Genome-based classification of *Micromonospora craterilacus* sp. nov., a novel actinobacterium isolated from Nemrut Lake

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Introduction

The family *Micromonosporaceae* (Krasil'nikov 1938) includes aerobic, Gram-stain positive, non-acid-fast organisms that form a non-fragmenting, branched, and septate mycelium that rarely carry scant aerial hyphae (Trujillo et al. 2014). According to the latest revisions based on whole genome data (Nouioui et al. 2018; Li et al. 2019), the family encompasses 30 validly named genera which can be distinguished by a combination of chemotaxonomic, morphological and phylogenetic characteristics (Carro et al. 2018). Microorganisms classified in this family have been isolated from various habitats such as soil (Gao et al. 2014), sediment (Phongsopitanun et al. 2015), rhizosphere (Wang et al. 2011) and plant tissues (Li et al. 2013).

Members of the family *Micromonosporaceae* are well known for their ability to produce clinically important bioactive secondary metabolites such as actaplanin (Debono et al. 1984), abyssomicins (Riedlinger et al. 2004), arenimycin (Asolkar et al. 2010), daunorubicin (Cassinelli et al. 1980), gentamicin (Lancini and Lorenzetti 1993), rifamycins (Huang et al. 2009; Kim et al. 2006) and teicoplanin (Coronelli et al. 1984). Considering that the isolation of novel species significantly increases the chances of the discovery of novel chemical entities, investigation of unexplored habitats to isolate novel actinobacteria is one of the most promising strategies to discover new bioactive secondary metabolites (Müller and Wink 2014).

Nemrut Lake, the second largest crater lake in the world, is located in eastern Anatolia, Turkey, and it is one of the unexploited habitats waiting to be included in novel actinobacteria isolation programmes. Thus, the present study was established to isolate novel actinobacteria from coastal sediment sample of Nemrut Lake. One of the strains isolated, NA12^T, was found to be a novel species of the genus *Micromonospora* based on polyphasic approach.

Materials and methods

Source, maintenance and cultivation of strain

Strain NA12^T was recovered from a coastal sediment sample of Nemrut Lake, a crater lake in eastern Anatolia, Turkey. It was isolated on actinomycete isolation agar (Kumar et al. 2012) supplemented with cycloheximide (50 µg/ml) and nalidixic acid (10 µg/ml) following incubation at 28 °C for 10 days after being inoculated with suspension of a gram sediment in ¼ Ringer's solution (Oxoxid). After purification, the strain was maintained on GYM agar medium (DSMZ Medium No. 65) and as suspensions of hyphal fragments in 25 % (v/v) glycerol stock solutions at -20 °C. Biomass for chemotaxonomic and molecular systematic analyses was cultured in shake flasks of ISP 2 broth medium at 28 °C for 14 days, and after harvested, washed thrice in sodium chloride solution (0.8 %, v/v). Cells for chemotaxonomic analyses were freeze-dried and stored at room temperature. Wet biomass for fatty acid analysis was obtained after cultivation on TSB agar medium at 28 °C for a week.

Phylogeny

After cultivation in ISP 2 broth medium at 28 °C for 14 days, genomic DNA of strain NA12^T was extracted following the method described by Chun and Goodfellow (1995). PCR amplification of the 16S rRNA gene was carried out using the universal primers 27F (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1492R (5'-ACGG(CT)TACCTTGTTACGACTT-3') (Weisburg et al. 1991). Purified PCR products were sequenced using an ABI PRISM 3730 XL automatic sequencer. Almost complete 16S rRNA gene sequence (1479 bp) was deposited in the GenBank data library and aligned with corresponding sequences of closely related type strains retrieved by EzBioCloud server (<https://www.ezbiocloud.net/>) (Yoon et al. 2017). A multiple sequence alignment was generated with MUSCLE program (Edgar 2004). Neighbour joining tree (Saitou and Nei 1987) was constructed in MEGA 10 (Kumar et al. 2018) using the model of Jukes and Cantor (Jukes and Cantor 1969). Phylogenetic analyses for maximum likelihood (ML) and maximum parsimony (MP) methods were conducted by the GGDC web server using the DSMZ phylogenomics pipeline (Meier-Kolthoff et al. 2014) adapted to single genes after pairwise sequence similarities were calculated according to Meier-Kolthoff et al. (Meier-Kolthoff et al. 2013a) via the GGDC web server (Meier-Kolthoff et al. 2013b) available at <http://ggdc.dsmz.de/>. ML tree was inferred from the alignment with RAxML

(Stamatakis 2014) using rapid bootstrapping in conjunction with the autoMRE criterion (Pattengale et al. 2010) and MP tree was inferred from the alignment with TNT (Goloboff et al. 2008) using 1000 bootstraps in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence additional replicates. The sequences were checked for compositional bias using the X^2 test as implemented in PAUP* (Swofford 2002).

Whole genome sequence analysis

The genome of strain NA12^T was sequenced using the Illumina HiSeq 2500 next generation sequencing platform with a 250-bp paired end protocol (MicrobesNG, Birmingham, United Kingdom) and the draft genome sequence was submitted to NCBI under the accession number POTY000000000. Then, the genome sequence was annotated on the Rapid Annotations Using Subsystems Technology (RAST) server (Aziz et al. 2008). For a more comprehensive phylogenetic analysis, the genome sequences of strain NA12^T, *M. radicis* AZ1-13^T, *M. zingiberis* PLAI 1-1^T and *M. craniella* LHW63014^T were uploaded to Type Strain Genome Server (TYGS) available at <https://tygs.dsmz.de/> (Meier-Kolthoff and Göker 2019). The results were provided by the TYGS on 2019-10-26. Briefly, the 16S rDNA gene sequences were extracted from the uploaded genomes using RNAmmer (Lagesen et al. 2007) and each sequence was subsequently BLASTed (Camacho et al. 2009) against the 16S rRNA gene sequence of each of the currently 10213 type strains available in the TYGS database, which was used as a proxy to find the best 50 matching type strains for each genome uploaded and to subsequently calculate precise distances using the Genome BLAST Distance Phylogeny approach (GBDP) and distance formula d5 (Meier-Kolthoff et al. 2013a). The distances were finally used to determine the 10 closest type strain genomes for each of the uploaded genomes. All pairwise comparisons among the set of genomes were conducted using GBDP and intergenomic distances inferred under the algorithm 'trimming' and distance formula d5 (Meier-Kolthoff et al. 2013a). 100 distance replicates were calculated each. Digital DDH values and confidence intervals were calculated using the recommended settings of the GGDC 2.1 (Meier-Kolthoff et al. 2013a). The resulting intergenomic distances were used to infer a balanced minimum evolution tree with branch support via FASTME 2.1.4 including SPR postprocessing (Lefort et al. 2015). Branch support was inferred from 100 pseudo-bootstrap replicates each. The trees were

rooted at the midpoint (Farris 1972) and visualized with PhyD3 (Kreft et al. 2017). The type-based species clustering using a 70% dDDH radius around each of the 18 type strains was done as previously applied (Liu et al. 2015). Subspecies clustering was done using a 79% dDDH threshold as previously introduced (Meier-Kolthoff et al. 2014). In addition, the level of pairwise genome-based similarities was evaluated based on the average nucleotide identity (ANI) value determined by using online OrthoANu algorithm on EzBioCloud server as described by Yoon et al. (Yoon et al. 2017) available at <https://www.ezbiocloud.net/tools/ani>.

Chemotaxonomy

Strain NA12^T was examined for chemotaxonomic properties found to be of value in *Micromonosporaceae* systematics (Trujillo et al. 2014). Standard chemotaxonomic techniques were used to detect the isomers of diaminopimelic acid (Staneck and Roberts 1974), menaquinones (Collins et al. 1985), polar lipids (Minnikin et al. 1984) and whole cell sugar composition (Lechevalier and Lechevalier 1970). In addition, fatty acid methyl esters (FAMES) were prepared by saponification, methylation and extraction from wet biomass following Miller (1982) with minor modifications from Kuykendall et al. (1988). The FAMES were analysed by gas chromatography (Agilent 6890 instrument) and the resultant peaks automatically integrated. The identities of the peaks were determined using Microbial Identification System (MIDI) Sherlock software version 6.1 and library RTSBA6 according to technical instructions provided by this system (Sasser 1990).

Growth, cultural properties and phenotypic tests

Cultural characteristics were observed on media from the International *Streptomyces* Project (ISP; Shirling and Gottlieb (1966)), modified Bennett's agar (Jones 1949), Czapek's and tryptic soy agar (Difco) after incubation at 28 °C for 14 days. The ISCC-NBS Colour Charts Standard Samples No. 2106 (Kelly 1964) was used to determine the colours of substrate mycelium and diffusible pigments. Micromorphological characteristics were observed by scanning electron microscopy (JEOL JSM 6060) using cultures grown on GYM medium at 28 °C for 21 days. Growth at different temperatures (4, 10, 20, 28, 37, 45 °C), at pH 4.0–12.0 (at intervals of 1.0 pH unit) and in the presence of 0 – 10 % NaCl

(w/v) were determined on ISP 2 medium. $\text{KH}_2\text{PO}_4/\text{HCl}$, $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ and $\text{K}_2\text{HPO}_4/\text{NaOH}$ buffer systems were used for pH adjustment. Degradation tests for casein, gelatin, hypoxanthine, xanthine, xylan, starch and L-tyrosine were conducted according to the methods described by Williams et al. (1983) using ISP 2 (Shirling and Gottlieb 1966) as basal medium. The capacity of the strain to metabolise Tween 40 and 80 was examined using the basal medium outlined by Sierra (1957). In addition, the strain was tested for its ability to oxidise diverse carbon and nitrogen sources and to show resistance to inhibitory compounds using GENIII microplates in an OmniLog device (Biolog Inc., Hayward, USA). To this end, the microplates were inoculated, in duplicate, using IF-C inoculating fluid provided by the manufacturer and a cell density of 85 % transmittance (T). The microplates were incubated at 28 °C for 7 days. The exported data were analysed using the opm package version 1.0.6 for R (Vaas et al. 2013; Vaas et al. 2012).

Results and discussion

The morphological and chemical properties of strain NA12^T are consistent with its classification in the genus *Micromonospora* (Genilloud 2015). Strain NA12^T exhibited good growth on ISP 1, ISP2, ISP3, ISP 4, ISP 6, Bennett's and tryptic soy agar media while poor growth on ISP 7 agar medium. The strain could not grow on ISP 5 and Czapek's agar media. The colour of the substrate mycelia was yellow-orange colour series on all media tested. Well-developed substrate hypha was observed to carry single warty-surfaced spores with diameter of approximately 0.7 – 0.8 µm (Fig. 1). No aerial mycelium was developed. The strain only produced orange-coloured soluble pigment on ISP 7 medium. In addition, strain NA12^T was able to metabolise L-alanine, L-arginine, D-aspartic acid, D-galactose, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, glycerol, D-mannitol, D-melibiose, D-raffinose, L-serine, stachyose and to grow in presence of lincomycin. The strain could tolerate NaCl up to 3 % (w/v). Temperature and pH ranges for the strain were observed as 20 – 37 °C (optimum 28 °C) and 6 – 12 (optimum 7 – 8), respectively. The detailed physiological and biochemical characteristics can be seen in Table 1.

Strain NA12^T contained 3-hydroxydiaminopimelic acid and *meso*-diaminopimelic acid in the cell wall. The whole cell sugars were identified as arabinose, fucose, glucose, mannose, rhamnose and xylose.

The predominant menaquinones were found to be MK-9(H₆) (60 %) and MK-9(H₄) (40 %). The diagnostic polar lipids were identified as phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylglycerol (PG), glycerophospholipid (GPL), amino-phospholipid (amino-PL) and two unidentified phospholipids (PL), corresponding to phospholipid type PII of Lechevalier et al. (1977) (Fig. S1). The cellular fatty acid profile was determined to be composed of *iso*-C_{16:0} (44.9 %), C_{17:1ω8c} (14.8 %), *iso*-C_{15:0} (7.2 %), 10-methyl C_{17:0} (4.5 %), anteiso-C_{15:0} (4.0 %), C_{17:0} (4.0 %), *anteiso*-C_{17:0} (4.0 %), C_{18:1ω8c} (3.0 %) and *iso*-C_{18:0} (1.4%) (Table S1).

The almost complete 16S rRNA gene sequence (1479 bp) of strain NA12^T was deposited in the NCBI GenBank data library under accession number KR780759. A pairwise sequence analysis on EzBiocloud server showed that the strain closely related to *M. radialis* AZ1-13^T, *M. zingiberis* PLAI 1-1^T, *M. craniella* LHW63014^T and *M. endophytica* 202201^T with pairwise sequence identity values ranging from 99.4 % to 99.3 %. Phylogenetic analysis based on ML and MP tree-making algorithms revealed that strain NA12^T is closely related to *M. endophytica* 202201^T and *M. radialis* AZ1-13^T. In the ML tree, strain NA12^T located in a distinct branch at the periphery of the cluster encompassed members of the genus *Phytohabitans* (Inahashi et al. 2010) and the type species, *Salinispora pacifica* CNR-114^T (Ahmed et al. 2013) as well as recently emended species within the genus *Micromonospora* (Nouioui et al. 2018; Li et al. 2019; Kuncharoen et al. 2019) (Fig. 2). These results are in line with the topology of the neighbour joining tree (Fig. S2).

The draft genome sequence of strain NA12^T has been deposited in GenBank with accession number of POTY000000000 and annotated with the RAST server. The draft genome is composed of 609 contigs with N50 value of 23584. The numbers of coding sequences and RNAs are 6504 and 93, respectively. The total genome size is 7.3 Mb and the G + C content is 71.5%. The phylogenomic tree constructed on TYGS (<https://tygs.dsmz.de/>) showed that strain NA12^T formed a distinct cluster within the clade composed of its close neighbours indicated by pairwise 16S rRNA gene sequence analysis as well as the type species of *Micromonospora phaseoli* CGMCC 4.7038^T, *Micromonospora sediminimaris* CGMCC 4.3550^T and *Micromonospora maris* AB-18-032^T (Fig. 3). To confirm the taxonomic position of strain NA12^T as a novel species, *in silico* DNA-DNA hybridization values between strain NA12^T and

its close phylogenetic neighbours were calculated and found well below the threshold value of 70 % recommended by Wayne et al. (1987) to delineate prokaryotic genomic species (Table 2). In addition, the average nucleotide identity values (ANI) between strain NA12^T and its close phylogenetic neighbours found to be 86.9 % for *M. radialis* AZ1-13^T, 87.3 % for *M. zingiberis* PLAI 1-1^T, 86.5 % for *M. craniella* LHW63014^T and 86.6 % for *M. endophytica* 202201^T, which are clearly below the 95 – 96 % threshold used for prokaryotic species delineation (Chun and Rainey 2014; Richter and Rosselló-Móra 2009). Both digital DDH and ANI values as well as phylogenetic trees confirmed that strain NA12^T is a novel member of the genus *Micromonospora*, for which the name *Micromonospora craterilacus* sp. nov. is proposed.

Description of *Micromonospora craterilacus* sp. nov.

Micromonospora craterilacus (cra.te.ri.la'cus. L. masc. n. crater a crater; L. masc. n. lacus a lake; N.L. gen. n. craterilacus of a crater lake)

Aerobic, Gram-positive actinobacterium which forms extensively branched substrate mycelium carrying single warty-surfaced spores with diameter of approximately 0.7 – 0.8 µm. Grows well on ISP 1, ISP2, ISP3, ISP 4, ISP 6, Bennett's and tryptic soy agar media. The substrate mycelium colour is yellow to orange on all media tested. The temperature range for growth is 20 – 37 °C (optimum 28 °C) and pH range is 6 – 12 (optimum 7 – 8). Tolerates NaCl up to 3 %. Positive for hydrolysis of aesculin and reduction of nitrate while negative for hydrolysis of allantoin, arbutin and urea. Casein, gelatin, starch, Tween 40 and 80 are degraded but hypoxanthine, L-tyrosine, xanthine and xylan are not. Metabolizes L-alanine, L-arginine, D-aspartic acid, D-galactose, L-galactonic acid-γ-lactone, D-galacturonic acid, N-acetyl-D-galactosamine, D-gluconic acid, D-glucuronic acid, N-acetyl-D-glucosamine, D-glucose-6-phosphate, glucuronamide, glycerol, D-lactic acid methyl ester, D-mannitol, D-melibiose, N-acetyl-neuraminic acid, quinic acid, D-raffinose, L-serine and stachyose and grows in the presence of lincomycin. The cell wall peptidoglycan contains 3-hydroxydiaminopimelic acid and meso-diaminopimelic acid. Whole cell sugars are arabinose, fucose, glucose, mannose, rhamnose and xylose. The major fatty acids (>10 %) are *iso*-C_{16:0} and C_{17:1ω8c}. Predominant menaquinones (≥ 40%) are MK-9(H6) and MK-9(H4). The polar lipids are phosphatidylethanolamine (PE),

diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylglycerol (PG), glycerophospholipid (GPL), amino-phospholipid (amino-PL) and two unidentified phospholipids (PL). The G+C content of the type strain is 71.5 % and total size of its genome is 7.3 Mb.

The type strain NA12^T (DSM 100982^T= KCTC 39647^T) was isolated from coastal sediment of Nemrut Lake. The GenBank accession number for the 16S rRNA gene and whole genome sequences of strain NA12^T are KR780759 and POTY000000000, respectively.

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Author contributions

HA, NS and KI designed the study. HA and IN conducted physiological experiments. HPK, HA, IN, JMI designed chemotaxonomic analyses. HA carried out genome sequence analysis. DC carried out scanning electron microscopy analysis.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary material

Fig. S1 Two-dimensional thin-layer chromatography of polar lipid pattern of strain NA12^T stained with molybdotophosphoric acid spray (Sigma P1518). Key: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; APL, aminophospholipid; GPL, glycerophospholipid; PL1-2, unidentified phospholipid. Solvent 1: chloroform : methanol : distilled water (65:25:4); solvent 2: chloroform : glacial acetic acid : methanol : distilled water (80:12:15:4).

Fig. S2 Neighbour joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between strain NA12^T and the type strains of the most closely related species of the family *Micromonosporaceae*. *Catellatospora citrea* DSM 44097^T was used as outgroup. The numbers above the branches are support values when larger than 50% from bootstrapping. The branches are scaled in terms of the expected number of substitutions per site

Table S1 Cellular fatty acid profiles (%) of strain NA12^T and its close relatives

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