**Halobium palmae** gen. nov., sp. nov., an extremely halophilic archaeon isolated from a solar saltern

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A novel and extremely halophilic archaeon, designated strain **2a**_47_2^T, was isolated from a solar saltern sample collected in Indonesia. Cells of the strain were Gram-stain-negative, non-motile and pleomorphic and formed orange–red pigmented colonies. Strain **2a**_47_2^T grew at 20–48 °C (optimum 38–41 °C), pH 6.0–8.5 (optimum pH 7.5), > 1.7 M NaCl (optimum 2.6 M) and < 0.5 M MgCl2 (optimum 0.3 M). The major polar lipids were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, two phospholipids and sulfated diglycosyl diether. The cells mainly contained menaquinone-8. The G+C content in the genomic DNA of the strain was 67.0 mol%.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain **2a**_47_2^T represents a member of the family *Halorubraceae* and is different from any other known halophilic archaea. This finding was also demonstrated by phylogenetic analyses based on deduced *rpoB* amino acid sequences. Collectively, these results show that strain **2a**_47_2^T represents a novel genus and species in the family *Halorubraceae*, and the name *Halobium palmae* gen. nov., sp. nov. is proposed. The type strain is **2a**_47_2^T (=NBRC 111368^T=InaCC Ar341^T).

Extremely halophilic archaea are taxonomically a single group of prokaryotes and belong to the class *Halobacteria* (Gupta et al., 2015, 2016; Oren, 2012). They are widely distributed in various hypersaline environments, from which they have been isolated. Solar salterns are one of the hypersaline environments inhabited by halophilic archaea and remain one of the primary sources of novel strains (Henriet et al., 2014; Viver et al., 2015). There are many solar salterns worldwide and natural salt is produced by slightly different methods at each site on a large or small scale. For these reasons, it is likely that various halophilic archaea inhabit the different environments found in each solar saltern. In Indonesia, salt is one of the key exports, and some salts are produced by local farmers using traditional methods. Recently, an extremely halophilic archaeon, designated strain **2a**_47_2^T, was isolated from a palm tree vessel used for the complete evaporation process before harvesting of salt in an Indonesian solar saltern. In this study, based on phenotypic characteristics as well as phylogenetic analyses, a novel taxon is proposed to accommodate the isolate, *Halobium palmae* gen. nov., sp. nov.

Sampling was performed in September 2013 at a solar saltern in Dawan, Bali, Indonesia (08°33′53″S 115°27′50″E). A fiber of palm tree, used as the vessel (34 °C, pH 6.2) for the evaporation process, was used as the inoculant, and the microbial isolation and purification procedures were performed at 30 °C on basal medium (Mori et al., 2015) supplemented with (per litre): 1 g sodium glutamate, 1 g trisodium citrate, 2 g Bacto Yeast Extract (Difco) and 2 g Bacto Casamino Acids (Difco). After incubation for 2 weeks, small orange–red colonies were observed, and a pure isolated strain, designated **2a**_47_2^T, was obtained by repeated colony pick-up.

Cells of strain **2a**_47_2^T were pleomorphic (0.3–0.6 µm wide and 0.5–2.0 µm long) under optimal growth conditions (Fig. S1, available in the online Supplementary Material). Cell motility was not observed by microscope, and the colonies were orange–red. Cells did not lyse in distilled water. The cells were Gram-staining-negative (conventional Gram staining), catalase-positive (Holding & Nicholas, 1971) and oxidase-positive (cytochrome oxidase paper; Nissui Pharmaceutical). Strain **2a**_47_2^T could hydrolyse Tween 80 but not starch, casein or gelatin. Production of H2S was tested in a tube with the liquid medium.
supplemented with Na$_2$SO$_3$ and cysteine and detection was performed using a filter-paper strip impregnated with lead acetate. H$_2$S was not produced from the culture of strain 2a$_{47}$-2$^T$. Production of indole was tested in a tube with the medium supplemented with Hipolypeptone (Nihon Pharmaceutical), and its formation was not detected in the culture of strain 2a$_{47}$-2$^T$ by using Kovac’s reagent. Enzyme activities were tested and identified by using the API ZYM system (bioMerieux) at 40°C; the strain showed positive reactions for alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. The anti-microbial susceptibilities of the strain were determined in liquid medium containing anti-microbial compounds (µg 1$^{-1}$, unless otherwise indicated), and the strain was shown to be sensitive to anisomycin (35), chloramphenicol (30), nitrofurantoin (50), novobiocin (25), rifampicin (25), bacitracin (30) and trimethoprim (30). The strain was resistant to ampicillin (25), erythromycin (25), gentamycin (25), kanamycin (25), nalidixic acid (30), neomycin (30), penicillin G (10 U), polymyxin B (100 U), streptomycin (25), tetracycline (30), nystatin (30), norfloxacin (30), ciprofloxacin (30) and vancomycin (30).

The optimal NaCl and MgCl$_2$ concentrations, temperature, and pH for growth were determined by using previously described procedures (Mori et al., 2015), and the results are shown in Fig. S2. Strain 2a$_{47}$-2$^T$ grew optimally in medium containing 2.6 M NaCl, and growth was observed at more than 1.7 M NaCl and at saturated NaCl. The strain grew with MgCl$_2$ concentrations of up to 0.5 M, with an optimal concentration of 0.3 M MgCl$_2$, although slight growth was observed in the absence of MgCl$_2$. The strain was able to grow at 20–48°C, with optimal growth occurring at 38–41°C. The pH range for growth was pH 6.0–8.5, with optimal growth occurring at pH 7.5. The specific growth rate and doubling time under the optimal growth conditions were 0.1 h$^{-1}$ and 6.9 h, respectively.

The utilization of different energy and carbon sources was determined by measuring the OD$_{660}$ of the culture during incubation at 40°C. As sole carbon and energy sources, various substrates were added to the modified basal medium (with 2.6 M NaCl) supplemented with 50 mM HEPES (pH 7.5). Acid production was tested in the modified basal medium and was determined by measuring the initial and final pH of the medium (Makhdoumi-Kakhki et al., 2012). In the presence of 0.1 g yeast extract 1$^{-1}$ and 0.1 g casamino acids 1$^{-1}$, strain 2a$_{47}$-2$^T$ could not utilize any substrates nor produce acids from any substrates although the following substrates were examined: (mM unless otherwise stated) D-glucose (10), D-fructose (10), D-mannose (10), D-galactose (10), D-melibiose (10), maltose (10), lactose (10), D-trehalose (10), sucrose (10), D-sorbitose (10), D-cellobiose (10), D-raffinose (10), D-arabinose (10), L-rhamnose (10), D-xyllose (10), D-ribose (10), ribitol (10), D-mannitol (10), D-sorbitol (10), glycerol (20), citrate (20), pyruvate (20), succinate (20), malate (20), L-glutamate (20), lactate (20), fumarate (20), acetate (20), starch (5 g l$^{-1}$), L-alanine (20), L-arginine (20), L-asparagine (20), L-aspartic acid (20), L-cysteine (20), glycine (20), L-lysin (20), L-methionine (20), ornithine (20), L-phenylalanine (20), L-proline (20), L-serine (20), L-tyrosine (20) and L-valine (20). Strain 2a$_{47}$-2$^T$ could grow well on 1 g yeast extract 1$^{-1}$ and 1 g casamino acids l$^{-1}$. The strain was unable to grow under anaerobic conditions using nitrate, dimethyl sulfoxide (DMSO), or trimethylamine N-oxide (TMAO) together with yeast extract and casamino acids. In the growth test with nitrate, formation of nitrite was not observed with a colorimetric assay (Hewitt & Nicholas, 1964). Formation of nitrite and N$_2$ gas from nitrate were tested by using the API20NE system (bioMerieux) at 40°C; the strain showed negative reactions. Anaerobic growth with L-arginine was not observed.

Genomic DNA was extracted by using a previously described procedure (Mori et al., 2000). The G+C content of the genomic DNA was determined by HPLC (Kamagata & Mikami, 1991; Mori et al., 2015). The G+C content of the genomic DNA of strain 2a$_{47}$-2$^T$ was 67.0 mol%. Respiratory quinones were extracted from the cells by using a previously described method (Nakagawa & Yamazato, 1993) and analyzed with an LCMS-QP 8000 spectrometer (Shimadzu). Strain 2a$_{47}$-2$^T$ contained mainly menaquinone-8 (98% of total quinones), and menaquinone-8(H) was detected as a minor quinone (2% of total quinones). The polar lipids of strain 2a$_{47}$-2$^T$ were extracted and examined by one- and two-dimensional TLC by using a previously described method (Hamada et al., 2010), and the patterns were visualized by spraying the TLC plates with 5% phosphomolybdic acid, Dittmer–Lester reagent (Dittmer & Lester, 1964), Schiff reagent and anisaldehyde. In the two-dimensional TLC analysis, the first and second solvents were chloroform/methanol/water (65:25:4, by volume) and chloroform/acetic acid/methanol/water (80:18:12:5, by volume), respectively, and the polar lipids of strain 2a$_{47}$-2$^T$ and 2 reference strains (Haloterrigens daqingensis NBRC 105739$^T$ and Haloarcula japonica NBRC 101032$^T$) were developed (Fig. S3a; data for reference strains not shown). In the one-dimensional TLC analysis, chloroform/acetic acid/methanol/water (80:18:12:5, by volume) was used as the solvent, and the polar lipids of strain 2a$_{47}$-2$^T$ and five reference strains (Halopenitus malékzadehii NBRC 110929$^T$, Halobacterium salinarum NBRC 102687$^T$, Haloterrigens daqingensis NBRC 105739$^T$, Halogranum salarium NBRC 110682$^T$ and Haloarcula japonica NBRC 101032$^T$) were developed (Fig. S3b). The identifications of lipids were comprehensively performed using spray-visualization and the spot positions of reference strains. Phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (Me-PGP), sulfated diglycosyl diether (S-DGD) and two unidentified phospholipids were detected as the major polar lipids of strain 2a$_{47}$-2$^T$. An almost complete 16S rRNA gene sequence for strain 2a$_{47}$-2$^T$ was determined using previously described methods (Mori et al., 2008a, b). Because several different types of 16S rRNA genes were detected in the strain, sequencing was
performed after the cloning procedures by using the pTBlue T-Vector (Novagen) and DNA Ligation Kit Version 1 (Takara). Phylogenetic trees based on 16S rRNA gene sequences were reconstructed by the neighbor-joining method using the CLUSTAL x program (Saitou & Nei, 1987; Thompson et al., 1997), the maximum-likelihood method by using the NucML program in the MOLPHY package (Adachi & Hasegawa, 1995; Hasegawa et al., 1985; Mori et al., 2003) and Bayesian inference using MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) after alignment using ARB software (Ludwig et al., 2004). The posterior probabilities of branching points were estimated by Bayesian inference with the default settings and the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites (nst, 6; rates, invgamma). A total of 200,000 generations were calculated for the data sets, and a cladogram with the posterior probabilities with each split and a phylogram with mean branch lengths were obtained with a parameter burn-in of 5000. Two different 16S rRNA gene sequences (denoted rrnA and rrnB) were obtained from strain 2a_47_2T with a sequence similarity of 99.4%. The phylogenetic analysis revealed that strain 2a_47_2T represented a member of the order Haloferales but was distant from all known genera, with less than 92% sequence similarities. The most closely related genus was Halogranum with 91.2–91.8% sequence similarity, but strain 2a_47_2T formed a cluster with the genera Halopenitus and Halorubrum in the family Halorubraceae.

![Phylogenetic Tree](image)

**Fig. 1.** Maximum-likelihood phylogenetic trees based on 16S rRNA gene (a) and deduced RpoB (b) sequences of strain 2a_47_2T and relatives. Bootstrap percentages are indicated at branch points. GenBank/EMBL/DDBJ accession numbers are shown in parentheses.
The maximum-likelihood tree reconstructed based on the sequences of strain 2a_47_2T and relatives is shown in Fig. 1a. The topology of the trees reconstructed by using the neighbor-joining and Bayesian methods (Fig. S4) were similar to that obtained by using the maximum-likelihood method, and strain 2a_47_2T was the sole lineage in the family Halorubraceae.

The rpoB' (B' subunit of RNA polymerase) gene of strain 2a_47_2T was amplified and its sequence was determined by using a previously described procedure (Minegishi et al., 2010). A phylogenetic tree based on the deduced RpoB' amino acid sequences was reconstructed by using the neighbor-joining method (CLUSTAL X) and the maximum-likelihood method (ProtML in MOLPHY) after alignment with the CLUSTAL X program. The trees are shown in Figs 1(b) and S4, respectively. The RpoB' sequence similarities between strain 2a_47_2T and the species with validly published names were less than 92%. The sequences of the following species were comparatively similar to that of strain 2a_47_2T: species of the genus Halorubrum (sequence similarities 91.3–91.6%), Salinigranum rubrum (91.4%), Haloquadratulina natans (91.4%), Halofex volcanii (90.8%), Salinirubrum litoreum (90.4%) and Halogeometricum palmae (90.3%).

Characteristics of strain 2a_47_2T and related genera in the family Halorubraceae are summarized in Table 1. Strain 2a_47_2T was phenotypically orange–red pigmented, mesophilic, neutrophilic and extremely halophilic, and these features are common in the family Halorubraceae. However, the phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain 2a_47_2T was distantly related to all known genera in the family Halorubraceae and the sequence similarities between the strain and the species in the family were less than 92%. Its characterization as phylogenetically solitary based on the 16S rRNA gene sequence was supported by the analysis based on the RpoB' amino acid sequences and the sequence similarities with the most closely related genera were just 91.3–91.6%. In addition, in phenotypic features, the isolate clearly differed from the members of the genera of the family Halorubraceae with respect to the following characteristics: (i) strain 2a_47_2T was resistant to lysis in distilled water unlike the members of the other genera; (ii) with few exceptions, nitrate reduction is observed in the members of the other genera but not for strain 2a_47_2T; (iii) the genomic G+C content of strain 2a_47_2T was relatively high. Based on the phylogenetic and phenotypic analysis results, a novel taxon, Halobium palmae gen. nov., sp. nov., belonging to the family Halorubraceae, is proposed for strain 2a_47_2T.

An interesting feature of strain 2a_47_2T is its requirement for relatively large amounts of yeast extract and casamino acids for growth, and no growth was observed on sole substrates at low concentrations of yeast extract and casamino acids. In this study, we did not determine which component(s) of yeast extract and casamino acids is/are necessary

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Table 1. Differential characteristics between strain 2a_47_2T and related genera in the family Halorubraceae

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell shape</strong></td>
<td>Pleomorphic</td>
<td>Pleomorphic</td>
<td>Pleomorphic</td>
<td>Pleomorphic</td>
<td>Rod</td>
<td>Rod</td>
<td>Pleomorphic</td>
<td>Rod</td>
<td>Pleomorphic</td>
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<tr>
<td><strong>Pigmentation</strong></td>
<td>Orange–red</td>
<td>Orange, red, pink</td>
<td>Pale-pink, cream, red</td>
<td></td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
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<tr>
<td><strong>Motility</strong></td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>±</td>
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<td><strong>Optimum growth conditions</strong></td>
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<tr>
<td><strong>Temperature (°C)</strong></td>
<td>38–41</td>
<td>25–30</td>
<td>35–40</td>
<td>40–45</td>
<td>30–40</td>
<td>37–42</td>
<td>45</td>
<td>37</td>
<td>37</td>
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<tr>
<td><strong>pH</strong></td>
<td>7.5</td>
<td>7.0–9.5</td>
<td>7.0–7.5</td>
<td>6.0–7.0</td>
<td>7.0–7.5</td>
<td>7.0–8.0</td>
<td>7.0–7.5</td>
<td>7.0</td>
<td>6.5–7.5</td>
</tr>
<tr>
<td><strong>NaCl (M)</strong></td>
<td>2.6</td>
<td>3.2–4.5</td>
<td>3.0–3.5</td>
<td>1.5–2.6</td>
<td>2.6–3.1</td>
<td>3.4–4.3</td>
<td>3.4–4.1</td>
<td>3.1</td>
<td>2.6–3.9</td>
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<tr>
<td><strong>Anaerobic growth</strong></td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>–</td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td><strong>Nitrate reduction</strong></td>
<td>–</td>
<td>±</td>
<td>±</td>
<td>–</td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>±</td>
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<tr>
<td><strong>Lysis in distilled water</strong></td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Mg²⁺</strong> requirement (M)</td>
<td>–</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>±</td>
</tr>
<tr>
<td><strong>DNA G+C content (mol %)</strong></td>
<td>67.0</td>
<td>60.2–71.2</td>
<td>63.8–66.0</td>
<td>67.0–70.0</td>
<td>62.4–62.9</td>
<td>65.1–68.0</td>
<td>58.4</td>
<td>62.9</td>
<td>55.7–64.4</td>
</tr>
</tbody>
</table>

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for the growth of strain 2a_47_2T. Unfortunately, because only one strain was isolated in our study, we could not ascertain whether this feature is the characteristic of the whole lineage. More isolates will be required to elucidate the characteristics of this genus of the family Halorubraceae.

Description of Halobium gen. nov.

*Halobium*(Ha.lo’bi.um. Gr. n. hals halos salt; Gr. n. bios life; N.L. neut. n. *Halobium* halophilic organism).

Cells are non-motile and pleomorphic under optimal growth conditions. Gram-staining-negative. Colonies are orange–red pigmented. Strictly aerobic and chemoorganoheterotrophic. Growth occurs at 20–48 °C, at pH 6.0–8.5 and with more than 1.7 M NaCl. Polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated diglycosyl diether and two phospholipids. Menaquinone-8 is the major respiratory quinone, with menaquinone-8(H2)2 detected as a minor component. Phylogenetically affiliated to the family Halorubraceae. The type species is *Halobium palmae*. Recommended three-letter abbreviation of the genus: Hbm.

Description of Halobium palmae sp. nov.

*Halobium palmae* (pal’mae. L. fem. gen. n. palmae of a palm tree).

Exhibits the following properties in addition to those given in the genus description. The NaCl concentration for growth is more than 1.7 M, with an optimum at 2.6 M. Mg2+ is not required for growth. The Mg2+ concentration for growth is less than 0.5 M, with an optimum at 0.3 M. Cells do not lyse in distilled water. Catalase- and oxidase-positive. Hydrolyses Tween 80 but not starch, casein or gelatin. Formation of H2S and indole are negative. API ZYM tests are positive for activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase. Anaerobic growth with nitrate, DMSO, TMAO and l-arginine is not observed. Growth occurs on yeast extract and casamino acids. No growth occurs on sole substrates such as sugars, short-chain fatty acids and amino acids with low concentrations of yeast extract and casamino acids. Optimally grows at 38–41 °C, pH 7.5, 1.7 M NaCl and 0.3 M MgCl2.

The type strain, 2a_47_2T (NBRC 111368T=InaCC Ar34T), was isolated from a solar saltern in Dawan, Bali, Indonesia. The genomic DNA G+C content of the type strain is 67.0 mol%.

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