**Hoeflea olei** sp. nov., a diesel-oil-degrading, anoxygenic, phototrophic bacterium isolated from backwaters and emended description of the genus **Hoeflea**

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A Gram-stain-negative, diesel-oil-degrading, rod-shaped bacterium (designated JC234ᵀ) was isolated from a water sample collected from diesel-oil-contaminated backwaters in Kerala, India. Strain JC234ᵀ was oxidase- and catalase-positive, and grew at 20–35 °C and at pH 7–9. Cells contained bacteriochlorophyll-α, hydroxydemethylspheroidene and three unidentified carotenoids. Growth occurred under aerobic, microaerobic and phototrophic anaerobic conditions. Strain JC234ᵀ could utilize diesel-oil as a sole source of carbon and energy. Based on the 16S rRNA gene sequence analysis, strain JC234ᵀ belonged to the genus **Hoeflea** within the family **Phyllobacteriaceae**, and was closely related to **Hoeflea alexandrii** AM1V30ᵀ (98.1 % 16S rRNA gene sequence similarity), **Hoeflea halophila** JG120-1ᵀ (97.6 %) and other members of the genus **Hoeflea** (<96.4 %). Strain JC234ᵀ showed 22 ± 2 % and 28 ± 1.5 % DNA–DNA hybridization with **Hoeflea alexandrii** KCTC 22096ᵀ and **Hoeflea halophila** KCTC 23107ᵀ, respectively. The DNA G+C content of strain JC234ᵀ was 54.3 mol %. The major cellular fatty acids were C₁₈:1ω7c/C₁₈:1ω6c, C₁₆:0 and C₁₆:1ω7c/C₁₆:1ω6c. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and phosphatidylglycerol were the major polar lipids. Strain JC234ᵀ contained Q10 as the predominant ubiquinone. On the basis of morphological, physiological, genetic, phylogenetic and chemotaxonomical analyses, we conclude that strain JC234ᵀ represents a novel species of the genus **Hoeflea**, for which the name **Hoeflea olei** sp. nov. is proposed. The type strain is JC234ᵀ (=KCTC 42071ᵀ=LMG 28200ᵀ). An emended description of the genus **Hoeflea** is also provided.

Reclassification of **Agrobacterium ferrugineum** LMG 128 (Ahrens, 1968; Rüger & Höfe, 1992) resulted in the creation of the genus **Hoeflea** (Peix et al., 2005) with **Hoeflea marina** as the type species. At the time of writing, the genus **Hoeflea** comprises six species with validly published names (http://www.bacterio.net/hoeflea.html): **Hoeflea marina** (Peix et al., 2005), **Hoeflea phototrophica** (Biebl et al., 2006), **Hoeflea alexandrii** (Palacios et al., 2006), **Hoeflea anabaenae** (Stevenson et al., 2011), **Hoeflea halophila** (Jung et al., 2013) and **Hoeflea suaedae** (Chung et al., 2013), all isolated from marine habitats. Members of the genus **Hoeflea** are Gram-stain-negative, aerobic or microaerobic, non-endospore-forming and halotolerant. The DNA G+C content of members of the genus ranges from 53 to 60 mol %. Diphosphatidylglycerol, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME), phosphatidylmonomethyl -ethanolamine (PEM), phosphatidylglycerol (PG), sulfoquinovosyl-syldiacylglycerol (SQDG), a few unidentified glycolipids, unidentified aminolipids and unidentified lipids are the major polar lipids of the genus **Hoeflea**. **Hoeflea phototrophica** is the only species of this genus reported to have bacteriochlorophyll-α (Bchl-α) and puf genes; however it lacks anaerobic growth in the dark or light (Biebl et al., 2006). In this study, we characterize a newly isolated strain (JC234ᵀ) as a novel species.

**Abbreviations:** Bchl-α, bacteriochlorophyll-α; DCM, dichloromethane; DDH, DNA–DNA hybridization; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmonomethylethanolamine; SQDG, sulfoquinovosyl-diacylglycerol

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and **puf** gene sequences of strain JC234ᵀ are HG965759 and LN811447, respectively.

Seven supplementary figures and a supplementary table are available with the online Supplementary Material.
of the genus Hoeflea which can utilize diesel-oil for growth, contains Bchl-a and puf genes and can grow anaerobically in the light.

Strain JC234T was isolated from an oil-contaminated backwater sample collected from Vembanad Lake, Kerala, India (GPS coordinates 9° 35’ N 76° 23’ E) during December 2013. The water sample that yielded strain JC234T had a pH of 7, a temperature of 29 °C and a salinity of 1.8 % (w/v). The water sample with traces of oil was inoculated into 250 ml conical flasks containing 100 ml mineral salts medium [comprising (g l⁻¹): KH₂PO₄ (0.5), MgSO₄·7H₂O (0.2), NH₄Cl (0.6), CaCl₂·2H₂O (0.05) and 5 ml ferric citrate solution (0.1 %, w/v)] with 2 % (v/v) diesel-oil as a carbon source, and was incubated at 30 °C with shaking at 100 r.p.m. for 3 days. Purification of bacteria was achieved by repeated streaking on nutrient agar. Strain JC234T was preserved as glycerol stocks and by lyophilization.

Genomic DNA was extracted and purified from strain JC234T according to the method of Marmur (1961) and the DNA G+C content was determined by HPLC (Mesbah et al., 1989). 16S rRNA gene amplification and sequencing was done as described previously (Subhash et al., 2013). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (Kim et al., 2012). The CLUSTAL W algorithm of the MEGA 5.2 software package was used for sequence alignments and MEGA 5.2 software (Tamura et al., 2011) was used for phylogenetic analysis of the individual sequences. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura, 1980). Neighbour-joining, maximum-likelihood and minimum-evolution methods in the MEGA 5.2 software were used to reconstruct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure based on 1000 replications.

The taxonomic relationship between strain JC234T and its closest phylogenetic neighbours was examined using DNA–DNA hybridization (DDH) studies. Genomic relatedness was determined by the membrane-filter technique as described previously (Seldin & Dubnau, 1985; Tourova & Antonov, 1987; Subhash et al., 2014). Hybridization was performed with three replications for each sample (control: reversal of strains was used for binding and labelling).

Morphological properties (cell shape, cell size, motility) of strain JC234T grown on nutrient broth was observed directly or after Gram-staining using Olympus BH-2 phase-contrast microscope. The hanging-drop method was performed as outlined in Cappuccino & Sherman (1998) to test motility. Malachite green was used for spore staining as described by Schaeffer & Fulton (1933). Internal membrane structures were viewed with a transmission electron microscope (H-7500; Hitachi), after the cells had been processed as described by Hanada et al. (2002). In vivo absorption spectra were measured with a Spectronic Genesys 2 spectrophotometer using sucrose solution for cell suspension (Trüper & Pfennig, 1981). Carotenoid composition was determined by C₁₅H₁₈-HPLC (Subhash et al., 2014). Amplification of the puf gene fragment was performed as described by Nagashima et al. (1997).

The pH range for growth was tested using nutrient broth, adjusted to different pH values (pH 4.0–11.0, intervals of 0.5 pH units) by using the appropriate biological buffers as described previously (Subhash et al., 2014). NaCl [0–10 % (w/v) at 0.5 % intervals] and temperature (4, 10, 15, 20, 25, 30, 35, 40 and 50 °C) ranges for growth were examined in nutrient broth and growth was measured turbidometrically at 540 nm in a colorimeter (Systronics).

Various biochemical tests such as hydrolysis of starch, casein, gelatin and Tweens 20 and 80, oxidase, catalase, nitrate reduction, nitrite reduction, H₂S production, methyl red and Voges–Proskauer tests were performed by the procedures as outlined in Cappuccino & Sherman (1998). Arginine dihydrolase, phenylalanine deaminase, ornithine decarboxylase and lysine decarboxylase activities were determined as described by Smibert & Krieg (1981).

Utilization of organic carbon compounds as carbon and energy sources for organo-heterotrophic growth was tested in a mineral medium as previously described (Lakshmi et al., 2011) replacing sodium pyruvate with specific organic compounds (0.35 % (v/v or v/v); growth was measured turbidometrically at 540 nm after 48 h. Nitrogen source utilization was tested by replacing ammonium chloride with 7 mM (final concentration) different nitrogen sources (NaNO₃, NaNO₂, glutamate, aspartate, glutamine and urea). Utilization of glutamate, glutamine, methionine, aspartate, peptone, Casamino acids and urea as sole source of carbon, nitrogen and energy was also determined. Phototrophic growth (light anaerobic) was tested in nutrient broth in fully filled screw-cap test tubes incubated at 2400 lx at 28–30 °C and chemotrophic growth was determined in nutrient broth incubated in aerobic dark at 100 r.p.m. at 28–30 °C.

Cellular fatty acids, polar lipids and quinone composition were analysed from cultures that attained 70 % maximal optical density (at their late exponential growth phase). Fatty acid analysis was done as described previously (Sasser, 1990; Subhash et al., 2014). Polar lipids analysis was performed as described previously (Tindall, 1990; Tindall et al., 1987; Oren et al., 1996; Subhash et al., 2014). Respiratory quinones were analysed as described by Xie & Yokota (2003).

To determine the diesel-oil degradation capability, strain JC234T was grown in 250 ml conical flasks containing 100 ml mineral salts medium with 5 % (v/v) diesel-oil as a carbon source and was incubated at 30 °C under shaking at 100 r.p.m. for 5 days. Cells were harvested by centrifugation (10 000 g for 15 min at 4 °C). The diesel-oil in the supernatant was extracted with 50 ml dichloromethane (DCM) and collected by centrifugation at 10 000 g, 4 °C for 10 min. The DCM fraction containing diesel-oil was concentrated and the extracted hydrocarbons were analysed.
by GC-MS. A control containing diesel-oil and no cells was used to determine background baseline of growth and degradation.

GC-MS analysis was done on a Pegasus HT TOF (high-throughput time-of-flight)-MS system (Leco) equipped with an Agilent series (7890) gas chromatograph. One microlitre of sample was injected into a HP-5 column (30 m, internal diameter 0.32 mm, thickness 0.25 μm), with helium as the carrier gas at a constant flow of 1.2 ml min⁻¹. The initial oven temperature of 60 °C was ramped to 300 °C at 3 °C min⁻¹ and held for 5 min. Inlet temperature was 250 °C, ion source temperature 250 °C and ionization energy −70 eV. Mass spectra were recorded at 50–1000 m/z. LecoChromaTOF software (version 4.21) was used to process the chromatograms and the metabolites were identified based on mass spectral comparison to a standard NIST (National Institutes of Standards and Technology) 98 library. Mass spectral peaks showing similarity above 70% were accepted, with maximum match equal to 100% for statistical analysis.

The EzTaxon-e server search analysis revealed that strain JC234ᵀ was most closely related to members of genus *Hoeflea*, and the highest sequence similarity was observed with *Hoeflea alexandrii* AM1V30ᵀ (98.1%), *Hoeflea halophila* JG120-1ᵀ (97.6%) and other members of the genus *Hoeflea* (<96.4%). The results of phylogenetic analysis of the 16S rRNA gene sequences suggested that strain JC234ᵀ clustered with the members of the genus *Hoeflea* and formed a separate clade along with the type strains of *Hoeflea alexandrii* and *Hoeflea halophila* (Fig. 1).

The DNA–DNA reassociation value between strain JC234ᵀ and *Hoeflea alexandrii* KCTC 22096ᵀ was 22 ± 2%, and between strain JC234ᵀ and *Hoeflea halophila* KCTC 23107ᵀ was 28 ± 1.5%; these hybridization values are well within the recommended standards to delineate a bacterial species based on DDH (Stackebrandt & Goebel, 1994).

The DNA G+C content of strain JC234ᵀ was 54.3 mol% (by HPLC).

On nutrient agar, colonies of JC234ᵀ were round, flat to raised, initially colourless or light reddish-brown but later...
are more and less tolerant, respectively (Table 1). The tempera-
ture range for growth further differentiates strain JC234T from its closest phylogenetic neighbours. Other
physiological and biochemical results are presented in the species description and Table 1.

The major fatty acids of strain JC234T were C18:1ω7c, C18:1ω6c, C16:0, and C16:1ω7c/C16:ω6c, with minor
amounts of C18:1ω7c 11-methyl and C18:0. Strain JC234T
shared the presence of major fatty acids with Hoeflea alexandrii KCTC 22096T and Hoeflea halophila
KCTC 23107T. However, significant differences in the
relative amounts of C16:1ω7c/C16:ω6c and C18:1ω7c 11-
methyl were found between strain JC234T and the type
strains of Hoeflea alexandrii and Hoeflea halophila (Table S1).

The polar lipid profile of strain JC234T contained PC, PME,
PG, PE, SQDG and unidentified lipids L1, L2 and L3 (Fig.
S5). Identification of SQDG in strain JC234T was based on
the motility of the spot as demonstrated by Chung et al. (2013) in
Hoeflea suaedae YC6899T, Hoeflea marina KACC
12993T and Hoeflea alexandrii KACC 12994T. The spot
resulting from SQDG was eluted from the TLC plate,
dissolved in chloroform/methanol (2 : 1, v/v) and the
Fourier transform infrared (FTIR) spectrum of the chloro-
form/methanolic extract was recorded on a Bruker FTIR
spectrometer in the wave number range of 3500–
500 cm⁻¹. The infrared spectrum of the chloroform/metha-
nolic extract showed characteristic absorption bands for sulfur-containing compounds at 959 cm⁻¹ and
792 cm⁻¹ indicating the presence of strong dehydration
of SO3 and symmetrical C-O-S association with a C-O-SO3
group. The above two characteristic bands and the other
absorption bands indicating the -CH3 bending and C-H
stretching confirmed the presence of SQDG (Fig. S6). The
polar lipid profile of strain JC234T was well in consonance
with the type species of the genus Hoeflea (Hoeflea
marina) which contained PC, PME, PG and PE as major
lipids as reported by Chung et al. (2013) and data from
this study (data not shown). The major quinone of strain
JC234T was ubiquinone Q10, which is in line with members
of the genus Hoeflea.

Based on the GC-MS analysis of diesel layer, various hydro-
carbons were seen to decrease in abundance sequentially
based on their carbon chain length and branching. Strain
JC234T degraded almost all the hydrocarbon present in
diesel (C10–C28) which suggests that this strain could be
efficiently used for the treatment processes of various
hydrocarbon-contaminated sites (Fig. S7).

Distinct phylogenetic, genotypic, chemotaxonomic and
phenotypic properties thus justify the description of strain
JC234T as representative of a novel species of the genus
Hoeflea, for which the name Hoeflea olei sp. nov. is pro-
posed. An amended description of the genus Hoeflea is
also provided. Phototrophy is a recognized genus-specific
character (Imhoff & Caumette, 2004) and hence it is deba-
table if a new genus needs to be described in future to
accommodate this anoxygenic phototrophic bacterium
based on multiple strains. Such description of a new
genus based on phototrophy was recently proposed for

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**Fig. 2.** Whole-cell absorption spectrum of strain JC234T

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Gemmobacter changlensis reclassifying into Cereibacter changlensis (Suresh et al., 2015). Similarly a chemotrophic bacterium, Rhodobacter massiliensis (Greub & Raoult, 2003) was reclassified as Haematobacter massiliensis (Helsel et al., 2007).

Description of Hoeflea olei sp. nov.

Hoeflea olei (o’le.i. L. gen. n. olei of oil)

Colonies are circular with entire margin, and texture is moist with flat to raised elevation. On nutrient agar, colonies are reddish-brown and measure 1–3 mm in diameter. Cells are straight rods (1–2 μm × 2–5 μm), Gram-stain-negative, motile and non-endospore-forming. Aerobic, microaerobic and phototrophic anaerobic growth is possible. Whole-cell absorption spectra of phototrophically grown cells exhibit maxima at 475, 512, 589, 802, 846 and 877 nm. Phototrophically grown cells have Bchl-a. Hydroxydemethylspheroidene and three unidentified carotenoids are present in phototrophically grown cells. Growth occurs on diesel-oil. Growth temperature range is 20–35 °C (optimum 30 °C). Growth pH range is pH 7–9 (optimum pH 7). NaCl is not required for growth but can be tolerated up to 8 % (w/v). Catalase- and oxidase-positive. Negative for hydrolysis of casein, gelatin, starch and Tweens 20 and 80. Tests for arginine dihydrolase, phenylalanine deaminase, lysine decarboxylase, ornithine decarboxylase and lysine decarboxylase. +, Positive; w, weakly positive; −, negative.

Table 1. Differentiating characteristics between strain JC234T and the type strains of phylogenetically related species of the genus Hoeflea

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Isolation source</td>
<td>Hydrocarbon-contaminated backwaters</td>
<td>Marine sediment</td>
<td>Dinoflagellate</td>
</tr>
<tr>
<td>NaCl tolerance range (optima) for growth (%, w/v)</td>
<td>0–8 (0–1)</td>
<td>0–10 (0–2)</td>
<td>0–5 (1)</td>
</tr>
<tr>
<td>Temperature range (optima) for growth (°C)</td>
<td>20–35 (30)</td>
<td>10–40 (30)</td>
<td>15–30 (25)</td>
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<tr>
<td>pH range for growth</td>
<td>7–9</td>
<td>6–9</td>
<td>6–9</td>
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<tr>
<td>Oxidase activity</td>
<td>+</td>
<td>w</td>
<td>+</td>
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<tr>
<td>Anaerobic phototrophic growth</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td>Hydrolysis of:</td>
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<tr>
<td>Tween 20</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>Tween 80</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Degradation of diesel-oil</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td>Organic substrates utilized for growth</td>
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<tr>
<td>Acetate</td>
<td>+</td>
<td>−</td>
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<td>Arabinose</td>
<td>−</td>
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<tr>
<td>Butyrate</td>
<td>+</td>
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<td>Citrate</td>
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<td>Fumarate</td>
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<td>Galactose</td>
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<td>Glucose</td>
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<tr>
<td>Pyruvate</td>
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<tr>
<td>Sucrose</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Bacteriochlorophyll-a</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>54.3</td>
<td>58.2</td>
<td>57.1</td>
</tr>
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</table>

*Hoeflea olei* sp. nov.

Taxa: 1, JC234T; 2, *Hoeflea alexandrii* KCTC 22096T; 3, *Hoeflea halophila* KCTC 23107T. All data from this study. All strains are Gram-stain-negative, motile, non-spore-forming rods, positive for catalase activity and show optimal growth at pH 7.0. All strains are negative for nitrate and nitrite reduction, H₂S production, methyl red, Voges–Proskauer, hydrolysis of starch, gelatin and casein, and activity of arginine dihydrolase, phenylalanine deaminase, ornithine decarboxylase and lysine decarboxylase. +, Positive; w, weakly positive; −, negative.
The type strain JC234\(^T\) (=KCTC 42071\(^T\)=LMG 28200\(^T\)) was isolated from a diesel-oil-contaminated backwaters of Vembanad Lake, Kerala, India. The DNA base composition of the type strain is 54.3 mol %.

**Emended description of the genus *Hoeflea* Peix et al. 2005**

The description of the genus is as given by Peix et al. (2005) with the following modifications. Some species show microaerobic growth and lack motility. Oxidase activity is negative in some species. Some members of the genus contain photosynthetic reaction-centre genes *puf* and form photosynthetic pigments such as bacteriochlorophyll-\(a\) and carotenoids. Chemo-organotrophic with or without photosynthetic pigments, but some species are phototrophic. Phosphatidylcholine, unidentified glycolipids, unidentified aminolipids and unidentified lipids are also present in some species. \(C_{16}:1\, \omega 7c/C_{16}:1\, \omega 6c\) can also be present in major amounts (>3 %). \(C_{18}:1\, \omega 7c\) 11-methyl and \(C_{16}:0\) can be present in minor amounts (<3 %). \(C_{19}:0\, \omega cyc\, \omega 8c\) is absent in some species. Some species require NaCl for growth and can tolerate concentrations up to 10–11 % (w/v) and pH of 5–10. Temperature range for growth is 4–42 °C. Some species can degrade diesel-oil. The DNA G+C content is 53.1–59.7 mol %.

The type species is *Hoeflea marina*.

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**References**


