


# *Nonomuraea insulae* sp. nov., isolated from forest soil

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**Abstract** Strain H2R21<sup>T</sup>, a novel actinobacterium, isolated from a forest soil sample collected from Heybeliada, Istanbul, Turkey, and a polyphasic approach was used for characterisation of the strain. Chemotaxonomic and morphological characterisation of strain H2R21<sup>T</sup> indicated that it belongs to the genus *Nonomuraea*. 16S rRNA gene sequence similarity showed that the strain is closely related to *Nonomuraea purpurea* 1SM4-01<sup>T</sup> (99.1%) and *Nonomuraea solani* CGMCC 4.7037<sup>T</sup> (98.4%). DNA–DNA relatedness

values were found to be lower than 70% between the isolate and its phylogenetic neighbours *N. purpurea* 1SM4-01<sup>T</sup>, *N. solani* CGMCC 4.7037<sup>T</sup> and *Nonomuraea rhizophila* YIM 67092<sup>T</sup>. The whole cell hydrolysates of strain H2R21<sup>T</sup> were found to contain meso-diaminopimelic acid as the diagnostic diamino acid and glucose, madurose, mannose and ribose as the cell sugars. The polar lipids were identified as phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, hydroxy-phosphatidylethanolamine, dihydroxy-phosphatidylethanolamine, phosphatidylinositol, gly-cophosphatidylinositol, two gly-cophospholipids and two unidentified lipids. The predominant

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menaquinones were identified as MK-9(H<sub>4</sub>) and MK-9(H<sub>6</sub>). The major fatty acids were found to be *iso*-C<sub>16:0</sub>, *iso*-C<sub>16:0</sub> 2OH and C<sub>17:0</sub> 10-*methyl*. On the basis of DNA–DNA relatedness data and some phenotypic characteristics, it is evident that strain H2R21<sup>T</sup> can be distinguished from the closely related species in the genus *Nonomuraea*. Thus, it is concluded that strain H2R21<sup>T</sup> represents a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea insulae* sp. nov. is proposed. The type strain is H2R21<sup>T</sup> (= DSM 102915<sup>T</sup> = CGMCC 4.7338<sup>T</sup> = KCTC 39769<sup>T</sup>).

**Keywords** *Actinobacteria* · *Nonomuraea insulae* sp. nov. · Polyphasic taxonomy · 16S rRNA gene

## Introduction

The genus *Nonomuraea*, containing aerobic, Gram-stain positive and non-acid-fast bacteria was proposed by Zhang et al. (1998) and belongs to the family *Streptosporangiaceae*. Members of the genus *Nonomuraea* form extensively branched substrate and aerial mycelia, which differentiate into hooked, spiral, or straight chains of spores (Kämpfer et al. 2005; Fang et al. 2017). Currently, the genus consists nearly fifty and 2 subspecies with validly published names (<http://www.bacterio.net/nonomuraea.html>). The members of the genus are characterised by the presence of *meso*-diaminopimelic as the diamino acid and madurose as the diagnostic sugar in whole cell sugar patterns (chemotype IIIB sensu Lechevalier and Lechevalier 1970). Characteristically, members of the genus are rich in *iso*-C<sub>16:0</sub> and C<sub>17:0</sub> 10-methyl branched chain fatty acids; contain MK-9(H<sub>4</sub>), MK-9(H<sub>2</sub>) and MK-9(H<sub>0</sub>) as predominant menaquinones; and have diphosphatidylglycerol, phosphatidylethanolamine and hydroxylated phosphatidylethanolamine as predominant phospholipids (Kämpfer 2012). Members of the genus *Nonomuraea* have been recently isolated from various habitats such as soil (Wang et al. 2014; Sriprechasak et al. 2017), rhizosphere (Shen et al. 2016; Wang et al. 2017), cave (Nakaew et al. 2012; Fang et al. 2017), root (Rachniyom et al. 2015; Li et al. 2017), coastal sediment (Xi et al. 2011), plant tissues (Li et al. 2011; Niemhom et al. 2017) and mangrove sediment (Suksaard et al. 2016). Additionally, it is known that some secondary metabolites such as

maduramycin, pyralomicin, the glycopeptide antibiotic A40926 and brartemicin are produced by members of the genus *Nonomuraea* (Sungthong and Nakaew 2015; Dalmastri et al. 2016).

In this study, we aimed to isolate novel actinobacteria from soil samples collected from Heybeliada island. The results of a polyphasic characterisation of strain H2R21<sup>T</sup>, one of the isolated strains, indicated that it represents a novel species in the genus *Nonomuraea*, here named *Nonomuraea insulae* sp. nov.

## Materials and methods

### Isolation and maintenance of strains

A forest soil sample was collected from Heybeliada (GPS coordinates 40°52′22.30″N and 29°04′33.80″E), İstanbul, Turkey. The sample was air-dried at room temperature for 14 days, heated at 60 °C for 20 min and suspended with Ringer’s solutions (Oxoid) to prepare serial dilutions. The suspension was spread on humic acid-vitamin (HV) agar (Hayakawa and Nonomura 1987) supplemented with filter sterilised cycloheximide (50 µg/ml), nalidixic acid (10 µg/ml) and rifampicin (0.5 µg/ml). After incubation at 28 °C for 2 weeks, strain H2R21<sup>T</sup> was purified on N-Z Amine agar (DSMZ Medium 554) for further study and stored in glycerol (20%, v/v) at – 80 °C for long term preservation. The reference strains *Nonomuraea solani* DSM 45729<sup>T</sup> and *Nonomuraea rhizophila* DSM 45382<sup>T</sup> were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures) and *Nonomuraea purpurea* 1SM4-01<sup>T</sup> was obtained from Dr. Kannika Duangmal (Kasetsart University).

### Morphological, cultural and physiological characteristics

The micromorphological properties of the strain were observed by examining gold coated dehydrated specimens grown on ISP 3 agar medium for 4 weeks using a JEOL JSM 6060 instrument. Cultural characteristics including growth, colours of the substrate mycelia and aerial mycelia, and soluble pigmentation were determined on ISP media 2–7 (Shirling and Gottlieb 1966), modified Bennett’s agar (MBA; Jones 1949), Czapek’s agar (Waksman 1967), nutrient agar (Waksman

1961) and tryptic soy agar (TSA; Difco) after 14 days of incubation at 28 °C using ISCC-NBS colour charts (Kelly 1964). Growth was determined at various temperatures (4, 10, 20, 28, 37, 40, 45, 50 and 55 °C), different pH (4.0–12.0) and tolerance of NaCl (up to 10%, w/v, at intervals of 1.0% unit) on yeast extract-malt extract (ISP 2) agar. The incubation time for temperature tests was 14 days, while the time for pH tests and tolerance of NaCl was 28 days. Degradations of Tweens 20, 40 and 80 were evaluated using the methods described by Nash and Krent (1991) while the other degradation tests (adenine, hypoxanthine, guanine, starch, xylan and xanthine) were carried out as described by Williams et al. (1983). Utilisation of carbohydrates as sole carbon sources at a final concentration of 1% (w/v) was performed according to the methods proposed by Shirling and Gottlieb (1966). Nitrogen source utilisation was examined according to Williams et al. (1983) with a final concentration of 0.1% (w/v) for each nitrogen source.

#### Chemotaxonomic characterisation

Biomass for chemotaxonomic studies of the isolate was prepared by growing cultures in N-Z Amine broth for 2 weeks at 28 °C with shaking at 150 rpm, harvested and washed three times with distilled water before freeze-drying. The diaminopimelic acid isomers and whole cell sugars of strain H2R21<sup>T</sup> were analysed according to Stanek and Roberts (1974) and Lechevalier and Lechevalier (1970) by thin layer chromatography (TLC), respectively. Polar lipids were extracted and analysed using the method of Minnikin et al. (1984) by two-dimensional TLC. Isoprenoid quinones were extracted and purified from freeze-dried cells by following the procedure of Collins (1985) and analysed by high performance liquid chromatography (HPLC) (Kroppenstedt 1982). The cellular fatty acids were extracted, methylated and analysed by gas chromatography using an Agilent Technologies 6890 N instrument, fitted with an autosampler and a 6783 injector, following the instructions for the Sherlock Microbial identification (MIDI) system (Sasser 1990; Kämpfer and Kroppenstedt 1996); the fatty acid methyl ester peaks were quantified using the TSBA 5.0 database.

#### DNA preparation, amplification and determination of 16S rRNA gene sequence

Genomic DNA extraction, PCR-mediated amplification and 16S rRNA gene sequencing were carried out as described by Chun and Goodfellow (1995) using an ABI PRISM 3730 XL automatic sequencer. An almost complete 16S rRNA gene sequence (1463 bp) was deposited in the GenBank database and aligned with corresponding sequences of representative type strains of the genus *Nonomuraea* retrieved from the EzBio-Cloud server (Yoon et al. 2017). Phylogenetic trees were constructed with the neighbour-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) algorithms in MEGA version 7.0 software (Kumar et al. 2016). Evolutionary distances were calculated using model of Jukes and Cantor (1969) and topologies of the resultant trees evaluated by bootstrap analysis (Felsenstein 1985) based on 1000 resamplings. *Thermopolyspora flexuosa* DSM 43186<sup>T</sup> (AY039253) was used as outgroup.

#### DNA base composition and DNA–DNA hybridization

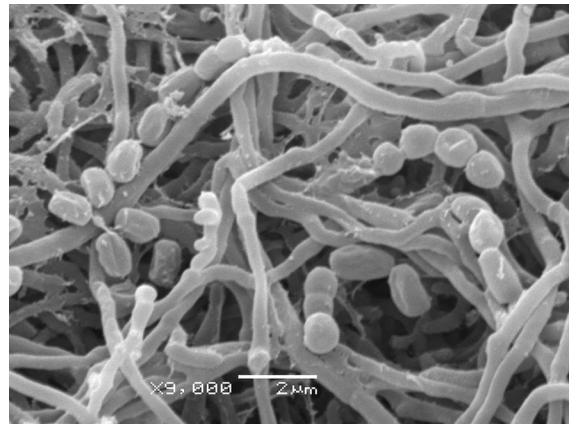
In order to determine G+C content, genomic DNA of strain H2R21<sup>T</sup> was extracted and purified by thermal denaturation ( $T_m$ ) method as described by Mandel and Marmur (1968) using *Escherichia coli* JM109 as the reference strain. DNA–DNA hybridization was performed spectrophotometrically by DNA reassociation kinetics as described by De Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983). Genomic DNAs from *N. purpurea* 1SM4-01<sup>T</sup>, *N. solani* DSM 45729<sup>T</sup>, *N. rhizophila* DSM 45382<sup>T</sup> and strain H2R21<sup>T</sup> were prepared according to the method of Marmur (1961); the purity and quality of each DNA preparation were checked by determination of the absorbance ratios at 260/280 nm (1.8–1.9) and at 260/230 nm (2.0–2.3) (according to Marmur 1961) and by the melting curves, which include hyperchromicity values from 30 to 40% (Mandel and Marmur 1968). Purified genomic DNAs were sonicated using a Sartorius Labsonic M to generate DNA fragments of between 400 and 800 bp in size. Sheared genomic DNA (about 100 mg) from each sample was added to 2 X SSC buffer and then denatured by raising the temperature to 100 °C and

cooled to the melting temperature in the spectrophotometer. The samples were kept at the optimal renaturation temperature for 3 min and the absorbance at 260 nm was recorded at 30 s intervals for a total of 30 min. The initial reassociation kinetics were determined by using linear regression analysis. The DNA hybridization values (%) for these microorganisms were calculated using the equation described by De Ley et al. (1970). Hybridizations were replicated five times for each sample. The highest and lowest values for each sample were excluded and the remaining three values were used for calculation of the mean level of DNA–DNA relatedness.

## Results and discussion

Strain H2R21<sup>T</sup> was observed to show good growth on ISP 2, ISP 3, ISP 6, TSA, MBA and nutrient agar media, moderate growth on ISP 7, weak growth on ISP 5 and Czapek's agar media but no growth on ISP 4 medium. The colour of the aerial mycelia was white on ISP 2, ISP 3 and Czapek's agar media (Table S1). It was determined that strain H2R21<sup>T</sup> produces short spore chains with smooth surfaces on aerial mycelia (Fig. 1). The extensively branched substrate mycelium was observed to be in the cream to brown colour series on all tested media. No soluble or melanin pigments were observed on any of the tested media. The strain was found to grow at a temperature range of 20–37 °C and optimally at 28 °C. Growth was observed in the pH range of 6.0–9.0 (optimum at pH 7.0). Strain H2R21<sup>T</sup> was found to tolerate NaCl up to 2% (w/v). Detailed physiological and biochemical properties are given in Table 1 and the species description.

The chemotaxonomic properties of strain H2R21<sup>T</sup> were found to be consistent with the characteristics of members of the genus *Nonomuraea* (Lechevalier and Lechevalier 1970; Kämpfer 2012). The novel strain contains *meso*-diaminopimelic acid in the cell wall peptidoglycan and glucose, mannose, ribose and madurose in whole cell hydrolysates. The main menaquinones were identified as MK-9(H<sub>4</sub>) (58.4%), MK-9(H<sub>6</sub>) (9.7%) and MK-9(H<sub>2</sub>) (5.5%), whilst other components could not be definitively identified. The



**Fig. 1** Scanning electron micrograph of strain H2R21<sup>T</sup> grown on ISP 3 agar after 4 weeks at 28 °C showing aerial mycelium differentiating into smooth surfaces oval-shaped spore chains. Bar, 2 μm

polar lipids were identified as phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, hydroxy-phosphatidylethanolamine, dihydroxy-phosphatidylethanolamine, phosphatidylinositol, glycoposphatidylinositol, two glycopospholipids and two unidentified lipids (Fig. S1). The major cellular fatty acids ( $\geq 10\%$ ) were identified as *iso*-C<sub>16:0</sub> (26.2%), *iso*-C<sub>16:0</sub> 2OH (24.9%) and C<sub>17:0</sub> 10-methyl (12.5%); the detailed fatty acid profile is listed in Table S2 in comparison with those of closely related type strains. Mycolic acids were absent.

Phylogenetic analysis based on 16S rRNA gene sequences of strain H2R21<sup>T</sup> and closely related type strains showed that the strain forms a subclade with *N. purpurea* 1SM4-01<sup>T</sup> (Fig. 2), which shares high 16S rRNA gene sequence similarity with the strain (99.1% similarity; 13 nt differences at 1441 sites). The 16S rRNA gene sequence similarity between strain H2R21<sup>T</sup> and *N. solani* CGMCC 4.7037<sup>T</sup> is 98.4% (23 nt differences at 1441 sites), and with both *Nonomuraea stahlianthi* SC1-1<sup>T</sup> and *N. rhizophila* YIM 67092<sup>T</sup> is 98.3% (24 nt differences at 1441 sites and 25 nt differences at 1443 sites, respectively). The association with *N. purpurea* 1SM4-01<sup>T</sup> was also supported by the maximum-likelihood and maximum-parsimony algorithms (Figs. S2, S3). DNA–DNA hybridization experiments were carried out between strain H2R21<sup>T</sup> and its phylogenetic neighbours, *N.*

**Table 1** Differential characteristics of strain H2R21<sup>T</sup> and type strains of closely related species of the genus *Nonomuraea*

Characteristics	1	2	3	4
Maximum NaCl tolerance (% w/v)	2	2	2	7
pH tolerance	6.0–9.0	7.0–9.0	7.0–9.0	6.0–8.0
Biochemical tests				
Allantoin	+	–	+	+
Degradation of (% w/v)				
Hyoxanthine (0.4%)	+	–	–	+
Starch (1%)	+	–	+	–
Guanine (0.05%)	+	–	+	–
Carbon source utilization (1.0% w/v)				
D-Arabinose	+	+	+	–
D-Fructose	–	+	+	+
D-Sorbitol	–	+	+	–
D-Galactose	–	+	+	–
D-Mannose	+	–	+	+
D-Melezitose	–	+	+	+
D-Melibiose	–	+	+	+
D-Ribose	–	+	–	–
Dextran	–	+	+	+
Inulin	–	–	+	+
L-Arabinose	–	+	+	+
Lactose	–	–	+	+
L-Glutamine	–	+	+	+
Maltose	–	+	+	+
Sucrose	+	+	+	–
Xylitol	–	–	+	+
Xylose	+	+	+	–
Use of sole nitrogen sources 0.1% (w/v)				
Glycine	+	–	+	+
L-Alanine	+	–	+	+
L-Arginine	–	–	+	+
L-Asparagine	–	–	+	+
L-Cysteine	+	–	+	+
L-Hydroxyproline	+	–	+	+
L-Serine	–	–	+	+
Major menaquinones (> 10%)	MK-9(H <sub>4</sub> )	MK-9(H <sub>4</sub> ) <sup>a</sup>	MK-9(H <sub>4</sub> ), MK-9(H <sub>6</sub> ) <sup>b</sup>	MK-9(H <sub>4</sub> ), MK-9(H <sub>6</sub> ) <sup>c</sup>
Polar lipid profile	DPG, PME, PE, OH-PE, 2OH-PE, PI, GPI	DPG, OH-PE, OH-PME, PE, PG, PI, PIM <sup>a</sup>	DPG, PME, PE, OH-PME, OH-PE, PI, PIM <sup>b</sup>	DPG, PME, PE, PG, PI <sup>c</sup>
Major fatty acids (> 10%)	<i>iso</i> -C <sub>16:0</sub> , <i>iso</i> -C <sub>16:0</sub> 2OH	C <sub>17:1cis</sub> 9, <i>iso</i> -C <sub>16:0</sub> 2OH, <i>iso</i> -C <sub>16:0</sub>	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:0</sub> 10-methyl, <i>iso</i> -C <sub>16:1</sub> G, <i>iso</i> -C <sub>16:0</sub> 2OH	C <sub>17:0</sub> 10-methyl, <i>iso</i> -C <sub>16:0</sub> 2OH
Whole-cell sugars	Glu, Man, Mad, Rib	Mad, Man, Rib <sup>a</sup>	Mad, Glu <sup>b</sup>	Mad, Glu, Man, Rib, Gal <sup>c</sup>

Strains: 1, H2R21<sup>T</sup>; 2, *N. purpurea* 1SM4-01<sup>T</sup>; 3, *N. solani* DSM 45729<sup>T</sup>; 4, *N. rhizophila* DSM 45382<sup>T</sup>. Symbols: +, positive; –, negative

DPG diphosphatidylglycerol, PME phosphatidylmethylethanolamine, PE phosphatidylethanolamine, OH-PE hydroxy-phosphatidylethanolamine, 2OH-PE dihydroxy-phosphatidylethanolamine, PI phosphatidylinositol, GPI glycoposphatidylinositol, OH-PME hydroxy-phosphatidylmonomethylethanolamine, PG phosphatidylglycerol, PIM phosphatidylinositol mannoside, Glu glucose, Man mannose, Mad madurose, Rib ribose, gal galactose

Data taken from: <sup>a</sup>Suksaard et al. (2016); <sup>b</sup>Wang et al. (2013); <sup>c</sup>Zhao et al. (2011)

*purpurea* 1SM4-01<sup>T</sup>, *N. solani* DSM 45729<sup>T</sup> and *N. rhizophila* DSM 45382<sup>T</sup>. The values between them were  $28.0 \pm 2.4\%$ ,  $38.1 \pm 3.1\%$  and  $40.4 \pm 2.9\%$ , respectively, which are lower than the cut-off value of 70%, the recommended threshold value for delineation of same prokaryotic genomic species (Wayne et al. 1987). The DNA G+C content of strain H2R21<sup>T</sup> was found to be 69.6 mol%.

Both phylogenetic and chemotaxonomic analyses, as well as phenotypic characterisation, indicated that the strain is a member of the genus *Nonomuraea*. However, strain H2R21<sup>T</sup> differs from its closely related neighbours in terms of some chemotaxonomic properties, such as the lack of *C*<sub>17:1cis9</sub> in its fatty acid profile, a major cellular fatty acid of *N. purpurea* 1SM4-01<sup>T</sup>, and having glucose and mannose in whole cell hydrolysates unlike *N. purpurea* 1SM4-01<sup>T</sup> and *N. solani* DSM 45729<sup>T</sup>, respectively. In addition, the strain can be differentiated from closely related species in terms of several phenotypic characteristics, such as inability to utilise D-melezitose, D-melibiose, dextran, L-arabinose, L-glutamine and maltose as sole carbon source, along with other phenotypic characters as given in Table 1.

It is evident on the basis of genotypic and phenotypic properties that strain H2R21<sup>T</sup> can be distinguished from its close neighbours *N. purpurea*, *N. solani* and *N. rhizophila*. Therefore, it is proposed that strain H2R21<sup>T</sup> be classified in the genus *Nonomuraea* as *Nonomuraea insulae* sp. nov. The Digital Protologue database TaxoNumber for strain H2R21<sup>T</sup> is TA00414.

### Description of *Nonomuraea insulae* sp. nov.

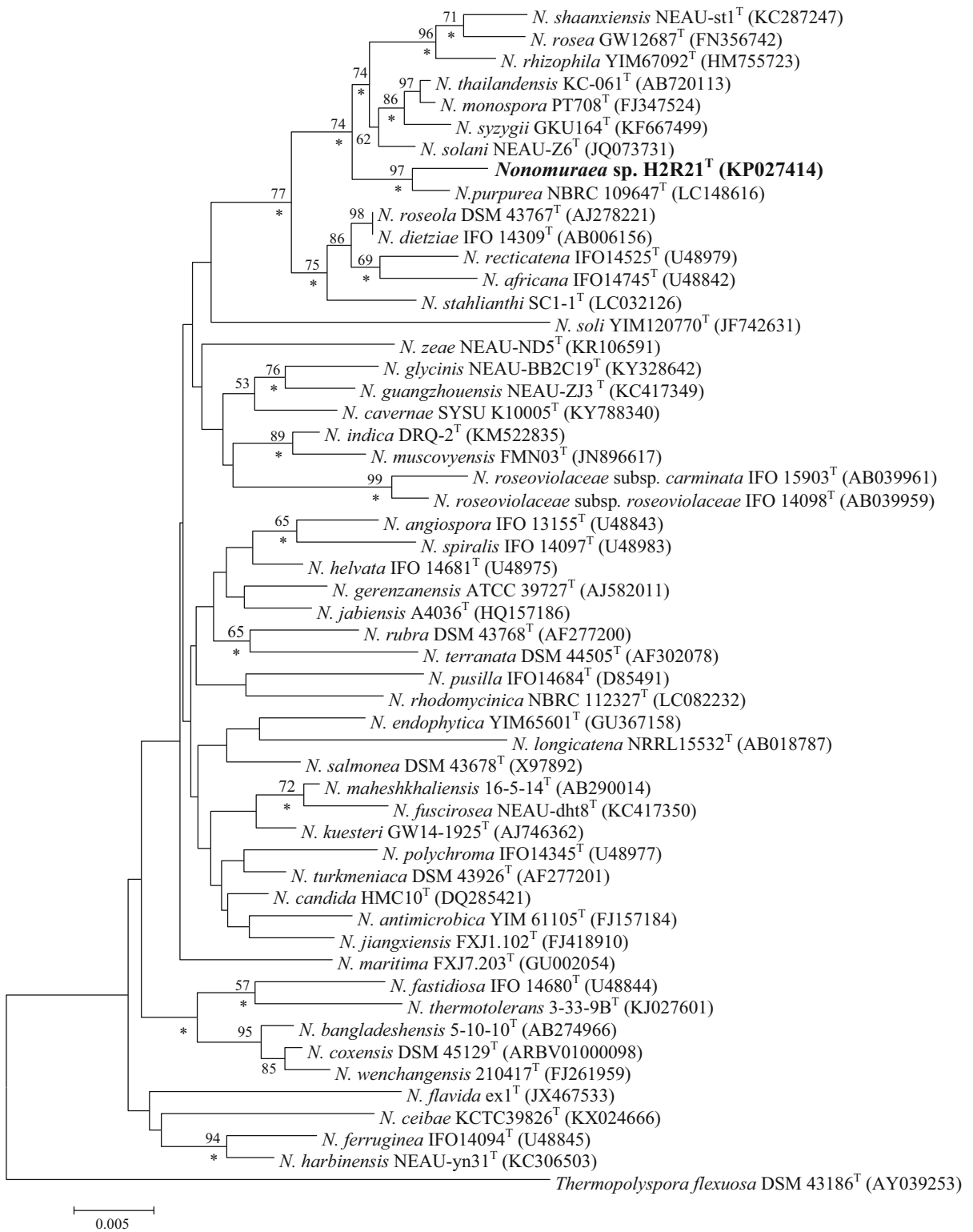
*Nonomuraea insulae* sp. nov. (in'su.lae. L. gen. n. *insulae*, belonging to the island of Heybeliada in the Marmara Sea, from where the type strain was isolated).

Aerobic, Gram-stain positive, non-acid-fast, non-motile actinobacterium that forms an extensively branched substrate mycelia. The aerial mycelium is white and forms short spore ( $0.6\text{--}0.8 \times 1.2\text{--}1.4 \mu\text{m}$ ) chains with smooth surfaces. Good growth occurs on ISP 2, ISP 3, ISP 6, modified Bennett's agar, tryptic soy agar and nutrient agar media, moderate growth on

**Fig. 2** Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the position of strain H2R21<sup>T</sup> within members of the genus *Nonomuraea*. Asterisks indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Numbers at the nodes indicate levels of bootstrap support (%); only values  $\geq 50\%$  are shown. *Thermopolyspora flexuosa* DSM 43186<sup>T</sup> (AY039253) was used as the outgroup. The GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per site

ISP 7 medium and weak growth on ISP 5 and Czapek's agar media; does not grow on ISP 4 medium. No diffusible pigment is observed on any of the above media and melanoid pigments are not produced on ISP 6 or ISP 7 media. Growth occurs at 20–37 °C (optimum at 30 °C), at pH 6.0–9.0 (optimum 7.0) and in the presence of 0–2% (w/v) NaCl. Allantoin and arbutin are hydrolysed, but not nitrate. Guanine, hypoxanthine, starch and xylan are degraded, but not adenine, xanthine or Tweens 20, 40 and 80. D-Arabinose, D-cellobiose, dextrin, glucose, myo-inositol, D-mannose, mannitol, L-rhamnose, sucrose and xylose are utilised as sole carbon and energy sources, but not L-arabinose, dextran, D-galactose, L-glutamine, D-fructose, inulin, lactose, maltose, D-melezitose, D-melibiose, D-ribose, D-sorbitol or xylitol. L-alanine, L-cysteine, glycine, L-histidine, L-hydroxyproline, Alfa-isoleucine, L-lysine, L-methionine, DL-phenylalanine, L-phenylalanine, L-proline, L-threonine and L-tyrosine are utilised as sole nitrogen sources, but not glycine, L-arginine, L-asparagine or L-serine. The predominant menaquinone is MK-9(H<sub>4</sub>), and MK-9(H<sub>6</sub>) and MK-9(H<sub>2</sub>) are also present as minor menaquinones. The polar lipids profile includes phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylethanolamine, hydroxy-phosphatidylethanolamine, dihydroxy-phosphatidylethanolamine, phosphatidylinositol, glycoposphatidylinositol, two glycopospholipids and two unidentified lipids. The whole cell sugars are glucose, mannose, ribose and madurose. The major fatty acids are *iso*-C<sub>16:0</sub>, *iso*-C<sub>16:0</sub> 2OH and C<sub>17:0</sub> 10-methyl. The G+C content of the genomic DNA of the type strain is 69.6 mol%.

The type strain, H2R21<sup>T</sup> (= DSM 102915<sup>T</sup> = CGMCC 4.7338<sup>T</sup> = KCTC 39769<sup>T</sup>), was isolated from a soil sample from Heybeliada, an island in the Marmara Sea, Turkey. The GenBank accession



number for the 16S rRNA gene sequence of the strain is KP027414.

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

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