Roseomonas elaeocarpi sp. nov., isolated from olive (Elaeocarpus hygrophilus Kurz.) phyllosphere

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An aerobic, Gram-stain-negative, coccobacillus-shaped, non-endospore-forming, pink-pigmented bacterium, designated PN2T, was isolated from an olive leaf. The strain grew at 15–35 °C with an optimum temperature for growth at 30 °C, and at pH 5.0–7.5 with an optimum pH for growth at 6.0. Growth was observed in the presence of up to 1.02 % (w/v) NaCl. The major fatty acids were C 19 : 0 cyclo ω8c, C 16 : 0 and C 18 : 1 ω7c. The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, unknown aminolipids, an unknown phospholipid and an unknown lipid. The respiratory quinone was ubiquinone-10. The DNA G + C content of strain PN2T was 70.4 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain PN2T was closely related to members of the genus Roseomonas and shared highest similarity with Roseomonas mucosa ATCC BAA-692 T (96.5 %), Roseomonas gilardii subsp. gilardii ATCC 49956 T (96.2 %) and Roseomonas gilardii subsp. rosea ATCC BAA-691 T (96.2 %). Furthermore, the DNA–DNA relatedness value between strain PN2T and the closest related species R. mucosa ATCC BAA-692 T was 27 %. These data allowed the phenotypic and genotypic differentiation of strain PN2T from its closest phylogenetic neighbour (R. mucosa ATCC BAA-692 T). Based on phenotypic and genotypic characteristics, strain PN2T is classified as representing a novel species of the genus Roseomonas for which the name Roseomonas elaeocarpi sp. nov. is proposed. The type strain is PN2 T (=BCC 44864 T =NBRC 107871 T).

Bacteria of the genus Roseomonas were first described by Rihs et al. (1993). They are Gram-stain-negative with plump coccus, coccobacillus or short rod shapes. Based on 16S rRNA gene sequence analysis, this genus belongs to the Alphaproteobacteria (Weyant & Whitney, 2005). At the time of writing, 23 species and two subspecies with validly published names are members of the genus Roseomonas, recently recognized species including Roseomonas aerophila (Kim et al., 2013), ‘Roseomonas musae’ (Nutaratat et al., 2013), Roseomonas tokyoensis (Furuhata et al., 2013), ‘Roseomonas aceris’ (Tonouchi & Tazawa, 2014), Roseomonas alkaliterae (Dong et al., 2014), Roseomonas rhizospherae (Chen et al., 2014), ‘Roseomonas sediminicola’ (He et al., 2014) and Roseomonas soli (Kim & Ka, 2014). There have been reports on the isolation of Roseomonas species from various sources such as medical and environmental samples including plant phyllosphere. Together, these findings indicate that members of the genus Roseomonas exist widely in nature. Rihs et al. (1993) reported that the DNA G + C content of the genus Roseomonas ranges from 65 to 71 mol%. However, Kim et al. (2013) showed the DNA G + C content of R. aerophila was 73.0 mol%, slightly above values previously reported, and Dong et al. (2014) reported a 63.0 mol% DNA G + C content for R. alkaliterae, a value that was slightly below values for species in the genus Roseomonas. From a literature review, the highest content for a
major fatty acid of this genus was for \( \text{C}_{18:1} \). \( \Delta^7 \) under the conditions studied (Gallego et al., 2006; Yoon et al., 2007; Furuhata et al., 2008, 2013; Yoo et al., 2008; Zhang et al., 2008; Venkata Ramana et al., 2010; Lopes et al., 2011; Kim et al., 2013; Nutaratat et al., 2013; Tonouchi & Tazawa, 2014; Chen et al., 2014; Dong et al., 2014; He et al., 2014, Kim & Ka, 2014) except Roseomonas lacus TH-G33\(^T\), which had iso-\( \text{C}_{16:0} \) as the predominant fatty acid (Jiang et al., 2006). Cells contained ubiquinone 10 (Q-10) as the major quinone (Kim et al., 2013; Dong et al., 2014; Chen et al., 2014). With regard to the polyamine pattern, members of the genus Roseomonas have spermidine as the predominant compound (Sánchez-Porro et al., 2009).

During the course of isolation and characterization of phyllospheric methylotrophic bacteria, strain PN2\(^T\) was obtained as a result of a selective isolation technique used to isolate such bacteria in our laboratory. However, methylotrophic growth was not observed after cultivation in liquid medium containing methanol as sole carbon source. According to Nutaratat et al. (2013), strain PN2\(^T\) is therefore considered to be a non-methylotrophic bacterium, even though it was isolated in methanol-containing medium. In this study, we report on the polyphasic taxonomic characterization of strain PN2\(^T\), isolated from an olive (Elaeocarpus hygrophilus Kurz.) leaf collected from Ubon Ratchathani province, Thailand. On the basis of phenotypic characteristics, chemotaxonomic data and phylogenetic analysis, the isolate is considered to represent a novel species of the genus Roseomonas.

Strain PN2\(^T\) was obtained after isolation on ammonium mineral salts (AMS) medium (Corpe, 1985) supplemented with 1 % (v/v) methanol by the leaf imprinting method (Chanprame, et al., 1996) and incubated at 30 °C for 3 days. The strain was maintained on glycerol peptone agar (GPA) medium (1 % glycerol, 1 % peptone and 1.5 % agar) or yeast extract mannitol agar (YMA) medium (0.1 % yeast extract, 1 % mannitol, 0.065 % K\( _2 \)HPO\(_4 \), 3H\(_2\)O, 0.02 % MgSO\(_4 \), 0.24 % beef extract). A positive result was evaluated as the blackening of the strip after 2 days of incubation. Physiological tests such as growth at different temperatures (10, 15, 20, 22, 25, 28, 30, 33, 35, 37, 40, 42, 45, 48 and 50 °C), pH (using biological buffers: citrate-phosphate buffer for pH 4.0–7.0, phosphate buffer for pH 7.5–8.0 and glycine-NaOH buffer for pH 8.5–9.0) and NaCl concentrations (0.02–10.02 %) were performed in glycerol peptone (GP) broth. Acid production from D-glucose, D-mannitol, D-sorbitol, D-xyllose, lactose and sucrose was determined in Andrade’s carbohydrate broth. Hydrolysis of casein (0.5 %, w/v), CM-cellulose (1.0 %, w/v), guanine (0.5 %, w/v), hypoxanthine (0.5 %, w/v), starch (1.5 %, w/v), tyrosine (0.5 %, w/v) and xanthine (0.5 %, w/v) was analysed by using the method of Gordon et al. (1974). Tween 80 (1.0 %, v/v) hydrolysis was analysed according to Sierra (1957) whereas chitin (0.4 %, w/v) hydrolysis was analysed via a protocol modified from that of Hus & Lockwood (1975). The oxidation of 71 carbon sources and tests for determining the sensitivity to different chemicals were determined by using Biolog GENIII Microplates. Enzyme activity and other biochemical characteristics were tested using the API ZYM and API 20NE galleries (bioMérieux), respectively, following the manufacturer’s instructions. Susceptibility to antibiotics was tested on Mueller–Hinton agar plates by using antibiotic discs (Kirby–Bauer method; Bauer et al., 1966). Disks containing the following amounts of antibiotic were used: 10 μg ampicillin, 30 μg chloramphenicol, 30 μg kanamycin, 10 U penicillin G, 10 μg streptomycin, and 10 and 30 μg tetracycline.

The 16S rRNA gene of strain PN2\(^T\) was PCR-amplified from bacterial genomic DNA by using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARC-3′) (Lane, 1991). PCR products were purified using a Gel/PCR DNA Fragment Extraction kit (Geneaid) and sequenced at the 1st Base Laboratory (Malaysia). The sequence obtained was edited using the Chromas 2.4.3 program (http://www.techneylium.com.au/chromas.html). For comparative purposes, the 16S rRNA gene sequence of strain PN2\(^T\) was compared with sequences of bacterial type strains from the database of the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012) and were aligned by clustal w version 1.83 (Thompson et al., 1994). Gaps in the sequences were deleted using the BioEdit program (Hall, 1999). Then, phylogenetic trees were reconstructed by using the neighbour-joining method (Saitou & Nei, 1987) with Kimura’s two-parameter model (Kimura, 1980), maximum-likelihood method (Felsenstein 1985) and maximum-parsimony method (Felsenstein 1988).
Table 1. Differential features between strain PN2T and the type strains of related Roseomonas species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<tr>
<td>NaCl tolerance (%)</td>
<td>1.02</td>
<td>3.02</td>
<td>3.02</td>
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<tr>
<td>Temperature for growth (°C)</td>
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<tr>
<td>Optimum</td>
<td>30</td>
<td>25</td>
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<tr>
<td>Range</td>
<td>15–35</td>
<td>15–37</td>
<td>12–48</td>
<td>12–42</td>
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<td>pH for growth</td>
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<tr>
<td>Optimum</td>
<td>6.0</td>
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<td>7.0</td>
<td>6.0</td>
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<tr>
<td>Range</td>
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<td>H2S production</td>
<td>–</td>
<td>+</td>
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<td>Simmons’ citrate utilization</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>Acid from lactose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Assimilation of (API 20NE):</td>
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<tr>
<td>Adipic acid</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>D-Glucose</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>L-Arabinose</td>
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<td>–</td>
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<tr>
<td>Potassium gluconate</td>
<td>–</td>
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<td>Trisodium citrate</td>
<td>–</td>
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<td>API ZYM results:</td>
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<tr>
<td>Acid phosphatase</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>Esterase lipase (C8)</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Leucine arylamidase</td>
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<td>+</td>
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<td>Carbon source oxidation (Biolog GENIII Microplates)</td>
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<tr>
<td>Acetic acid</td>
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<td>+</td>
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<tr>
<td>β-D-Glucose, L-rhamnose</td>
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<td>–</td>
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<td>β-Ketoglutaric acid</td>
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<td>+</td>
<td>–</td>
<td>–</td>
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<td>Citric acid, D-saccharic acid</td>
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<td>–</td>
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<tr>
<td>D-Galactose, D-fructose</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Gluconic acid, D-malic acid, D-mannose, mucic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Melibiose, glycerol, D-fucose, propionic acid</td>
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<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Quinic acid</td>
<td>–</td>
<td>–</td>
<td>+</td>
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Strains: 1, PN2T; 2, R. mucosa ATCC BAA-692T; 3, R. gilardii subsp. gilardii CCUG 33005T; 4, R. gilardii subsp. rosea CCUG 48655T. All the tests were performed in the present study under identical conditions. +, Positive; –, negative.

1981) and maximum-parsimony method (Nei & Kumar, 2000) with the program MEGA version 5 (Tamura et al., 2011). Bootstrap confidence values were obtained using 1000 resamplings (Felsenstein, 1985).

The DNA G + C content was determined at Techno Suruga Laboratory by HPLC as described by Katayama-Fujimura et al. (1984). Cellular fatty acid analysis was carried out by the Identification Service of the DSMZ using a gas chromatograph as described for the Sherlock Microbial Identification System (MIDI). Strains were cultivated in 50 ml GP broth (1 % glycerol and 1 % peptone) in 250 ml Erlenmeyer flasks and incubated at 30 °C on an orbital shaker (1 g, 200 r.p.m.) for 48 h prior to cell harvest for fatty acid analysis. To analyse for polar lipids, strains were grown on GPA medium and incubated at 30 °C for 48 h. Polar lipids were analysed by TLC using the method described by Minnikin et al. (1977). The extraction and purification of ubiquinones were carried out according to Yamada & Kondo (1973) and Kuraishi et al. (1985) and compounds were then identified by HPLC as described previously (Limityng et al., 2012). The level of DNA–DNA relatedness between strain PN2T and R. mucosa ATCC BAA-692T was examined on nylon membranes according to the method of Wang et al. (2011) under optimal hybridization conditions (at the optimal hybridization temperature of 53 °C).

The pink colonies of strain PN2T grown on GPA medium were circular, convex, smooth and with entire margins. Cells were Gram-stain-negative, non-motile and non-endospore-forming. Scanning electron microscopy of strain PN2T showed that cells were coccobacilli (Fig. S1, available in the online Supplementary Material). Optimal temperature for growth was 30 °C (range 15–35 °C) and optimal pH for growth was 6.0 (range pH 5.0–7.5). Strain PN2T grew in the presence of up to 1.02 % (w/v) NaCl. Strain PN2T did not produce acid from D-glucose, D-mannitol, D-sorbitol, D-xylose, lactose or sucrose. Strain PN2T did not produce H2S based on the sterile lead acetate paper strip test. In addition, antibiotic susceptibility tests indicated that strain PN2T was sensitive to (per disc) chloramphenicol (30 μg), kanamycin (30 μg), streptomycin (10 μg) and tetracycline (10 and 30 μg) but resistant to ampicillin (10 μg) and penicillin.
Morphological, physiological and biochemical characteristics of strain PN2<sup>T</sup> are shown in the species description. Detailed differential test reactions of the phenotypic characteristics between strain PN2<sup>T</sup> and the closest species are summarized in Table 1.

Based on biochemical characteristics, strain PN2<sup>T</sup> was differentiated from the closely related species *R. mucosa* ATCC BAA-692<sup>T</sup>, *Roseomonas gilardii* subsp. *gilardii* CCUG 33005<sup>T</sup> and *Roseomonas gilardii* subsp. *rosea* ATCC BAA-691<sup>T</sup>. Differential features between strain PN2<sup>T</sup> and these closely related species are shown in Table 1. Differential results were observed for assimilation of several compounds and for enzyme production. Only strain PN2<sup>T</sup> produced esterase lipase (C8) but not leucine arylamidase or acid phosphatase. Moreover, strain PN2<sup>T</sup> was the only strain that utilized D-gluconic acid, D-malic acid, D-mannose and D-mucic acid but did not utilize acetic acid or citrate (Simmons’ method).

The 16S rRNA gene sequence of strain PN2<sup>T</sup> (1457 bp) was compared with those of type strains in the EzTaxon-e database. Comparative analysis revealed that the closest relatives of strain PN2<sup>T</sup> were *R. mucosa* ATCC BAA-692<sup>T</sup> (96.5 % 16S rRNA gene sequence similarity), *Roseomonas pecuniae* N75<sup>T</sup> (87 %), *Roseomonas aerophila* 173/96<sup>T</sup> (80 %), *Roseomonas terrae* JCM 14592<sup>T</sup> (76 %), *Methylobacterium aquaticum* GR16<sup>T</sup> (73 %), and *Roseomonas frigidaquae* CW67<sup>T</sup> (72 %).
Cells are coccobacilli (0.7–1.0 μm in width, 1.0–1.3 μm in length). Gram-stain-negative, non-endospore-forming and non-motile. Colonies are pink-pigmented, round and convex with clear margins on GPA medium at 28–30°C. Growth occurs at 15–35°C (optimum 30°C) and pH 5.0–7.5 (optimum pH 6.0). Tolerates up to 1.02% (w/v) NaCl. Catalase and oxidase reactions are positive whereas methylen blue and Voges–Proskauer tests are negative. Does not produce acid from D-glucose, D-mannitol, D-sorbitol, D-xylene, lactose or sucrose. Nitrate is not reduced; urease is produced. H₂S and indole are not produced. Arginine, casein, chitin, CM-cellulose, aesculin, gelatin, guarine, hypoxanthine, starch, Tween 40, Tween 80, tyrosine and xanthine are not hydrolysed. Utilizes D-galactose, D-fructose, D-fucose, D-mannose, D-fructose 6-phosphate, acetoacetic acid, citric acid, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, α-ketoglutaric acid, D-malic acid, L-malic acid, mucic acid, D-saccharic acid, dextrin and glucuronamide, but not L-arabinose, D-glucose, α-D-glucose, L-fucose, lactose, maltose, L-rhamnose, D-xylene, gentiobiose, cellobiose, D-lactose, raffinose, melibiose, sucrose, stachyose, trehalose, turanose, D-arabitol, glycerol, D-mannitol, D-sorbitol, myo-inositol, N-acetyl-D-galactosamine, N-acetylgalactosamine, D-glucose 6-phosphate, N-acetyl-β-D-mannosamine, 3-methyl glucose, methyl β-D-glucoside, D-glucose 6-phosphate, adipic acid, acetic acid, N-acetyl neuraminic acid, γ-aminoobutyric acid, D-aspartic acid, L-aspartic acid, L-glutamic acid, bromosuccinimide, capric acid, formic acid, β-hydroxybutyric acid, β-hydroxy-β-D-butyric acid, D-hydroxyphenylactic acid, α-ketoglutaric acid, L-lactic acid, D-lactic acid methyl ester, phenylacetic acid, propionionic acid, β-pyroglutamic acid, quinic acid, L-alanine, L-arginine, L-histidine, glycyrl-L-proline, L-serine, methyl pyruvate, pectin, potassium gluconate, D-salicin, inosine, Simmons’ citrate or trisodium citrate. Alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase are present whereas β-galactosidase, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, β-galactosidase, α-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. The major fatty acids are C₁₉₀:0 cyclo ω9c, C₁₆₀:0 and C₁₈₁ω7c. The major fatty acids are consistent with those found for the type strains of the closest related species characterized under the conditions used in this study (Table S1).

The polar lipid profile of strain PN2ᵀ consisted of diphosphatidyglycerol, phosphatidylethanolamine, phosphatidylincholine, phosphatidylglycerol, three unknown aminolipids, an unknown phospholipid and an unknown lipid (Fig. S2). The profile indicates that diphosphatidyglycerol is the major lipid of strain PN2ᵀ. The polar lipid profile of strain PN2ᵀ was almost the same as that of R. mucosa ATCC BAA-692ᵀ (Fig. S3), Roseomonas gilardii subsp. rosea CCUG 48655ᵀ (Fig. S4) and Roseomonas gilardii subsp. gilardii CIP 104026ᵀ (Sánchez-Porro et al., 2009).

The major quinone of strain PN2ᵀ was Q-10, as also shown for Roseomonas gilardii subsp. gilardii KACC 11652ᵀ (Dong et al., 2014) and other members of the genus Roseomonas (Gallego et al., 2006). The level of DNA–DNA relatedness between strain PN2ᵀ and the closest related species, R. mucosa ATCC BAA-692ᵀ, was 27%, which is well below the 70% cut-off point for recognition of genomic species (Wayne et al., 1987). Consequently, we suggest that strain PN2ᵀ represents a novel species of the genus Roseomonas, for which the name Roseomonas elaecarpi sp. nov. is proposed.

### Description of Roseomonas elaecarpi sp. nov.

Roseomonas elaecarpi (e.lae.o.car’pi. N.L. gen. n. elaecarpi of the olive *Elaeocarpus* sp.).

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