Actinomadura*, for which the name *Actinomadura physcomitrii* sp. nov. is proposed. The type strain is LD22^T (= CCTCC AA 2018050^T = JCM 33455^T).

Keywords Endophyte · Pigmented · Rudentiformis · Genome · *Thermomonosporaceae* · Actinobacteria · *Funariaceae* · *Funariales*

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Abbreviations

ANI	Average nucleotide identity
dDDH	Digital DNA:DNA hybridization
ISP	International *Streptomyces* Project
ISCC-NBS	Inter-society color council-national bureau of standards
SSC	Saline-sodium citrate
GC-MS	Gas chromatography-mass spectrometer
MEGA	Molecular evolutionary genetics analysis
CCTCC	China center for type culture collection
JCM	Japan collection of microorganisms

Introduction

The genus *Actinomadura* was first proposed by Lechevalier and Lechevalier (1968). Species of the genus *Actinomadura* are non-acid-alcohol-fast, non-motile actinomyces that form an extensively branched non-fragmenting substrate mycelium. Aerial mycelium are absent or moderately developed, differentiating to spore chains with various morphologies. Members of the genus *Actinomadura* contain *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell wall peptidoglycan and madurose as the characteristic sugar in whole cell hydrolysates. The predominant menaquinones are MK-9(H_4) and MK-9(H_6) and MK-9(H_8). The major fatty acids are identified as branched saturated and unsaturated fatty acids and tuberculostearic acid. The major phospholipids are characterized diphosphatidylglycerol and phosphatidylinositol (Trujillo and Goodfellow 2012). Members of the genus *Actinomadura* are widely distributed in soil. Recently, some strains were also isolated from plant tissues (Qin et al. 2009; Rachniyom et al. 2015) and marine environments (He et al. 2012; Li et al. 2019). At the time of writing, 73 species with validly published names (www.bacterio.net/actinomadura.html) have been described. Moss, as an environmental indicator plant, is widely used in various ecological fields. However, few studies have been carried out on the endophytic actinomycetes of mosses. As part of a programme to research the diversity of endophytic actinomycetes in moss, strain LD22^T was isolated from *Physcomitrium sphaericum* (Ludw) Fuernr, a species of moss. In this study, we performed a polyphasic taxonomic analysis on this strain, and propose strain LD22^T represents a new species of the genus *Actinomadura*.

Materials and methods

Isolation and maintenance of the organism

Strain LD22^T was isolated from *P. sphaericum* (Ludw) Fuernr collected in Kunming, Yunnan Province, Southwest China (25° 21′ N, 102° 92′ E). The plant sample was processed as described by Jia et al. (2013) and incubated on dulcitol-proline agar (DPA) (Liu et al. 2019) supplemented with cycloheximide (50 mg l^{-1}) and nalidixic acid (20 mg l^{-1}). After 28 days of aerobic incubation at 28 °C, the single colony was transferred and purified on International *Streptomyces* Project (ISP) 3 medium (Shirling and Gottlieb 1966) and maintained as glycerol suspensions (20%, v/v) at − 80 °C. The reference strains *Actinomadura montaniterrae* CYP1-1B^T and *Actinomadura rudentiformis* HMC1^T were obtained from Japan Collection of Microorganisms (JCM). These strains were cultured under the same conditions as reference strains for comparative analyses.

Phenotypic characterization

Morphological characteristics were observed by light microscopy (Nikon ECLIPSE E200) and scanning electron microscopy (Hitachi SU8010) with cells grown in ISP 3 medium for 28 days. Samples for scanning electron microscopy were prepared as described by Jin et al. (2019). Spore motility was assessed by light microscopic observation of cells. Cultural characteristics were observed on ISP 1-7 (Shirling and Gottlieb 1966), Czapek's agar (CA) (Waksman 1967), modified Bennett's agar (MBA) (Jones 1949) and Nutrient agar (NA) (Waksman 1961) after 14 days at 28 °C. Colour determination was done by using colour chips from the ISCC-NBS colour charts standard samples No 2106 (Kelly 1964). Growth at different temperatures (10, 15, 20, 25, 28, 30, 37, 40, 45 and 50 °C) was determined on ISP 3 medium after incubation for 14 days. The pH range for growth (pH 3.0–12.0, at intervals of 1 pH unit) was tested in ISP 2 broth using the buffer system described by Zhao et al. (2019). Tolerance of various NaCl concentrations (0–10.0%, with an interval of 1%, w/v) was tested in ISP 2 broth as the basal medium at 28 °C for 14 days in shake flasks (250 rpm). Hydrolysis of Tweens (20, 40 and 80) and production of urease were tested as described by Smibert and Krieg (1994). The

utilisation of sole carbon and nitrogen sources, decomposition of cellulose, hydrolysis of starch and aesculin, reduction of nitrate, coagulation and peptonisation of milk, liquefaction of gelatin and production of H_2S were examined as described previously (Gordon et al. 1974; Williams et al. 1989; Yokota et al. 1993).

Chemotaxonomic analyses

Biomass for chemotaxonomic studies was prepared by growing the strain in GY broth (Jia et al. 2013) in Erlenmeyer flasks for 5 days at 28 °C with shaking at 250 rpm. Cells were harvested by centrifugation, washed twice with distilled water and freeze-dried. The isomer of diaminopimelic acid in the cell wall was derivatised according to McKerrow et al. (2000) and analysed by the HPLC method described by Liu et al. (2013). Analysis of the muramic acid residue *N*-acyl type was determined using the method of Uchida and Aida (1984). The whole cell sugars were analysed according to the procedures developed by Lechevalier and Lechevalier (1980). Phospholipids in cells were extracted and identified by the method of Minnikin et al. (1984). Menaquinones were extracted from freeze-dried biomass, purified according to Collins (1985) and analysed by a HPLC–UV method (Wu et al. 1989). To determine cellular fatty acid compositions, strain LD22^T, the reference strains *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T were cultivated in GY broth for 5 days in Erlenmeyer flasks at 28 °C and detected under the same conditions. Freeze-dried biomass were soaked with 10 ml methanol for 30 min and centrifuged (3000 rpm, 10 min). The supernatant was concentrated and placed in test tubes. To each tube, 3 ml of 1 M NaOH in methanol was added, and the tubes were sealed with nitrogen gas, vortexed, and placed in a 70 °C water bath for 30 min. The tubes were cooled to room temperature for 2–3 min, 3.5 ml of 20% boron trifluoride was added, then the tubes were sealed, vortexed, and placed in water bath with the same condition above. The tubes were cooled to room temperature, 5 ml of saturated NaCl and 2 ml *n*-heptane was added and centrifuged (4000 rpm, 10 min). The heptane phase was analysed by GC–MS performed on a GC instrument (GC-2010 Plus; Shimadzu) coupled with a mass selective detector (GCMS-QP; Shimadzu) and an autosampler injector (AOC-20i; Shimadzu). A capillary column DB-5MS with dimensions of 30.0 m × 0.25 mm × i.d. 0.25 mm film thickness (Shimadzu) was used for the separation of fatty acid methyl esters. The initial temperature of 40 °C was maintained for 2 min, raised to 320 °C at the rate of 6 °C min^{-1}, and then kept at 320 °C for 1 min. Helium was used as a carrier gas with a flow rate of 1.42 ml min^{-1}. The injector temperatures was 280 °C. The MS was operated in the electron impact (EI) mode at 70 eV in the scan range of 28–500 m/z. The fatty acids were identified using the NIST 14 database.

Molecular analysis

Phylogenetic analyses

Extraction of chromosomal DNA and PCR amplification of the 16S rRNA gene sequence were carried out using a standard procedure (Kim et al. 2000). The PCR product was purified and cloned into the vector PMD19-T (Takara) and sequenced using an Applied Biosystems DNA sequencer (model 3730XL). An almost full-length 16S rRNA gene sequence (1509 bp) was An almost full-length 16S rRNA gene sequence (1509 bp) was obtained and compared in the EzBioCloud server (https://www.ezbiocloud.net/; Yoon et al. 2017a) and retrieved using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi;). The phylogenetic trees were constructed based on the 16S rRNA gene sequences of strain LD22^T and related reference species. Sequences were multiple aligned in Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 using the Clustal W algorithm and trimmed manually where necessary. Phylogenetic trees were constructed with the neighbour-joining (Saitou and Nei 1987), maximum likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) algorithms using MEGA 7.0 (Kumar et al. 2016). The stability of the topologies of the phylogenetic trees was assessed using the bootstrap method with 1000 repetitions (Felsenstein 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). 16S rRNA gene sequence similarities between strains were calculated on the basis of pairwise alignment using the EzBioCloud server (Yoon et al. 2017a). The *gyr*B gene was

obtained from the whole genome. Phylogenetic analysis was performed as described above. Whole-genome phylogeny was generated using TYGS server (http://tygs.dsmz.de) (Meier-Kolthoff and Göker 2019).

Genomic analysis, DNA–DNA hybridization and DNA G + C content

For draft genome sequencing and assembly, the genomic DNA of strain LD22^T, *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T were extracted with the SDS method (Nikodinovic et al. 2003). The harvested DNA were detected by the agarose gel electrophoresis and quantified by Qubit. Whole-genome sequencings were performed on the Illumina NovaSeq PE150 platform. A-tailed, ligated to paired-end adaptors and PCR amplified with a 350 bp insert was used for the library construction at the Beijing Novogene Bioinformatics Technology Co., Ltd. Illumina PCR adapter reads and low quality reads from the paired-end were filtered using the readfq (Version 10) remove reads with less than a certain percentage of low-quality bases (mass value $\leq$ 38 or default is 40 bp), a certain percentage of reads with N bases (default is 10 bp), overlap exceeds a certain threshold (default is 15 bp) and the possibility reads originating from the host. All good quality paired reads were assembled using the SOAP denovo (Li et al. 2008, 2010). (http://soap.genomics.org.cn/soapdenovo.html) into a number of scaffolds. Two genomic metrics are now available to distinguish between orthologous genes of closely related prokaryotes, including the calculation of average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH). The dDDH and ANI values were determined between the genomes of strain LD22^T and *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T online at http://ggdc.dsmz.de using the Genome-to-Genome Distance Calculation (GGDC 2.0) (Meier-Kolthoff et al. 2013) and the ChunLab's online ANI Calculator (www.ezbiocloud.net/tools/ani) (Yoon et al. 2017b), respectively.

Results and discussion

Phenotypic characteristics

Morphological observation of 28-day culture of strain LD22^T grown on ISP 3 medium revealed that it forms well-developed, branched substrate mycelium and aerial hyphae that differentiates into long, flexuous spore chains consisting of oval spores (0.7–0.9 × 1.0–1.2 μm) with warty surfaces (Fig. 1a, b). The spores are non-motile. Cultural characteristics of the isolate are shown in Fig. S1 and Table S1. Good growth was observed on ISP 1, ISP 2, ISP 3, ISP 5, ISP 6 and MBA media; moderate growth on ISP7, NA and CA media; but no growth on ISP 4 medium. Aerial mass colour is white and that of the substrate mycelium varied from pale yellow to brilliant greenish yellow. Pale yellow diffusible pigment was observed

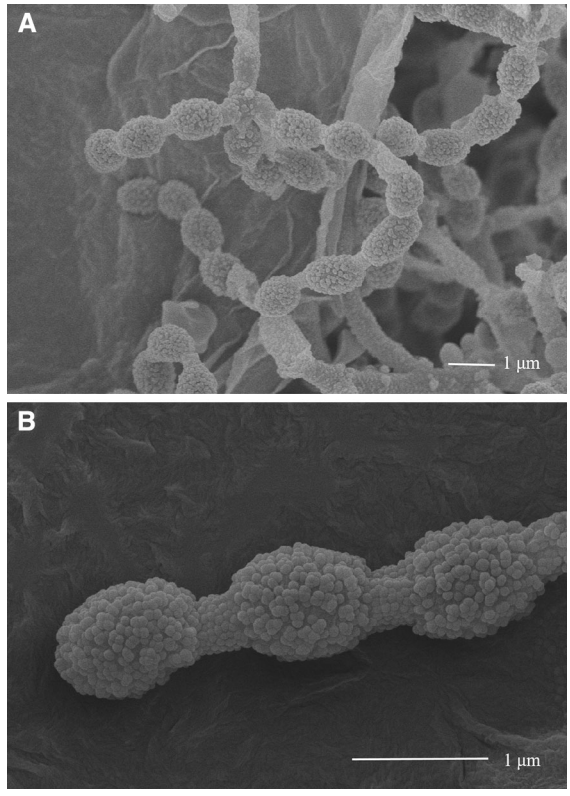

Fig. 1 Scanning electron microscopy of strain LD22^T grown on ISP3 for 28 days at 28 °C. *Bar*, 1.0 μm

on ISP1 medium. Growth of strain LD22^T was found to occur at pH 4.0–8.0 (optimum, 7.0) and in the presence of 0–2.0% (w/v) NaCl. The temperature range for growth was found to be 20–45 °C, with optimum growth at 28 °C. Positive results were obtained for the utilisation of dulcitol, hydrolysis of starch and Tweens (40 and 80), D-fructose, D-glucose, lactose, D-maltose, D-mannitol, L-rhamnose, D-raffinose and D-sucrose as sole carbon sources, and L-asparagine, L-alanine, L-arginine, L-proline, L-serine, L-threonine and L-tyrosine as nitrogen sources. Detailed physiological and biochemical properties are presented in the species description and the differential characteristics of strain LD22^T and two closely related species of the genus *Actinomadura* are summarised in Table 1.

Table 1 Characteristics allowing differentiation of strain LD22^T from its closely related species, *A. rudentiformis* CYP1-1B^T and *A. rudentiformis* HMC1^T

Characteristics	1	2	3
Growth pH	4–8	5–8	5–8
Tolerance of NaCl (%, w/v)	0–2	0–5	0–3
liquefaction of gelatin	–	–	+
Hydrolysis of			
Aesculin	–	+	+
Tween 20	–	–	+
Tween 80	+	–	+
Carbon utilisation			
L-Arabinose	–	+	+
D-Fructose	+	+	+
D-Galactose	–	–	+
Inositol	–	–	+
D-Maltose	+	–	–
D-Mannose	–	–	+
D-Xylose	–	+	+
Nitrogen utilisation			
L-Arginine	+	–	+
Creatine	–	+	+
L-Glutamic acid	–	+	+
L-Glutamine	–	+	+
Glycine	–	+	+

Strains: 1, LD22^T; 2, *A. montaniterrae* CYP1-1B^T; 3, *A. rudentiformis* HMC1^T; All data presented are from this study. +, positive; –, negative

Chemotaxonomic characteristics

Strain LD22^T was found to contain *meso*-diaminopimelic acid in cell wall hydrolysates. The acyl type of muramic acid in peptidoglycan was determined to be the acetyl group. The whole-cell sugars were glucose, madurose, galactose and ribose. The polar lipid profile was determined to consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside and an unidentified phospholipid (Fig. S2). Menaquinones of strain LD22^T were identified as MK-9(H_4) (40.2%), MK-9(H_8) (26.0%), MK-9(H_6) (20.7%) and MK-9(H_2) (13.1%). The major cellular fatty acids (> 10%) detected were $C_{16:0}$ (33.2%), 10-methly $C_{18:0}$ (25.8%) and $C_{18:1}\omega9c$ (17.1%). Detailed fatty acid profiles of strain LD22^T and the closely related type strains are given in Table S2. All these chemotaxonomic data showed that strain LD22^T should be assigned to the genus *Actinomadura*.

Molecular characteristics

Identification using the EzTaxon-e server revealed that strain LD22^T belongs to the genus *Actinomadura* and shared high sequence similarities with *A. montaniterrae* CYP1-1B^T (99.2%) and *A. madurae* DSM 43067^T (98.8%). 16S rRNA gene sequence similarities between strain LD22^T and other species of the genus *Actinomadura* were lower than 98.7%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain LD22^T formed a phyletic line with *A. rudentiformis* HMC1^T (98.6%) and clustered with *A. montaniterrae* CYP1-1B^T in the neighbour-joining tree (Fig. 2), a relationship also recovered by the maximum-likelihood (Fig. S3) and maximum-parsimony algorithms (Fig. S4). Further, Phylogenetic analysis based on *gyr*B gene (Fig. 3 and Fig. S5) and whole-genome sequences (Fig. S6) showed strain LD22^T formed a stable phyletic line with *A. montaniterrae* CYP1-1B^T. The isolate also showed highest *gyr*B gene sequence similarity with *A. montaniterrae* CYP1-1B^T (98.9%). Thus, based on the phylogenetic relationship, 16S rRNA and *gyr*B gene sequences similarities, the isolate was mostly related to *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T.

The assembled genome sequences of strain LD22^T, *A. montaniterrae* CYP1-1B^T and *A. rudentiformis*

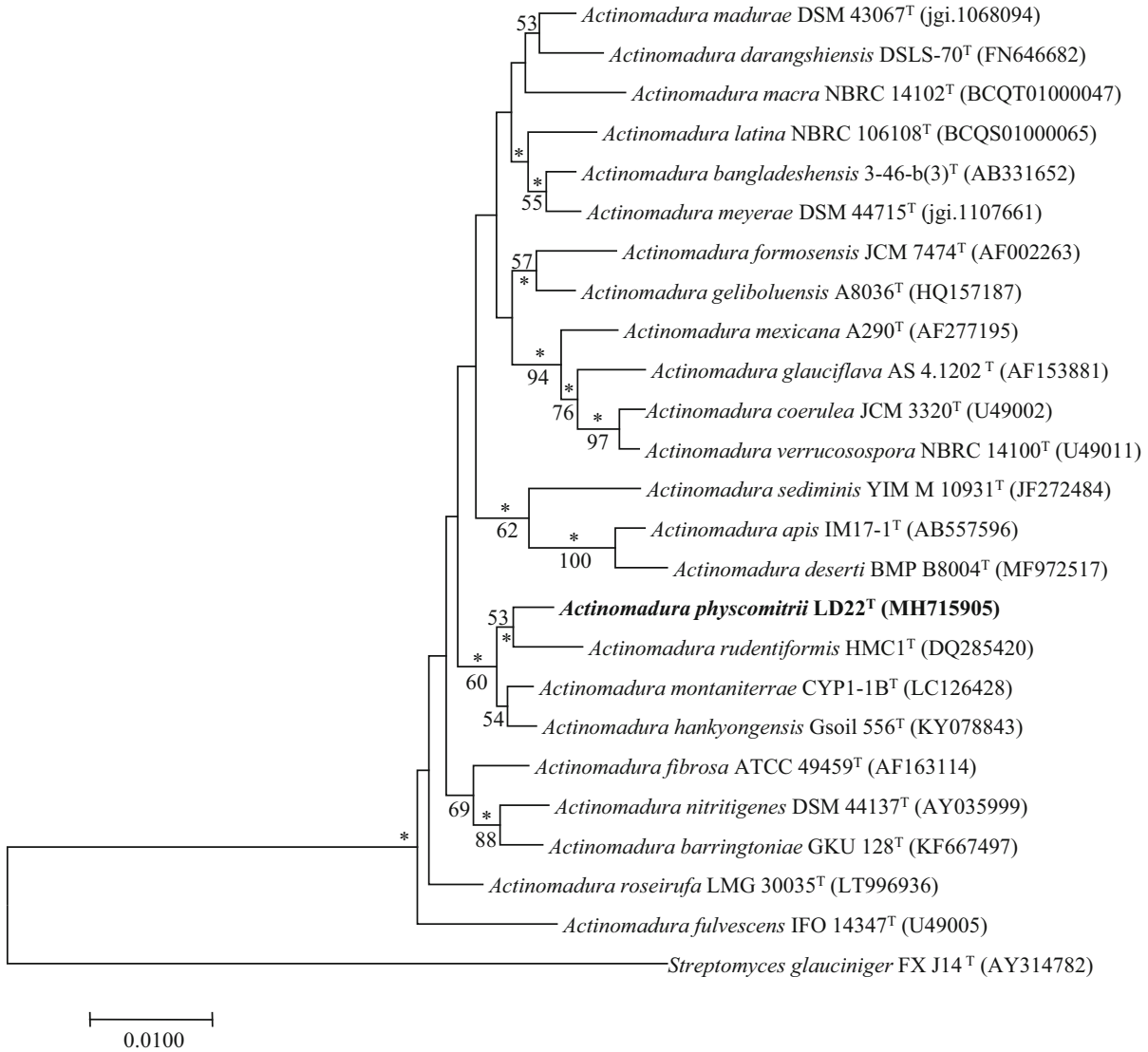

Fig. 2 Neighbour-joining tree based on 16S rRNA gene sequences showing relationships between strain LD22^T (1509 bp) and the 24 phylogenetically closely related representative species with validly-published names in the genus *Actinomadura*. The out-group used was *Streptomyces glauciniger* FXJ14^T. Only bootstrap values above 50% (percentages of 1000 replications) are indicated. *Asterisks* indicate branches also recovered in the maximum-likelihood tree; *Bar*, 0.01 nucleotide substitutions per site

HMC1^T were found to be 10.0 Mbp (111 scaffolds), 10.7 Mbp (506 scaffolds) and 10.7 Mbp (51 scaffolds), respectively. Genome sequencing showed the DNA G + C contents were 72.5, 73.0 and 69.6 mol %, respectively. The 16S rRNA gene sequence of strain LD22^T from whole genome sequence shared 100% similarity to that from PCR sequencing without inconsistent nucleotide, suggestting that the genome sequence was not contaminated. The draft genome sequences were deposited at the GenBank/EMBL/DDBJ under the accession numbers WBMS00000000, WBMR00000000 and WBMT00000000, respectively. The comparable features of the genome sequences between the strain LD22^T and *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T are presented in Table S3.

The dDDH and ANI values were employed to further clarify the relatedness between strain LD22^T

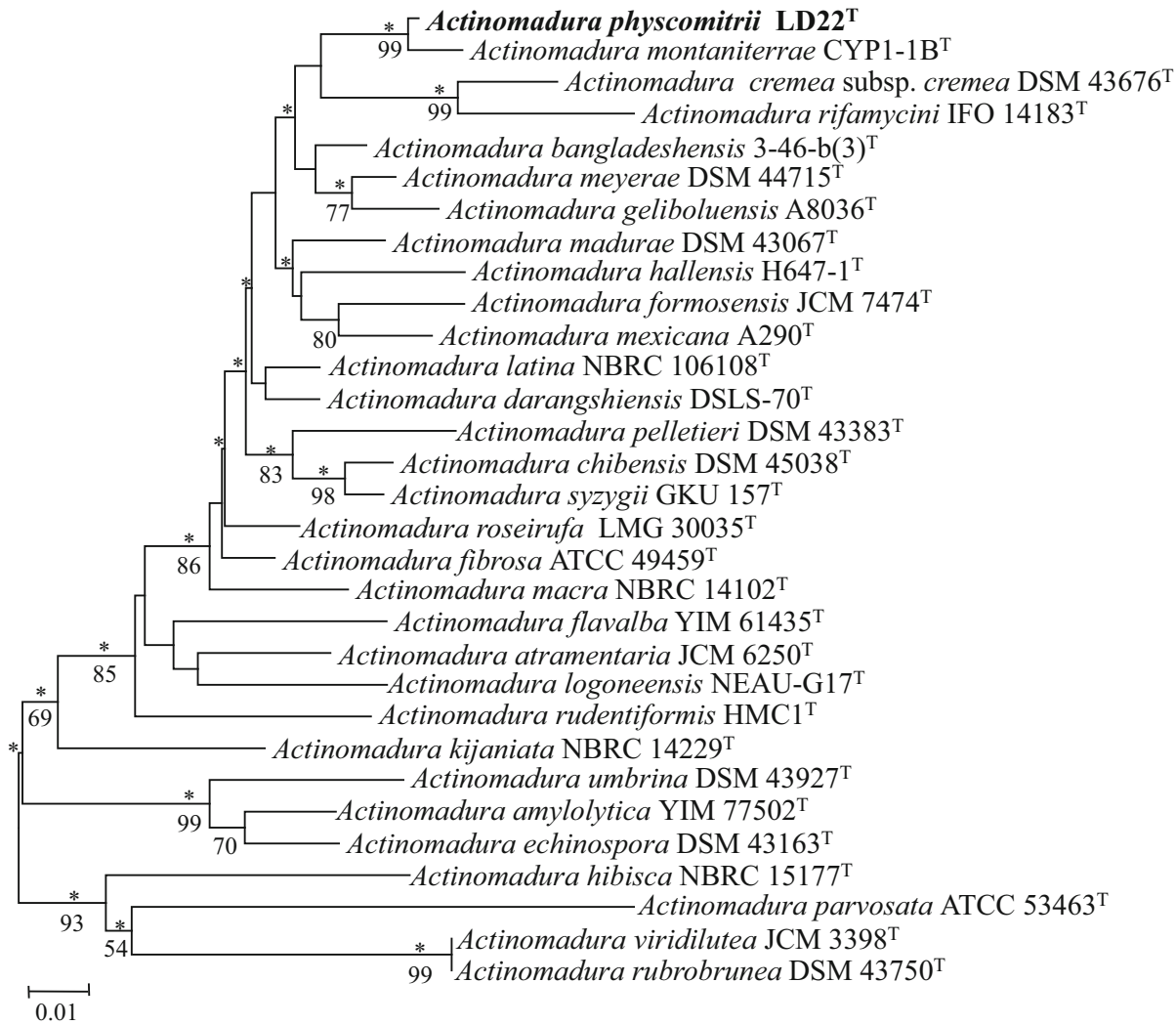

Fig. 3 Neighbour-joining tree based on *gyr*B gene sequences showing relationships between strain LD22^T and the 30 related representative species with validly-published names in the genus *Actinomadura*. Only bootstrap values above 50% (percentages of 1000 replications) are indicated. *Asterisks* indicate branches also recovered in the maximum-likelihood tree; *Bar*, 0.01 nucleotide substitutions per site

and *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T. The levels of dDDH between them were 46.7–53.6% and 20.5–25.3%, which are below the threshold value of 70% recommended by Wayne et al. (1987) for the delineation of prokaryotic species. Similarly, the ANI values of 93.3% and 78.8% were found between LD22^T and *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T, a result well below the threshold used to delineate prokaryote species (Richter and Rossello-Mora 2009; Chun and Rainey 2014).

Besides the genotypic evidence above, comparison of phenotypic characteristics between strain LD22^T and its closely related species, *A. montaniterrae* CYP1-1B^T and *A. rudentiformis* HMC1^T, was performed to differentiate them (Table 1). For instance, strain LD22^T could grow at pH 4.0, while the closely related strains could not; NaCl tolerance of strain LD22^T was up to 2%, which is lower than that of closely related strains. Other phenotypic differences included liquefaction of gelatin, hydrolysis of aesculin, Tween 20 and 40, and patterns of carbon and nitrogen utilisation. In addition, The colony colours

and production of diffusible pigments on different test media also could make a contribution to differentiate the isolate from its closely related species (Fig. S1 and Table S1). Therefore, it is evident from the phenotypic and genotypic data that strain LD22^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura physcomitrii* is proposed.

Description of *Actinomadura physcomitrii* sp. nov.

Actinomadura physcomitrii (phys.co.mi'tri.i. N.L. gen. n physcomitrii of the moss Physcomitrium)

Aerobic, Gram-positive actinomycete. Forms well-developed and extensively branched substrate mycelia. Aerial mycelia form flexuous spore chains; spores are oval, warty and non-motile. Negative for decomposition of cellulose, hydrolysis of aesculin and Tween 20, liquefaction of gelatin, peptonization and coagulation of milk, production of H_2S and reduction of nitrate. The diagnostic cell wall diamino acid is *meso*-diaminopimelic acid. Contains diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside as polar lipids. The menaquinones are MK-9(H_4) (40.2%), MK-9(H_8) (26.0%), MK-9(H_6) (20.7%) and MK-9(H_2) (13.1%). Major fatty acids are $C_{16:0}$ (33.2%), 10-methly $C_{18:0}$ (25.8%) and $C_{18:1}\omega9c$ (17.1%). The G + C content of the DNA of the type strain is 72.5 mol %.

The type strain, LD22^T (= CCTCC AA 2018050^T = JCM 33455^T), was isolated from moss [*Physcomitrium sphaericum* (Ludw) Fuernr] collected in Kunming, Yunnan Province, Southwest China. The GenBank accession number for the 16S rRNA gene sequence and the draft genome sequence of the type strain are MH715905 and WBMS00000000, respectively.

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Author contributions XZ performed the laboratory experiments, analysed the data, and drafted the manuscript. CP and ZW contributed to the biochemical characterisation. JZ and YS contributed to the fatty acids determination. CL and WX designed the experiments and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants and/or animals performed by any of the authors. The formal consent is not required in this study.

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