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Polyphasic identification of *Lechevaliera fradia* subsp. *Iranica*, A rare actinomycete isolated from Loshan region of Iran

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Abstract

Introduction: Actinomycetes are widely distributed in natural and man-made environments and have capability for degradation of organic matter. They are also well known as a rich source of antibiotics and bioactive molecules and are of considerable importance in industry.

Materials and methods: In this study, a rare actinomycete was isolated and subjected to polyphasic identification. Identification of this rare actinomycetes was carried on according to polyphasic taxonomic approach which has been outlined by International Committee on Systematics of Prokaryotes (ICSP).

Results: The cell wall of strain LO5 contained *meso*-diaminopimelic acid as diaminoacid and galactose, mannose and rhamnose as diagnostic sugars. The phospholipids consisted of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Phylogenetic analysis based on nearly complete *16S rRNA* gene sequence comparison revealed affiliation to the family *Pseudonocardiaceae* and its similarity to the most closely related neighbor the *Lechevalieria fradiae* CGMCC 4.3506^T was 98.6%. The level of DNA–DNA relatedness between the novel strain and *Lechevalieria fradiae* CGMCC 4.3506^T was only 75%. So according to the recommendations of a threshold value of 70% DNA-DNA similarity, LO5 belongs to the species *Lechevalieria fradiae* CGMCC 4.3506^T. On the basis of genomic and phenotypic properties, the subspecies *Lechevalieria fradiae* subsp. *Iranica* IBRC-M 10378 is proposed**.**

Discussion and conclusion: A polyphasic approach based on phenotypic, chemotaxonomic and phylogenetic investigations has been proposed for categorizing of a rare actinomycete in subspecies level. The techniques used to obtain the data required for determination of the taxonomic status of this isolate are based on minimal standards that have been established by some of the taxonomic subcommittees of the International Committee on Systematics of Prokaryotes (ICSP) for specific groups of organisms.

Key words: Polyphasic taxonomy, Rare actinomycetes, Chemotaxonomy, Phylogenetic analysis

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Introduction

Actinomycetes are widely distributed in natural and man-made environments, and play an important role in the degradation of organic materials. They are also well known as a rich source of antibiotics and bioactive molecules, and are of considerable importance in industry. Rare actinomycetes have usually been regarded as strains of actinomycetes whose isolation frequency by conventional methods is much lower than that of genus *Streptomyces* (1). Consequently basic knowledge of the habitat, physiology and productivity of molecules of rare actinomycetes gradually increased (2). The genus *Lechevaliera* is a rare actinomycete that belongs to the family *Pseudonocardiaceae* (3) and displays type III cell wall composition (*meso*-diaminopimelic acid along with galactose and ribose as whole-cell sugar pattern), type II phospholipid pattern and MK-9 (H_4) as a major menaquinone. In the course of our investigation for screening of phytotoxin producing actinomycetes, a rare actinomycetes, strain LO5, was isolated from a rhizospheric soil sample collected around rice farm at Loshan, a city located in Gilan province in the north of Iran.

Materials and methods

Sampling and pretreatment of soil

Soil samples were collected from the rhizospheric layer of rice plant on Loshan city located in Gilan province in the north of Iran. The soil was air dried at room temperature for 7 days. For separation of spores from soil particles, it was well grinded and then subjected to heat drying process at 100°C for one hour (4).

Isolation and Culture conditions

Strain LO5 was isolated by diluting the treated soil sample in sterile 0.9% (w/v) sodium chloride solution, plating on glucose-asparagine-dipotassium phosphate (5). The pH was adjusted on 6.8 to 7.0. The isolation plates were incubated at 28°C aerobically for 3 weeks. It was maintained on yeast extract-malt extract agar (ISP 2) plate containing (per liter) malt extract (10.0 g), glucose (4.0 g), yeast extract (4.0 g) and agar (18.0 g) at 4°C and as glycerol suspensions (20%, v/v) at -20ºC and -80°C.

Polyphasic identification

All steps of polyphasic identification process were done at Iranian Biological Resource Center (IBRC), Microorganisms Bank. *Lechevalieria fradiae* CGMCC 4.3506 was obtained from Chinese General Microbiological Culture Collection (CGMCC), cultivated under the same growth conditions as strain LO5 and used as reference strain. Strain LO5

and *L. fradia*^{T} were subjected to polyphasic identification process simultaneously.

Morphological characterizations

The color of aerial mycelium (spore mass) and substrate mycelium (reverse color) was determined on yeast (ISP 3) containing (per liter) Oatmeal Agar (20.0 g), Ferric sulphate heptahydrate (0.001 g), Manganese chloride tetrahydrate (0.001 g), Zinc sulphate heptahydrate (0.001 g) and Agar (18.0 g) at 28ºC for 3 weeks using color chips from the ISCC-NBS (Inter-Society Color Council-National Bureau of Standards) charts (6). The ability for production of diffusible pigment was assayed on ISP 2, ISP 3, inorganic salts-starch agar (ISP 4) consisting (per liter) Soluble starch (10.0 g), Dipotassium phosphate (1.0 g), Magnesium sulfate (1.0 g), Sodium chloride (1.0), Ammonium sulfate (2.0 g), Calcium carbonate (2.0 g), Ferric sulphate heptahydrate (0.001 g), Manganese chloride tetrahydrate (0.001 g), Zinc sulphate heptahydrate (0.001 g), agar (18.0 g) and glycerol-asparagine agar (ISP 5) containing (per liter) L-asparagine (1.0 g), Dipotassium phosphate (1.0 g), Ferric sulphate heptahydrate (0.001 g), Manganese chloride tetrahydrate (0.001 g), Zinc sulphate heptahydrate (0.001 g), agar (18.0 g) and Agar (18.0 g). the pH for all media was adjusted at 7.4± 0.2. Spore chain morphology of a 7-day-old culture grown on ISP 2 agar were observed using Olympus BX51 light microscope. These cultural and morphological properties were examined on media recommended by Shirling & Gottlieb (7) and Waksman (8).

Physiological and Biochemical characterizations

Physiological and biochemical tests were carried out using standard methods (9 and 10) at 28°C which included catalase and oxidase determination, acid production from carbohydrate, decomposition of 80, hydrolysis of starch, pectin and DNA. Tolerance to NaCl was assessed in ISP 2 agar medium containing 0, 1, 3 and 5% NaCl. Reduction of nitrate and production of melanoid pigment were determined by the method of the ISP (7). Production of H_2S was tested by growing the strain LO5 in ISP 6 agar slant medium at 28°C. All tests were recorded after 7 to 21 days.

Chemotaxonomic characterizations

Cell biomass for comparative chemotaxonomic evaluations of strain LO5 and *Lechevalieria fradiae* CGMCC 4.3506^{T} was obtained by cultivation in shaken flasks containing ISP 2 broth medium for 5 days. The biomass was harvested by centrifugation at 5000 rpm for 20 min, washed twice in distilled water and dried at 70°C for 3 hours. Isomers of diaminopimelic acid and whole cell sugars were determined by established TLC methods (11 and 12). Phospholipids were extracted and identified by the method of Minnikin *et al.* (13). According to this method, 100 mg of dried cells were suspended in 2.0 ml of sodium chloride (0.3%, w/v).

The Purified methanol (20.0 ml) was added to the mixture and held at 100°C in a screw-capped tube. After cooling, chloroform (10.0 ml) and 6 ml of the sodium chloride (0.3%, w/v) were added to the mixture and shaked for 2 hours and the debries removed by filtration. Cholroforme and sodium chloride (0.3%, w/v) in same volume (10.0 ml) was added to the filtrate and chlroform layer was collected by decantour. The collected layer was concentrated at low air pressur at 37°C and used for chromatographic analysis.

Genomic analysis

Genomic DNA from strain LO5 was prepared using modification of salting out procedure introduced by Kieser *et al.* (14). Approximately 50 to 100 mg biomass of strain LO5 growth was taken from ISP 2 broth medium and transferred to a 150 µl microfuge tube. Biomass was resuspended in 567 µl SET buffer (NaCl 75 mM, EDTA 25 mM (pH 8.0), Tris-HCl 20mM (pH 7.5) and mixed with lysis solution buffer which contained 100 µl lysosyme (30 mg/ml in 10 mM Tris pH 8.0) and incubated at 37°C for one hour. Lysis was accomplished by adding 9 µl proteinase K (600 U/ml), 100 µl SDS 10% and 3 µl RNase (10 mg/ml) followed by brief mixing and incubation at 55°C for 2 hours. The lysate was centrifuged at 10,000 rpm for 15 min. The resulting preparation was extracted according to reference protocol. DNA was precipitated with 2-propanol and rinsed with ethanol 70% (v/v). The pellet was dried at room temperature and DNA dissolved in 50µl TE solution.

PCR amplification was carried out using: 10 F (5´-GAGTTTGATCCTGGCTCA-3´) and: 1500 R (5´-AGAAAGGAGGTGATCCAGC-3´) universal primers. PCR reaction conditions included three temperature cycles. It was involved initial denaturation at 94°C for 5 min followed by 25 cycles including denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 90 s. This is followed by a final extension in 72°C for 10 min. The purified PCR product was sequenced using an ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). Identification of phylogenetic neighbors was initially carried out by the BLAST (15) and mega BLAST (16) programs against the database of type strains with validly published prokaryotic names. The calculation of pairwise sequence similarity was done using global alignment algorithm, which was implemented at the EzTaxon-e server (17). Direct sequence determination of the PCR-amplified DNA was conducted on ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). Multiple alignments was carried out by CLUSTAL-X (18) and phylogenetic analysis was performed using the software package MEGA version 5 (19). Phylogenic tree was

constructed using neighbor-joining method and bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings.

DNA-DNA hybridization was carried out as described by De Ley *et al.* (20) incorporating the modifications described by Huss *et al.* (21), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with an in situ temperature probe (Varian).

Results

Strain LO5 was a Gram positive, strictly aerobic and non-acid fast actinomycete. The strain formed powdery-surfaced colonies and produced abundant vivid orange yellow substrate and aerial mycelium fragmented to ovoid, smooth and non-motile spores on ISP3. No diffusible pigments were produced only on ISP 3 medium. It grew at a temperature range of 20 to 40°C (optimum temperature 30°C), pH 6 to 8 (optimum pH 7.0) and NaCl concentrations of 0 to 3%. Urea was not hydrolysed. The strain did not reduce nitrate to nitrite despite nitrite reduction was not observed. Starch, tween 80, pectin and DNA were not decompsed. Melanin was not produced on ISP 6 and ISP 7. Moreover, this strain was not capable to produce H_2S. Acid is produced from D-glucose. All details of phenotypic features are included in Table1.

Strain contained major amounts of *LL*-diaminopimelic acid, galactose, mannose and rhamnose as diagnostic sugars in whole-organism hydrolysates (wall chemotype III) (Fig. 1). Significant quantities of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three unknown amino phosphloipid and phospholipid were found in the membrane of strain LO5 (Fig. 2).

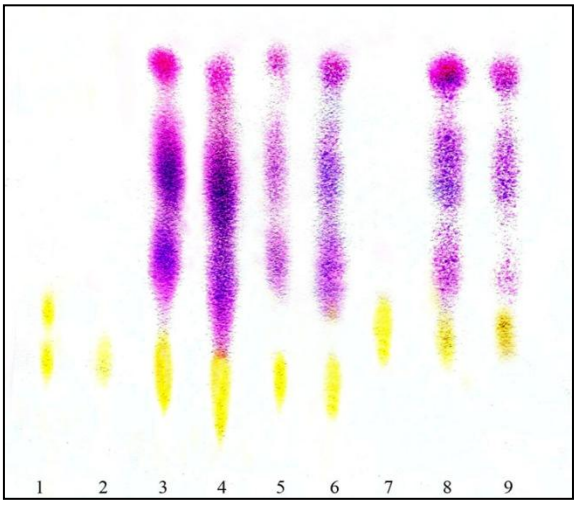

Fig. 1- Separation of diaminopimelic acid (DAP) isomers by TLC (the numbers at bottom are the origin). All of the following were hydrolysed except the standard solution. 1, DL-DAP (Sigma Chemical Co.); 2, D-DAP purified from DL-DAP (Sigma Chemical Co.); 3, LO5; 4, *Lechevalieria fradiae* CGMCC 4.3506; 5, *Nocardia* sp.; 6, *Micromonospora* sp.; 7, L-DAP purified from DL-DAP (Sigma Chemical Co.), 8 and 9, *Streptomyces* sp. The L-DAP is the faster-moving spot with more R_f value while D-DAP has lower R_f value. Note long smear of amino acids migrating ahead of DAP in hydrolysates.

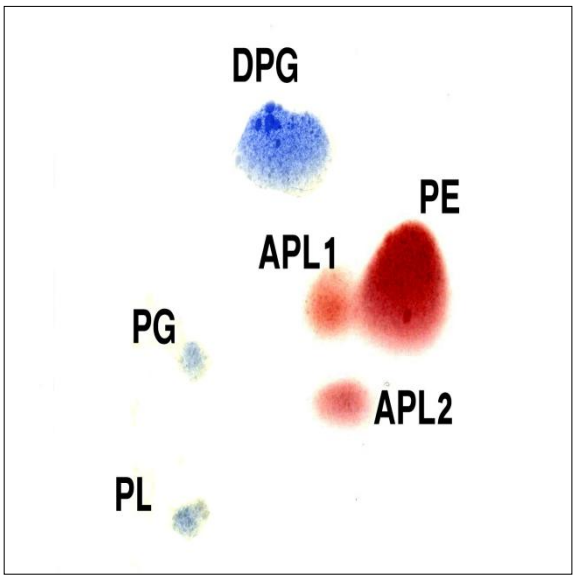

Fig. 2- Thin Layer Chromatography of polar lipids of strain LO5. Two-dimensional plate revealing the phospholipid pattern of strain LO5 confirming the presence of PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamin; PME, phosphatidylmethylethanolamine; PL, phospholipid.

Polar lipids were simoultanously stained in innovative manner with molybdenium-blue and Ninhydrin reagents, the specific dyes for phosphate and free amino group detection respectively. The lipids contain phosphate such PG, DPG and PL turn blue in a white background whereas lipids with free amino group turn purple in a white background. The plates of LO5 were interpreted against *Lechevalieria fradiae* CGMCC 4.3506 as reference type strains.

The *16S rRNA* gene sequence analysis using almost complete *16S rRNA* gene sequences of strain LO5 (1400nt) revealed that this strain is a member of the family *Pseudonocardiaceae* and the most related neighbours are *Lechevalieria fradiae* CGMCC 4.3506 (98.6%), *Lentzea albida* IFO 16102^T (98.5%), *Lentzea waywayandensis* NRRL B-16159^T (98.5%) (Fig. 3)

The level of DNA-DNA relatedness between *Lechevalieria* sp. Lo5 and *Lechevalieria fradiae* CGMCC 4.3506 was calculated about 75% which resembles that the tested strain and the reference one should be classified as the same genomic species.

Table 1- Phenotypic characters of LO5 and *Lechevalieria fradiae* CGMCC 4.3506^T

Characters	Strain Name	
	LO5	*Lechevalieria fradiae* CGMCC 4.3506^T
Nitrate Reduction	-	+
Hydrolysis of:		
Urea	-	+
Degradation of		
Tween 80	-	-
Starch	-	+
DNA	-	-
Acid production from:		
D-raffinose	-	+
D-cellobiose	+	-
D-fructose	+	-
D-glucose	+	-
Glycerol	+	-
D-mannitol	+	-
D-mannose	+	-
D-melibiose	+	-
L-rhamnose	+	-
D-sucrose	+	-
Adonitol	+	-
Galactose	+	-
Inositol	+	-
Lactose	+	-

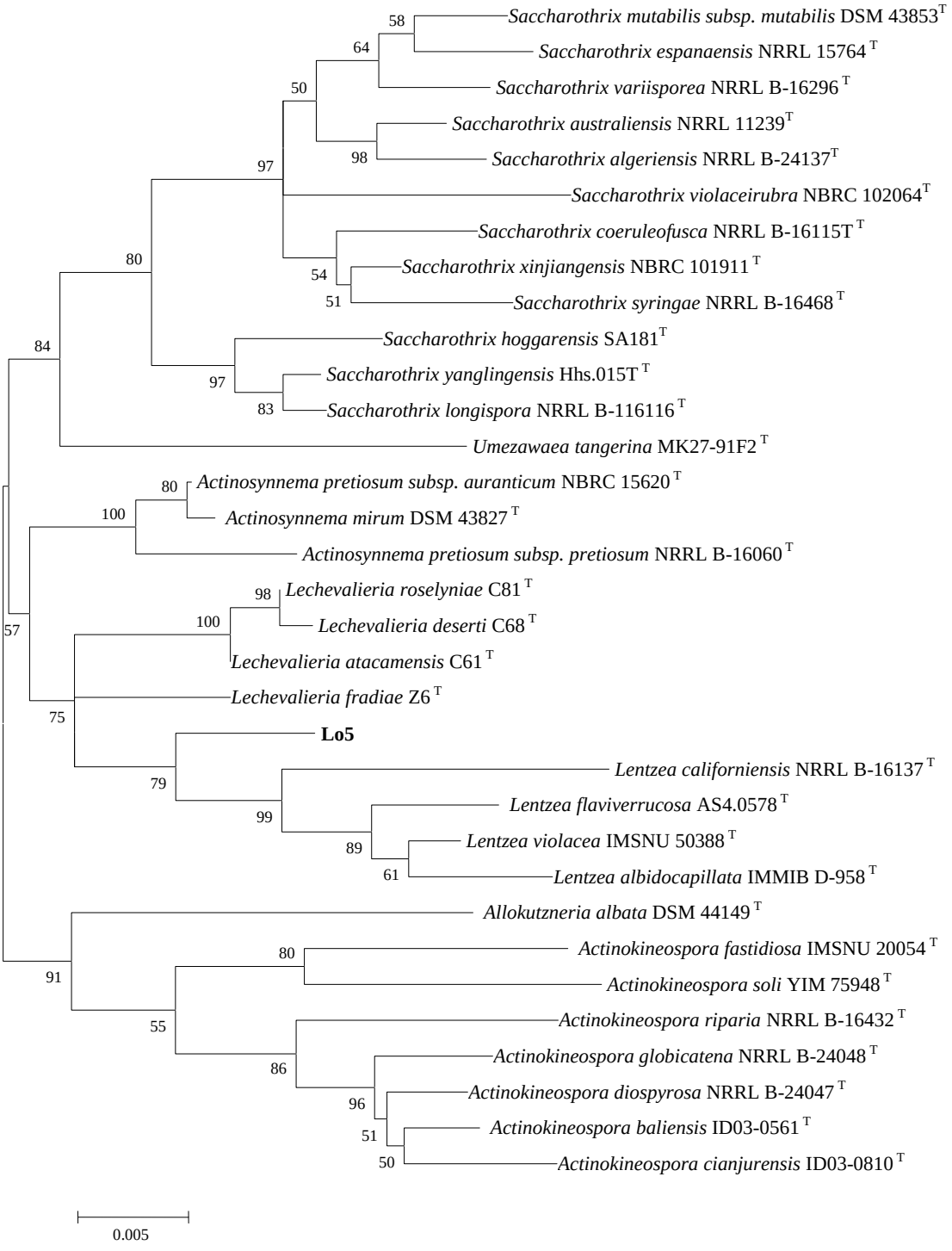

Fig. 3- Phylogenetic tree based on nearly complete *16S rRNA* gene sequences showing relationships between strain Lo5 and representatives of known species classified in genera assigned to the family *Pseudonocardiaceae*. Numbers at branch nodes are bootstrap *p values* (percentages of 100 replicates). Bar, 5 substitutions per 1000 nucleotide positions

Discussion and conclusion

In this report, we tried to outline the steps in the process of characterizing a rare actinomycete strain and presenting that information in the form of a taxonomic description. The techniques used to obtain the data required for the determination of the taxonomic status of this isolate are based on minimal standards that have been established by some of the taxonomic subcommittees of the International Committee on Systematics of Prokaryotes (ICSP) for specific groups of organisms. The prokaryotes are currently characterized using a polyphasic approach that brings together a variety of phenotypic, chemotaxonomic and genotypic data that comprise the formal description of a novel taxon. The minimal standards are defined on the basis of polyphasic approach should be consulted and applied when working with one of the groups for which such standards exist. In the case of actinomycetes, all steps of polyphasic identification process are applied. The first step in any characterization process is to place it into a phylogenetic context and determine its phylogenetic relationship with previously described type species. Depending on the proximity to other taxa, a strategy for its characterization and description can be designed. Determining DAP isomers in the cell hydrolysate of actinomycete strain is the first step of identification process. The presence of D-DAP or L-DAP is the main criteria to categorizing actinomycetes in rare group and others. However, the mixture of DAP isomers may be reported in some actinomycete strain such as genus *Kitasatospora* (22). Analysis of whole cell sugar content of cell can be a useful technique to diagnose rare ones. In the rare actinomycetes, the pattern of cell wall sugar plays a key role in their valid chemo-grouping. As the results revealed galactose, mannose and rhamnose are diagnostic sugar to identify genus *Lechevalieria* whereas in *Lentzea,* rhamnose is absent and ribose is a replacing sugar (23 and 24). Generally, analysis of polar lipids is recommended in identifying members of family *Pseudonocardiaceae,* but it brings lower valuable data than other chemical analysis. However phosphatidylethanolamine is widespread in the family's *Pseudonocardiaceae, Actinoplanaceae* and *Streptomycetacea,* so its determination does not provide sufficient and useful data for taxonomic purposes. Defining phylogenetic position for a new isolate according to *16S rRNA* gene sequence is the best way to reach a good and proper verdict about its taxonomic statues. Although similar values between variable taxonomic groups, were evaluated differently. In some cases, different species have *16S rRNA* gene sequence pairwise similar values more than 99%, but in other cases, that could not be treated as the separate taxonomic group. Moreover, DNA-DNA hybridization (DDH) or DNA-DNA reassociation technique, can use

as our last attempt to make raw comparisons of whole genomes between different organisms. According to the latest rules on polyphasic taxonomy, when *16S rRNA* gene sequence pairwise similar values are greater than 97%, the authors should carry out DNA-DNA hybridization studies. In this study, the *16S rRNA* gene similarity between strain LO5 and *Lechevalieria fradiae* CGMCC 4.3506^T was more than 97%, so DDH analysis was suggested for determination of strains relationship. The result was shown that the whole genome of these strains have a share of 75% similarity which is not sufficient dissimilarity to support the classification of the strain as a new taxon. So we can conclude that although phenotypic and chemotaxonomic differences between these strains were proposed that they may belong to distinct taxonomic groups, the genetic based methods did not support it. This result also accentuates the importance of polyphasic approach for characterization of a new isolate.

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شناسایی پلی فازی *Lechevaliera fradia* subsp. *Iranica*، یک اکتینومایست نادر جدا شده از منطقه لوشان ایران

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چکیده

مقدمه: اکتینومایست‌ها به طور وسیعی در محیط‌های طبیعی و ساخته دست بشر پراکنده شده‌اند و توانایی تجزیه مواد آلی را دارا هستند. همچنین، آن‌ها به عنوان یک منبع غنی برای تولید آنتی‌بیوتیک‌ها و مولکول‌های فعال زیستی شناخته می‌شوند و از اهمیت چشمگیری در صنعت برخوردار هستند.

مواد و روش‌ها: در این مطالعه، یک اکتینومایست نادر با نام LO5 جداسازی شد و تحت شناسایی پلی فازی قرار گرفت. این اکتینومایست نادر بر اساس دیدگاه تاکسونومی پلی فازی که به‌وسیله کمیته بین المللی سیستماتیک پروکاریوت‌ها (ICSP) مصوب شده است، شناسایی شد.

نتایج: دیواره سویه LO5 دارای دی‌آمینو پایملیک اسید نوع مزو یا دی(D) و همچنین، گالاکتوز، مانوز و رامنوز به‌عنوان قندهای تشخیصی است. محتوای فسفولیپیدی غشا شامل: دی فسفاتیدیل گلیسرول، فسفاتیدیل گلیسرول و فسفاتیدیل اتانول آمین است. تحلیل فیلوژنی بر اساس مقایسه توالی کامل ژن *16S rRNA* نشان داد که این سویه از اعضای خانواده *Pseudonocardiaceae* و دارای شباهت ۹۸/۶ درصدی به *Lechevalieria fradiae* CGMCC 4.3506^T است. ارتباط DNA-DNA بین سویه LO5 و *Lechevalieria fradiae* CGMCC 4.3506^T گویای مقدار ۷۵ درصدی است که با توجه به بالاتر بودن آن از حد ۷۰ درصد که برای معرفی گونه‌های جدید به آن استناد می‌شود، سویه LO5 به عنوان زیرگونه *Lechevalieria fradiae* CGMCC 4.3506^T با نام *Lechevalieria fradiae* subsp. *Iranica* IBRC-M 10378 معرفی می‌شود.

بحث و نتیجه‌گیری: یک روش پلی فازی مبتنی بر مطالعات فنوتیپی، شیمیوتاکسونومی و فیلوژنی برای طبقه‌بندی یک اکتینومایست نادر در حد زیرگونه پیشنهاد شده است. برای به دست آوردن اطلاعات مورد نیاز برای تعیین وضعیت تاکسونومیک سویه LO5، از تکنیک‌هایی استفاده شده است که با حداقل استانداردهای لازم برای طبقه‌بندی یک میکروارگانیسم خاص که توسط زیرکمیته‌های تاکسونومی کمیته بین المللی سیستماتیک پروکاریوت‌ها تصویب شده اند، مطابقت دارند.

واژه‌های کلیدی: شناسایی پلی فازی، اکتینومایست‌های نادر ، شیمیوتاکسونومی، مطالعات فیلوژنتیک

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* نویسنده مسؤول مکاتبات

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