 doi:10.20944/preprints202207.0157.v1

Article

Description of *Methylomonas Aquatica* Sp. Nov., Isolated from a Tropical Indian Rice Rhizosphere

Pranitha S. Pandit[1,2], Kumal Khatri[1,2], Jyoti A. Mohite[1,2] and Monali C. Rahalkar[1,2*]

[1]C2, Bioenergy group, MACS Agharkar Research Institute, G.G. Agarkar Road, Pune 411004, Maharashtra, India
[2]Savitribai Phule Pune University, Ganeshkhind Road, Pune 411007, Maharashtra, India
* Correspondence: author: monalirahalkar@aripune.org

Abstract

An aerobic methanotroph, strain Kb3^T, was isolated from a rhizospheric soil sample collected from a tropical Indian rice field. The cells were motile, Gram-negative bacilli, formed pink colonies and pink turbid/pellicles in the liquid medium. Biochemical characteristics showed that strain Kb3^T utilised only methane and methanol as its sole carbon and energy sources. The isolate's 16S rRNA gene sequence expressed 99.52% similarity to the recently described valid species *Methylomonas fluvii* EbB (*Mmf*), with 92% query cover. But examining the genome similarity between Kb3 and EbB, a DDH value of 44.20% [41.6 - 46.7%] and an Ortho-ANI value of 91.48 was observed that were below the current cut-off values for species differentiation. Also, the 16S rRNA gene phylogeny and the phylogenomic analysis branched the two species separately. The major fatty acid in *Methylomonas* sp. Kb3 was $C_{14:0,}$ followed by $C_{16:1}$ ω5c. The genome sequence revealed the size of strain Kb3 is 5.1 Mb, with the G + C content of 51.8%. Strain Kb3^T shared the closest relatedness with *Methylomonas* sp. LW13 reveals a 99.66% 16S rRNA gene similarity, an Ortho-ANI value of 97.9%, a DDH value of 87.3%, and a close branching in the phylogenomic tree Kb3 and LW13 together form a new species. The genomic and phylogenetic distinction between species *Mmf* and strain Kb3 supports Kb3^T to be described as a novel species within the genus

 doi:10.20944/preprints202207.0157.v1

Methylomonas, with the proposed name, *Methylomonas aquatica* sp. nov. (*Mma*) and the type strain being Kb3^T (=MCC 4012, =JCM 33634, =KCTC 72521).

Keywords: *Methylococcaceae*; methanotroph; Indian rice field; *Methylmonas*; novel species

Introduction

As estimated in 2012, the atmosphere's methane concentration has increased by about 2.5 times from 0.75 ppm to 1.81 ppm in the last 300 years since 1750 (Saunois et al. 2016). Methane is the second most important greenhouse gas after carbon dioxide (CO_2), having 28-36 times more global warming potential than CO_2 (IPCC 2014). The anthropogenic sources of methane include three primary sources, namely agriculture and waste, biomass and biofuel burning, and fossil fuels. Among the global anthropogenic methane emission sources, agriculture and waste contribute to 57% (195 $TgCH_4$/yr) of the total methane emissions (328 $TgCH_4$/yr), with maximum concentration in the South East Asian regions of the world (Saunois et al. 2016). Paddy or rice cultivation falling under the category of agriculture and waste contributes to about 30 $TgCH_4$/yr (range 24-36 $TgCH_4$/yr), averaging to 9% of the total global anthropogenic methane emissions (Saunois et al. 2016). Within Asia, India contributes about 3.9 $TgCH_4$/yr (3.3-4.5 $TgCH_4$/yr) (Ganesan et al. 2017), accounting for only 13% of the rice field methane emissions in the world despite being the second-largest rice producer in the world (Chauhan et al. 2017).

The 'methane oxidizing bacteria' (MOB) or methanotrophs are the key players in the methane cycle in the rice ecosystems (Conrad 1996; Conrad 2007). However, the methanotrophic ecology from such tropical rice ecosystems has long been neglected, with very few reports (Lüke et al. 2014). Our team has successfully studied several

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

tropical Indian rice fields for their culturable and molecular diversity. We have described two novel genera; *Methylocucumis oryzae* Sn10-6^T *(Pandit et al. 2018; Pandit and Rahalkar 2019)* and *Methylolobus aquaticus* FWC3^T (Rahalkar et al. 2020), and one species; *Candidatus* Methylobacter oryzae KRF1 (Khatri et al. 2020), so far. This culturable diversity from the tropical Indian rice ecosystems proves them to be an important habitat for inhabiting the key methane mitigating bacteria. In this study, we report the cultivation of another new member belonging to the genus *Methylomonas* of the *Methylococcaceae*. (*Methylomonadaceae* – not validly described) family.

The *Methylomonas* spp. are the most abundant and commonly occurring methanotroph in the rice fields (Dianou et al. 2009; Ferrando and Tarlera 2009; Shrestha et al. 2010). The genus *Methylomonas* consists of ten validated species to date (Bowman 2015); *Methylomonas methanica* (Whittenbury R 1984), *Methylomonas pelagica* (Sieburth et al. 1987), *Methylomonas aurantiaca*, *Methylomonas fodinarum* (Bowman et al. 1990), *Methylomonas scandinavica* (Kalyuzhnaya et al. 1999), *Methylomonas koyamae* (Ogiso et al. 2012), *Methylomonas paludis* (Danilova et al. 2013), *Methylomonas lenta* (Hoefman et al. 2014) and the recently described *Methylomonas albis* and *Methylomonas fluvii* (Bussmann et al. 2021). As per LPSN (https://lpsn.dsmz.de/genus/methylomonas), there are ten non validly described *Methylomonas* species. Considering this abundant *Methylomonas* spp. in nature, recently, there was a proposed '*Methylomonadaceae*' family within class *Gammaproteobacteria* which included type Ia methanotrophs (Cabrol et al. 2020; Houghton et al. 2019; Parks et al. 2018). However, this proposal yet awaits validation. These *Methylomonas* species have been isolated from sediments of freshwater lakes and rivers, wetland muds, activated sludge or denitrification tanks, deep groundwaters, and rice rhizospheres (Bowman 2015). *Methylomonas koyamae* was the first

 doi:10.20944/preprints202207.0157.v1

Methylomonas species reported from the rice field floodwater in Japan (Ogiso et al. 2012). The present study describes the polyphasic characterisation of another representative of the genus *Methylomonas* strain Kb3, isolated from an organically fertilised tropical India rice field. Based on the observations, we propose the methanotrophic strain Kb3 represents a novel species of the genus *Methylomonas*.

Materials and Methods

Cultivation and isolation of strain Kb3

The enrichment of a rhizosphere sample (Kb) collected from an organically fertilised tropical rice field was established in dilute Nitrate Mineral salts (dNMS) medium. The field was located in the Kalbhorwadi village, Urawade of Pune district (18.37°N, 73.45°E), India. In the 10^{-3} dilution of the enrichment set for the sample, Kb orange floating growth in the form of a pellicle was observed. The growth in the 10^{-3} dilution was observed in the enrichment that was set in the serum bottles with a headspace containing 20% methane and 25% air that was added after filtration through a syringe filter (0.2 microns, 25 mm). The remaining headspace had nitrogen gas flushed in the serum bottles before autoclaving and incubated at 25°C. A micro-aerophilic condition was maintained in the serum bottles. The growth in the bottle was further diluted upto 10^{-8} dilutions in microtitre plates in order to obtain only a pure methanotroph in the higher dilutions. In the microtitre plate, similar growth was observed in the dilutions 10^{-3} to 10^{-7}. The growth in the higher dilutions was observed under the phase-contrast microscope. The culture was preserved at -20°C and later used for the preliminary identification of the *pmo*A (particulate methane monooxygenase ß subunit) sequence to identify the bacteria. The preserved growth sample's direct *pmo*A gene amplification was performed, and the obtained sequence was submitted to the NCBI database for BLAST analysis.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

For isolation, the pellicle was carefully extracted with the help of the thick gauge needle and streaked onto the dNMS agarose (2%) plates. These plates were incubated at 25°C in a desiccator with the headspace flushed with nitrogen gas and addition of methane and air such that the percentages corresponded to 20% and 25%, respectively. The plates were observed for growth every 2-3 days until the colonies appeared. The methanotrophic bacteria always had some closely associated heterotrophic bacteria growing with them. These heterotrophic bacteria were removed by repeated streaking of the methanotrophic colonies until the axenic culture was obtained. The purity of the culture was confirmed by microscopic observation of the cells, and no growth on the 1/10 diluted nutrient agar plates supplemented with 0.1% glucose. The strain was named Kb3, as it was isolated from the 3rd dilution of the Kb sample enrichment bottle.

Morphological examinations of strain Kb3

The Kb3 cells were observed under 40X and 100X magnification of the phase-contrast microscope (Nikon 80i, Japan microscope with a camera). The cell structure and accurate measurements were observed using the Scanning Electron Microscopy (SEM) (Zeiss model EVO-MA-15 SEM). For SEM observation, the cells were processed in the following manner: - The pelleted cells were washed with 0.1% sodium cacodylate solution before fixing the cells in 4% (v/v) glutaraldehyde prepared in 0.2 M sodium cacodylate buffer (pH 7.2) and incubated at 4°C for 1 - 2 days. The next day the glutaraldehyde was removed by centrifugation at 8000rpm for 5 min at 4°C in a Hermle centrifuge (Hermle Labortechnik Z 36 HK). The pellet was then washed thrice in 0.1 M sodium cacodylate buffer (pH 7.2) with intermittent 10 min incubation at room temperature. The washing step was carried out by centrifugation at 8000 rpm for 10 min. The washing step was followed by the fixation of the cells in 1% (w/v) osmium

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

tetraoxide prepared in 0.1 M sodium cacodylate buffer with incubation for 2 hrs at room temperature. The fixed cells were then washed thrice with the buffer and then further processed for dehydration. The dehydration process of the cells was carried out with ascending concentrations of acetone (30%, 50%, and 75%). For each concentration of acetone, the cells were centrifuged at 8000 rpm for 10 min with an interval of 10 min incubation at room temperature. An addition of 95% acetone was done before storing the sample at 4°C until the next processing. The cell pellet that was obtained after centrifugation of the stored sample was washed twice in 100% acetone and resuspended the third time in 100% acetone, and about 50 µl of the 100% acetone cell suspension was loaded onto clean, surface-sterilised metal stubs that were further dried at room temperature before being sputtered with gold dust. These loaded gold-sputtered stubs were then placed in the SEM tray and the observations were done under expert supervision.

For studying the motility of the cells, the wet mount of a well-grown cell suspension was observed under 100X magnification of the phase-contrast microscope. The Gram character of the cells was examined using the standard Gram staining protocol.

Physiological characterisation of Kb3

A 10% of freshly grown Kb3 inoculum with an OD of 0.1-0.2 was used for all the physiological characterisation experiments. All the tests were performed in triplicates. The growth of strain Kb3 was tested with various sugars as carbon (C) sources where filter-sterilised 0.1% (w/v) maltose, xylose, glucose, fructose, sucrose, arabinose, and raffinose were supplemented to the dNMS medium in microtitre plates. Additionally, growth was also checked in 0.1% formate and formamide in microtitre plates that served as C1 acid and amide groups, respectively. The microtitre plates were incubated in a desiccator at 25°C containing 20% methane and remaining air as the gas mixture.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

Alternately, the substitution of methanol for methane was tested at varying concentrations from 10 mM to 1000 mM in liquid dNMS medium in sealed serum bottles. Similarly, the utilisation of formaldehyde from 1 mM to 50 mM was tested in the liquid medium. The utilisation of acetate in the range of 2-10 mM was also tested by adding sodium acetate in a liquid dNMS medium in the micro-titre plates. These plates were incubated directly at 25°C without placing in a desiccator with a gas environment.

The strain Kb3 to utilise organic and inorganic nitrogen sources was examined by substituting KNO_3 in a liquid dNMS medium.

The substrates tested were; - NH_4Cl (2.5mM to 100mM), urea (2.5mM to 100mM), (rest 0.05%) peptone, yeast extract, and amino acids like glycine, serine, valine, asparagine, aspartate, L-glutamic acid, and glutamate, in microtitre plates with liquid medium. Growth of the strain Kb3 was also tested under a micro-oxic condition with 25% air (~ 4% oxygen) in a nitrogen-free liquid medium. The ability of the strain to utilise and reduce nitrite was also examined by supplementing the medium with the respective salts under hypo-oxic (0% air) and micro-oxic (5% air) conditions with 20% methane as the headspace gas or 200mM methanol.

The growth and tolerance of strain Kb3 were also tested in several pH ranges using several combinations of buffers and acid-base systems. The growth was analysed in microtitre plates through a wide range of pH values from 3 to 9.6 in triplicates. The phosphate buffer was replaced with the respective buffer combinations for attaining the desired pH. The citrate- phosphate was used for the pH range of 3 – 6.8. Likewise, for attaining the alkaline pH 8, 9 and 9.6, the glycine buffer was added to the dNMS medium. Non-buffered conditions were also tested wherein the pH range of 3 to 10 was set by using HCl and NaOH, acid and base combination. The incubations of these

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

differently buffered inoculated mediums were the same as that of the regular dNMS medium. The microtitre plates were incubated in the desiccator with 20% methane and the remaining air environment at 25°C.

The optimum temperature for the growth of strain Kb3 was determined by incubating the inoculated serum bottles at various temperatures like 15°C, 20°C, 25°C, 30°C, 37°C, and 40°C. The test was performed in triplicates, and OD600 measurements estimated the growth.

The ability of the strain to grow or tolerate salt stress was studied by inoculating the culture in various salt concentrations like 0.1%, 0.5%, 1% and 2.0%, w/v, added to the mineral medium.

The culture was exposed to a range of oxygen concentrations like hypo-oxic (0% oxygen), micro-oxic (1% and 5% oxygen), partial oxic (10% oxygen), and complete oxic (20% oxygen), to test its ability to grow under these varying oxygen concentrations. For these conditions, the dNMS medium in the serum bottles was flushed with nitrogen gas before autoclaving. A calculated percentage of filtered air, i.e. 0, 5, 20, 50 and 80%, was added in ml volumes with a syringe alongwith 20% methane in all bottles. The bottles were incubated at 25°C.

A well-grown culture of strain Kb3 was preserved at -80°C and under liquid nitrogen in 15% glycerol, 5% DMSO in the mineral medium and 5% DMSO in trypticase soy broth containing 1% tryptone (1% TT) (Hoefman et al. 2012). The culture was revived at intervals of 1 month, three months and six months.

Chemotaxonomic analysis

A 5-day old Kb3 culture grown in dNMS agarose plates (5 nos.) was submitted for Fatty Acid Methyl Ester (FAME) analysis. The culture in the late exponential phase

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

(3rd quadrant of streaking) was used mainly for estimating the cellular fatty acid concentrations. The culture was divided into two parts in 50 ml falcon tubes and centrifuged. The pellets were pooled and transported on ice to Pune, India's National Centre for Microbial Resources (NCMR) facility. The pellet was then washed and further processed in the following manner: - saponification, methylation, extraction and final injection of the processed sample into gas chromatography (7890A Agilent Technologies). The peaks of the separated methyl esters were then compared using the Sherlock Pattern, a peak identification software containing a pre-stored database of all the known methyl esters. The graph alongwith its respective percentage of fatty acids was then obtained from NCMR and used to interpret the data.

Phylogenetic analysis

DNA was extracted from a well-grown liquid cell culture using the Gram-negative protocol of the Sigma GenElute™ Kit and examined using gel electrophoresis. The extracted DNA was further used for carrying out the 16S rRNA gene and *pmo*A gene amplification and sequencing. The complete *pmo*A and 16S rRNA sequences were obtained and submitted to the NCBI (https://submit.ncbi.nlm.nih.gov/) to get their accession numbers. A clean 16S rRNA sequence confirmed the purity of the culture.

The 16S rRNA gene and *pmo*A gene sequences were analysed using the BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The related species sequences were extracted from NCBI to generate the phylogenetic trees using the respective sequences. The trees were constructed with the help of the MEGA X software (Kumar et al. 2018) after the sequences were aligned with the MAFFT server (https://mafft.cbrc.jp/alignment/server/), an online alignment tool. The phylogenetic analysis was performed using the maximum likelihood algorithm of the Tamura–Nei

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

(16S rRNA tree) and Poisson (*pmo*A tree) correction and 1000 replications to confirm the accuracy of the constructed tree.

Draft genome analysis

The extracted genomic DNA was submitted to Sandor Company, Hyderabad India, to carry out the whole-genome sequencing using the Illumina HiSeq platform. The *de-novo* assembly of the short length sequences obtained was carried out using Velvet 1.2.10 assembler. The contigs were then submitted for annotation to the Rapid Annotation using Subsystem Technology (RAST server) (Aziz et al 2008, Overbeek et al 2014) and NCBI. The amino acid files were extracted from the RAST server and submitted to the BlastKOALA (http://www.kegg.jp/blastkoala/) online server for determining the metabolic pathways using the KEGG database.

Comprehensive genome analysis was performed to determine the genome similarity with the closely related species using the digital DNA–DNA hybridisation (dDDH, http://ggdc.dsmz.de/) (Meier-Kolthoff et al. 2013). The Kb3 genome sequence was compared to the closely related species for their pairwise average nucleotide identity (ANI-b) using JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/#analyse) online server (Richter and Rosselló-Móra 2009; Sangal et al. 2016). Also, the Ortho average nucleotide identity (Ortho ANI) was calculated with the closely related species using the online ORTHO-ANI tool (https://www.ezbiocloud.net/tools/orthoani) (Lee et al. 2016; Yoon et al. 2017). The draft genome was also used for constructing the phylogenomic comparison tree using the PATRIC database.

The genome was also subjected to orthologous gene cluster comparative analysis between the closely related genomes using the Orthovenn 2 (https://orthovenn2.bioinfotoolkits.net/home), a web platform that is designed for easy

 doi:10.20944/preprints202207.0157.v1

annotation and comparison between the protein sequences of multiple species (Xu et al. 2019).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

Results and discussion

Isolation and morphological studies

A formation of thick pink/orange pellicle was observed in the 10^{-3} enrichment of the serum bottles that were further diluted in the microtitre plates. In the microtitre plate, pink/orange pellicular growth was observed in the dilutions 10^{-3} to 10^{-7}. This growth was observed under a phase-contrast microscope where the cells appeared as short motile rods. These rods were dark and coccobacilli shaped. In this preliminary observation these dark motile rods were observed alongwith many small lighter shaded rods and cocci, which were identified as heterotrophs based on some identifications that were performed on the heterotrophic cells.

This thick pink/orange pellicle formed pink coloured translucent mucoid colonies measuring 2 mm – 5 mm in diameter on the solid dNMS plates (Fig 1a). The colonies were irregularly shaped with a watery consistency. The pink Kb3 colonies appeared on the plates after 7 days of incubation at 25°C under micro-aerophilic condition with 20% methane, 25% air and remaining nitrogen atmosphere. These pink colonies were contained many other pin-point colonies. On observation, we found that these colonies belonged to the heterotrophic bacteria that were also observed in the enrichments earlier.

After several rounds of streaking and restreaking of the pink isolated colonies we successfully obtained the axenic culture of strain Kb3. The purity of the culture was confirmed by the microscopic observation of only the dark, motile coccobacilli shaped cells, with no small bacterial cells around them (Fig. 1d). The cells measured roughly about 1 – 2 µm × .0.5–1 µm. The cells stained Gram negative and were healthily motile. They did not form chains but occurred in singles or paired form.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

The pure culture of Kb3 when inoculated in liquid medium formed a floating pink pellicle at the surface of the liquid medium in the bottle (Fig. 1b) after about 5 days of incubation under 20% methane and remaining air environment. On shaking, the pellicle drifted and sank to the bottom of the bottle. This pellicle formation was typical when the phosphate buffer concentration of the medium was low initially. On increasing the buffer concentration of the medium, the growth of the culture transformed to a turbid suspension and no pellicle was observed (Fig.1c). This turbid growth helped in measuring the concentration such as OD of the culture that was otherwise not possible. The morphology and the dimensions of the Kb3 cells were confirmed by the SEM imaging wherein the short plumpy rods were observed which measured 1.3 µm × 0.6 µm as the average cell size (Fig.1e).

Physiological characteristics

The strain Kb3 was cultivated and isolated under 20% methane, 25% air and remaining nitrogen atmosphere. Thus, the strain successfully oxidised methane for growth under micro-oxic conditions. An OD of 0.3 was reached when the strain Kb3 was grown under a completely oxic environment with 80% air (16% oxygen) and 20% methane. Thus, the growth of Kb3 was favoured under complete oxic condition. Strain Kb3 could tolerate and grow in methanol concentrations from 50 mM to 800 mM with optimum growth between 200 mM to 500 mM methanol, expressing a maximum OD of 0.25. Among the other carbon sources analysed, strain Kb3 did not utilise or grow in any other carbon source except methane and methanol. Thus, its favoured carbon sources are limited to methane and methanol.

Strain Kb3 could grow in the presence of NH_4Cl upto 100mM concentration, but it could not tolerate urea even at the lowest concentration tested (2.55 mM). Kb3 sustained in all concentrations of KNO_3 (2.5 mM – 100 mM). Thus, the methanotrophs'

 doi:10.20944/preprints202207.0157.v1

tolerance to different nitrogen substrates could be co-related to their sustainability under the influence of nitrogen-based fertilisers in the rice fields (Vishwakarma and Dubey 2010). The extensive use of NH_4 based fertilisers for increasing the rice yield also impacts the methanotrophic flora (Shrestha et al. 2010). Therefore, the impact is controversial and could be stimulative (Bodelier et al. 2000, Vishwakarma and Dubey 2010) or inhibitory (Dubey and Singh 2000, Dubey et al. 2002).

Among the other nitrogen sources analysed, strain Kb3 could utilise and grow in L-serine, L-valine, peptone, and yeast extract. The culture failed to grow under the nitrogen-free medium under all tested conditions. It did not fix the nitrogen gas added in the bottle nor utilise the nitrite source for growth.

Kb3 expressed tolerance concerning growth for a wide range of pH from 5 – 10 under buffered as well as non-buffered conditions. However, the optimum pH was 6.5–7 (Table 1). The optimum temperature at which strain Kb3 flourished was 25 - 28°C (Table 1) under 20% methane and air environment, thus expressing its mesophilic nature. Kb3 could grow optimally at temperatures 20°C, 25°C and 30°C. Marginal growth was seen at 15°C and 37°C and no growth at 40°C. The maximum OD_{600} attained by strain Kb3 was 0.35 at 25°C followed by 0.32 at 28°C. The OD_{600} at 15, 20, 30, and 37°C were 0.05, 0.09,0.16, and 0.06, respectively.

Strain Kb3 was sensitive to the NaCl (salt) concentrations, as it was found to tolerate only upto 0.5% (w/v) NaCl. Beyond this concentration, there was no growth observed. The growth of the strain Kb3 under the hypo-oxic (1% oxygen) and micro-oxic (5% oxygen) conditions was not observed, even after ten days of incubation. Whereas the partial oxygen (20% air) condition favoured the growth of the strain bringing the OD to 0.18 in 6 days. However, the preferred growth condition was 80% air (16% oxygen), which attained an OD of 0.32 in about four days.

 doi:10.20944/preprints202207.0157.v1

The preservation of the strain Kb3 was attempted using three methods that gave varying results. The culture failed to revive from the frozen stocks prepared using 15% glycerol and 5% DMSO in 1% TT broth even after minimal preservation at freezing temperatures. However, the storage with 5% DMSO proved to be a successful preservation technique as the culture could be revived after several months of storage at -80°C. Another recommended method was storing the liquid culture at 4°C in refrigeration for 6-8 months, followed by its subculture could be used under laboratory conditions for regular experimentations.

The favourable growth conditions for the Kb3 culture could be summed up as 20% methane with complete oxic growth condition, incubation at 25°C, and pH 6.8. 250 - 400 mM methanol would also serve as an alternative for the C source. However, the preferred C source is methane as the aim of cultivation of these native methanotrophs is their future application for methane mitigation studies.

Table 1: - Differential characteristics of strain Kb3 and phylogenetically related Type I genera

Methylomonas sp. Kb3 (This study); *Methylomonas fluvii* (Bussmann et al. 2021); *Methylomonas albis* (Bussmann et al. 2021); *Methylomonas methanica* (Bowman et al. 1993); *Methylomonas koyamae* (Ogiso et al. 2012); *Methylomonas fodinarum* (Bowman et al. 1990); *Methylomonas scandinavica* (Kalyuzhnaya et al. 1999); *Methylomonas aurantica* (Bowman et al. 1990); *Methylomonas paludis* (Danilova et al. 2013); *Methylomonas lenta* (Hoefman et al. 2014)

(v – variable within strains; nd – not determined)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

Characteristics	***Methylomonas* sp. strain Kb3**	***Methylomonas fluvii***	***Methylomonas albis***	***Methylomonas methanica***	***Methylomonas koyamae***	***Methylomonas fodinarum***	***Methylomonas scandinavica***	***Methylomonas aurantiaca***	***Methylomonas paludis***	***Methylomonas lenta***
Cell Morphology	**Coccobacilli (short rods)**	Rod shaped	Rod shaped	Rods	Rods	Rods	Rod like ovoids	Rods	Rods	Rods
Cell size	**1 - 2 µm x 0.5 - 1 µm**	2.5 µm x 0.8 µm	2.5 µm x 0.8 µm	0.5 – 3 µm x 0.5 – 1 µm	1.2 -2.5 µm x 0.8 – 1.1 µm	0.5 – 3 µm x 0.5 – 1 µm	1.5 – 1.7 µm x 0.6 – 0.8 µm	0.5 - 3 µm x 0.5 - 1 µm	1 - 4 µm x 1 - 1.5 µm	1.3 – 2 x 0.6 – 0.9 µm
Colony color	**Pink/orange**	Pink	Pink	Pink	Pink/ orange	Orange	Pink	Orange	Pale pink	White to pink sheen
Motility	**+**	-	-	+	+	+	+	+	-	+
Optimum growth temperature (°C)	**25 - 30**	15	15	25 - 30	28 - 30	30 - 35	15 - 20	20 - 25	20 - 25	20 - 25
Optimum pH	**6.5 - 7**	6	6	7	6.5	7	6.8 – 7.6	7	5.8 - 6.4	6.8 – 7.3
nifH gene	**+**	+	+	v	nd	+	nd	+	+	-
CH_4 oxidation gene	**pMMO/sMMO**	pMMO/sMMO	pMMO/sMMO	pMMO/sMMO (v)	pMMO	pMMO/sMMO(nd)	pMMO	pMMO/ sMMO (nd)	pMMO	pMMO
G+C content (mol%)	**51.8**	51.5	51.07	51-54	57	58-59	53 - 54	55-58	48 - 49	47

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

Chemotaxonomic characteristics and phylogenetic analysis

C_{16} has been reported to be the major fatty acid in type Ia methanotrophs (Bowman 2016). The Type Ia methanotroph, *Methylomonas* sp. strain Kb3 possessed C_{14} as the major fatty acid, with the dominance of $C_{14:0}$ being 46.1%, followed by $C_{16:1}$ ω5c being 17.8% (Table 2). $C_{16:0}$ is the next highest concentration of fatty acid in strain Kb3. *Methylomonas fluvii*, the closest phylogenetic relative to strain Kb3, expressed a higher percentage of fatty acid $C_{14:0}$ (51.6%) followed by $C_{12:0}$ with 26.6% (Bussmann et al. 2021). *M. fluvii* and *M. albis* did not possess other C_{16} fatty acids except $C_{16:0}$ (Table 2) (Bussmann et al. 2021). Strain Kb3 also possessed other fatty acids like $C_{12:0}$, $C_{16:0}$ iso, $C_{17:0}$ iso, $C_{16:0}$ 3OH and $C_{18:1}$ ω7c / ω6c in minor percentages (Table 2). The strain Kb3 expressed a distinctive FAME profile compared to the other *Methylomonas* relatives of the type Ia methanotrophs, thus supporting the species level novelty of the strain Kb3 (Table 2).

Table 2: - FAME profile of *Methylomonas* sp. Kb3 in comparison to the related *Methylomonas* species

Fatty acids	***Methylomonas* sp. strain Kb3**	***Methylomonas fluvii* EbB**	***Methylomonas albis EbA***	***Methylomonas methanica* *Methylomonas aurantiaca* *Methylomonas fodinarum***	***Methylomonas koyamae***	***Methylomonas lenta***	***Methylomonas paludis***
12:0	**2.13**	26.6	19.2	-	-	0.6-2.5	-
13:0	-	3.7	5.1	-	-	0.6-0.9	-
13:1		3.7	4.8				
14:0	**46.1**	51.6	45.9	18.9-24.6	23	6.4-9.8	11.8
15:0	-			0-1.2	1.2	5.3-5.8	0.5
15:0 iso	-	4.3		0-2.5	-	-	-
15:0 anteiso	-			0-2.4	-	-	-
15:1 ω8c	-			-	-	1.0-2.3	-
15:1 ω6c	-		7.5	-	-	0.5-0.6	-
15:1 ω5c	-			-	-	1.0-1.1	-
16:0	**14.3**	7.9	8.6	4.3-8.7	7.7	5.0	5.6
16:0 iso	**0.95**						
16:1ω11c	-			-	-	-	-

16:1ω8c	-			**18.7-41.3**	**39.4**	**40.8-42.4**	**22.1**
16:1ω7c	-			7.7-15.3	4.35	9.1-10.5	13.9
16:1ω6c	-			4.5-13.3	-	-	5.0
16:1ω5c	**17.8**			1.9-6.3	16.7	11.7-18.3	1.8
16:1ω5t	-			7.9-16.6	-	-	34.8
17:0 cyclo	-			0-2.1	-	-	-
17:0 iso	**2.48**						
17:1 ω8c	-			-	-	0-0.8	-
17:1 ω7c	-			0-0.7	-	-	-
17:1 ω7t	-			0-0.3	-	-	-
18:0	-			0-0.1	0.72	-	1.2
18:1 ω7c	**2.02**			0.2-2.5	-	-	-
18:1 ω6c	**1.32**			-	-	-	-
18:1 ω5c	-			0-0.2	1.7	-	-
19:0 cyclo	-			0.2-0.4	-	-	-
19:1 branched	-			0-0.5	-	-	-
15:0 2-OH	-			-	1.43	-	-
16:0 3-OH	**12.9**	5.1	9.0	-	3.8	4.1-4.2	-

(Values are represented in percentages %)

The 16S rRNA gene sequence BLAST analysis in the NCBI server, selecting the dis-contiguous megablast option alongwith the option of only type species comparisons, strain Kb3 was found to be a closest relative of *Methylomonas fluvii* with 99.52%, but the query cover was 92%. The next closest type pecies is *Methylomonas methanica* $S1^T$ with 97.9% sequence similarity. The 16S rRNA gene of strain Kb3 also expressed a 99.1% sequence similarity with the recently described "*Methylomonas denitrificans*" FJG1 (Kits et al. 2015) and a 99.5% similarity with *Methylomonas* sp. LW13, a freshwater isolate (Kalyuzhnaya et al. 2015), when the type species option was deselected during NCBI BLAST analysis. The threshold for novel species demarcation based on the 16S rRNA gene similarity is ≤ 98.7% (Kim et al. 2014). However, the recent proposal for improving classification recommends the overall genome-related index (OGRI) as a tool for going beyond mere 16S rRNA based identification (Chun et al. 2018). The flowchart suggests calculating OGRIs when the

 doi:10.20944/preprints202207.0157.v1

16S rRNA gene similarity is ≥ 98.7% between closely related type species (Chun et al. 2018). Thus, in this case, the 16S rRNA gene relatedness between strain Kb3 and *Methylomonas fluvii* is 99.52% which recommends genome-level analysis for species-level identification of strain Kb3. The 16S rRNA gene sequence submitted to the NCBI database has accession number KP793700.

The phylogenetic tree of the 16S rRNA sequences of strain Kb3 and the phylogenetically affiliated species showed that Kb3 formed a distinct phylogenetic position from a branch of *Methylomonas fluvii* EbB and *Methylomonas methanica* S1, the closely related type species (Fig. 2). Kb3 shared a common branch of 46% bootstrap value with *Methylomonas* LW13, a Japanese rice field strain Stu-1B-Pr (Dianou et al. 2009), and another Indian rice field culture *Methylomonas* sp. Sn4 (Pandit et al. 2016), indicating that these strains could belong to the same species within the genus *Methylomonas*. *Methylomonas* sp. Sn4 was another closely related strain enriched and isolated from a different tropical Indian rice field in our lab. In the phylogenetic tree, all these strains branch away from *M. fluvii* with 86% bootstrap value and 95% bootstrap value with *M. methanica*. Freshwater sediments and flooded rice ecosystems both these habitats demonstrate methane and oxygen gradients (Knief 2015), thus explaining the similarities in the strain LW13 and strain Kb3.

Strain Kb3 also held a unique phylogenetic position in the *pmo*A gene-based tree, wherein it branched close to many *Methylomonas* strains that were not validly described (Fig. 3). The BLAST analysis of the *pmo*A gene expressed 97.35% similarity to *Methylomonas* LW13, 95.72% similarity to "*Methylomonas denitrificans*" FJG1, and 97.02% with strain Stu1B-Pr.

The closest type species most related to strain Kb3 was *Methylomonas fluvii EbB*^{T} with 95.15% similarity isolated from a cold river Elbe flowing through Germany. The *pmo*A (molecular marker gene) has been co-related to the 16S rRNA gene similarities for species and genus demarcation. The species delineation using the *pmo*A gene sequence was deduced to OTU_4, which meant a value ≤ 96% *pmo*A nucleotide sequence similarity would define a novel species (Knief 2015). Thus, considering the cut-off value for the *pmo*A gene similarity, supporting the 16S rRNA gene differentiation, strain Kb3 could be a putative novel species.

Genomic Characteristics

The Kb3^T draft genome was deposited to the NCBI database (PIZT00000000), https://www.ncbi.nlm.nih.gov/genome/13566?genome_assembly_id=357342 for getting additional information related to the genome and the annotated proteins. The draft genome size of strain KB3 is 5.12 Mb with a G+C content of 51.8% (Rahalkar and Pandit 2018). Strain Kb3 genome expressed a complete set of genes for methane oxidation pathway, which included pMMO genes (1+copy), soluble methane monooxygenase gene (1 copy), methanol dehydrogenase, tetrahydromethanopterin pathway genes for formaldehyde oxidation and formate dehydrogenase genes. The annotated proteins in the draft genome sequence of strain Kb3, analysed through the PGAP platform of the NCBI database, displayed similarity to *Methylomonas* sp. LW13/" *Methylomonas denitrificans*" FGJ1/ *Methylomonas methanica*. Kb3 also expressed genes for converting nitrite to nitric oxide, nitric oxide to nitrous oxide, and genes for nitrogen fixation, similar to most Type I methanotrophs. Even though genes for nitrogen fixation were present, significantly less growth was observed in nitrogen-free medium under micro-oxic conditions. Kb3 possessed genes encoding for chemotaxis that supported the mobility of the cells towards the CH_4 substrate. It also contained a gene expressing carotenoid pigment synthesis representing the orange/pink colony colour.

Genome data has been currently described as the basis for determining the overall genomic relatedness between two strains and the arbitrate for the species demarcation (Auman et al 2000, Lee et al 2016, Rosselló-Móra and Amann 2015). In addition to the 16S rRNA and other biochemical characterisation of strain Kb3, genome comparisons were made to determine that *Methylomonas* Kb3 formed a novel species. Based on the 16S rRNA gene similarities, the genome comparisons of Kb3 were performed with the genomes of *Methylomonas fluvii* EbB^T, *Methylomonas methanica* S1^T, and *Methylomonas* sp. strain LW13, using various genome-based parameters as mentioned in the materials and methods section. The comparison of Kb3 with *Methylomonas fluvii* EbB^T and *Methylomonas methanica* S1^T demonstrated the OrthoANI values of 91.48% and 86.30%, respectively, lower than the cut-off value of 95% (Lee et al. 2015; Lee et al. 2016). The digital DNA-DNA hybridisation (dDDH) calculated between strain Kb3, *Methylomonas fluvii* EbB^T, and *Methylomonas methanica* S1^{T}was 44.20% [41.6 - 46.7%] and 31.60% [29.2 - 34.1%], respectively, lower than

 doi:10.20944/preprints202207.0157.v1

the cut-off determined to be 70% (Auch et al. 2010; Meier-Kolthoff et al. 2013). The ANIb values were 90.95% with *Methylomonas fluvii* EbB^T and 85.67% with *Methylomonas methanica* S1^T barring the 95-96% demarcation value (Sangal et al. 2016). This data supported that Kb3 represented a new species member within the genus *Methylomonas*. The closest resemblance of Kb3 expressing 99.66% 16S rRNA gene similarity was observed with the freshwater lake sediment isolate *Methylomonas* sp. LW13. The genome comparisons of Kb3 and LW13 (NCBI accession number JNLB00000000) thus revealed some interesting facts. The OrthoANI comparison value of Kb3 with *Methylomonas* LW13 was 97.9%, followed by the dDDH value of 87% and the ANIb value of 97.46%. The two strains barred the cut-off values for each of the tests, and thus, together, they could form a novel species of the genus *Methylomonas*. Another supporting factor was that the G+C content for both *Methylomonas* LW13 and *Methylomonas* Kb3 was same i.e. 51.8%. It was also reported that the two strains, Kb3 and LW13, together represent a novel species within the genus *Methylomonas* (Orata et al. 2018)

The phylogenomic tree indicated that *Methylomonas* Kb3 and *Methylomonas* sp. LW13 (Kalyuzhnaya et al. 2015) grouped together and away from *Methylomonas fluvii* EbB, the closest type species (S Fig. 1), thus, forming a putative novel species within the *Methylomonas* genus.

The Venn diagram depicted 4334 orthologous gene clusters distributed among the five strains under consideration (S.Fig. 3a). The centre of the Venn diagram represented 3044 core orthologous gene clusters conserved in all five genomes, representing 70.23% of the total orthologous clusters shared among the five genomes being compared. *Methylomonas* sp. Kb3 contained 227 unique orthologous gene clusters (S.Fig. 3a), accounting for 5.23% of the total orthologous clusters compared with *Methylomonas fluvii* EbB. This gene-level uniqueness in the genomes supports the novelty of the *Methylomonas* sp. Kb3. This strain also possessed seven unique gene clusters that were absent in the other correlated species.

The HeatMap generated (S.Fig. 3b) by coinciding with the orthologous gene clusters of the five closely related *Methylomonas* genomes represented the values of the core gene clusters shared between the individual strains. 4332 of the 4334 orthologous gene clusters are common between the *Methylomonas* sp. Kb3 and the

 doi:10.20944/preprints202207.0157.v1

Methylomonas sp. LW13 accounting for about 99.9% similarity between the strains (shown with a dark brown shade). The gene cluster sharing between *Methylomonas* sp. Kb3 and the closest type species *Methylomonas fluvii* EbB is 4107 of the total 4334 genes explaining their relatedness with 94.76%. Followed by this, the values of gene cluster relatedness between *Methylomonas* sp. KB3 and *Methylomonas methanica* S1 is 93.88% (4069 genes), and with *Methylomonas albis* EbA the value is 93.37% (4047 genes) (S. Fig. 3b).

Strain Kb3 has been deposited in one Indian and two international culture collections; National Centre for Microbial Resources (NCMR), India, (=MCC 4012), Korean Collection for Type Cultures, Korea (=KCTC 72521), and Japanese Collection of Microorganisms, Japan (=JCM 33634). With the success in preservation at 5% DMSO and deposition in three repositories of international repute, strain Kb3 could be described as a novel species of the genus *Methylomonas*.

The similarities between the *Methylomonas* LW13 isolated from the freshwater sediment of Lake Washington and Kb3 isolated from a flooded rice field in India in their genomic characteristics are remarkable. Our previous publication which had a description of the Kb3 genome features (Rahalkar and Pandit 2018) mentioned the proposal of the name *Methylomonas aquatica*. Thus, we propose the name: *Methylomonas aquatica* strain Kb3 sp. nov. for this putative novel species considering the habitats of the closely related strains, Kb3 and LW13.

Description of *Methylomonas aquatica* sp. nov.

Methylomonas aquatica (a.qua'ti.ca. N.L. fem. n. aquatica, belonging to or living in water). Cells are Gram-negative, motile rods measuring 1 – 2 µm × .0.5–1 µm. Cells are singular or paired and form orange/pink pigmented surface pellicles or colonies. Culture grows only on methane or methanol as the sole carbon and energy source. RuMP is the central methane oxidation pathway. Grows optimally at the mesophilic condition at 25°C with pH 6.8, cells were sensitive to NaCl above 0.5%. The DNA G+C content is 51.8 mol%. Possesses C_{14} as the major fatty acid with the dominance of $C_{14:0}$ being 46.1%, followed by $C_{16:1}$ ω5c being 17.8% as the predominant fatty acids. The type strain, KB3 (=JCM 33634, =MCC 4012, =KCTC 72521), was isolated from

India's flooded rice paddy field. The 16S rRNA gene sequence submitted to the NCBI database has accession no. KP793700, and the *pmo*A gene sequence accession number is KP862532. The NCBI genome accession number is PIZT00000000.

Acknowledgements

MCR acknowledges the Department of Biotechnology, DBT BioCARe program (BT/BioCARe/06/840/2012) and SERB, Department of Science and Technology, (EMR/2017/002817) for providing the funds. PSP acknowledges the Department of Science and Technology for providing her women scientist (WOS-A) fellowship and funds (SR/WOS-A/LS-410/2017). Kumal Khatri acknowledges CSIR for the research fellowship. We thank Mr. Jaywant Kalbhor (rice farmer) for permitting us to sample the rice plants from his field and for his co-operation.

Authors' contributions

PSP carried out the laboratory studies, including sample collection, enrichment, isolation cultivation of the organism, performed all culture-based studies, molecular characterization and analysis. KK and JAM helped with experiments in the characterisation and maintenance of the organism. PSP wrote the manuscript, did the phylogenetic analysis and prepared the figures. MCR designed the study, collected the samples, supervised the experiments and carried out the initial experiments during isolation and characterization of the strain. All the authors reviewed the manuscript and approved the final version.

Conflict of interest

The authors confirm and declare that there is no conflict of interest.

Figure legends

 doi:10.20944/preprints202207.0157.v1

Figure 1: - Growth of strain Kb3 on solid plate (a); pellicle formation of the cutlure in liquid medium (b); turbid growth of the culture in the broth (c); microscopic images of Kb3 cells; phase contrast image (d) and SEM image of the cells (e).

Figure 2: - 16S rRNA gene-based phylogenetic tree of Kb3 and related species, constructed using the Maximum likelihood algorithm with the Tamura-Nei model and 1000 replicates. Bar represents 2% divergence.

Figure 3: Phylogenetic tree of the partial *pmo*A gene sequences of strain Kb3 and other phylogenetic relatives of the Type I methanotrophs. The tree was constructed using the maximum likelihood algorithm with the Poisson correction model and 1000 bootstraps. Bar represents 5% divergence.

Supplementary Figure 1: - Phylogenomic tree of strain Kb3 with the genomes of the closely related Type I methanotrophs constructed using PATRIC database with a 20% divergence bar

Supplementary Figure 2: - OrthoANI comparative tree of *Methylomonas* sp. strain Kb3 and genomes of closely related valid *Methylomonas* species.

Supplementary Figure 3: - (a) Venn diagram representing the shared and differentiating orthologous gene clusters between strain Kb3 and related *Methylomonas* species. (b) Heatmap table showing the values of gene clusters shared between each of the compared *Methylomonas* species.

References

Auch AF, von Jan M, Klenk H-P, Göker M (2010) Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison Standards in genomic sciences 2:117-134 doi:10.4056/sigs.531120

Bowman J, Sly L, Cox J, Hayward A (1990) Methylomonas fodinarum sp. nov. and Methylomonas aurantiaca sp. nov.: two closely related type I obligate methanotrophs Systematic and applied microbiology 13:279-287

Bowman JP (2015) Methylomonas. In: Bergey's Manual of Systematics of Archaea and Bacteria. pp 1-10. doi:10.1002/9781118960608.gbm01183

Bowman JP (2016) Methylococcaceae. In: Bergey's Manual of Systematics of Archaea and Bacteria. pp 1-8. doi:10.1002/9781118960608.fbm00225.pub2

Bowman JP, SLY LI, NICHOLS PD, Hayward A (1993) Revised taxonomy of the methanotrophs: description of Methylobacter gen. nov., emendation of Methylococcus, validation of Methylosinus and Methylocystis species, and a proposal that the family Methylococcaceae includes only the group I methanotrophs International Journal of Systematic and Evolutionary Microbiology 43:735-753

Bussmann I, Horn F, Hoppert M, Klings K-W, Saborowski A, Warnstedt J, Liebner S (2021) Methylomonas albis sp. nov. and Methylomonas fluvii sp. nov.: Two cold-adapted methanotrophs from the river Elbe and emended description of the species Methylovulum psychrotolerans Systematic and Applied Microbiology 44:126248 doi:https://doi.org/10.1016/j.syapm.2021.126248

Cabrol L et al. (2020) Anaerobic oxidation of methane and associated microbiome in anoxic water of Northwestern Siberian lakes Science of The Total Environment 736:139588 doi:https://doi.org/10.1016/j.scitotenv.2020.139588

Chauhan BS, Jabran K, Mahajan G (2017) Rice production worldwide vol 247. Springer,

Chun J et al. (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes International journal of systematic and evolutionary microbiology 68:461-466

Conrad R (1996) Soil microorganisms as controllers of atmospheric trace gases (H2, CO, CH4, OCS, N2O, and NO) Microbiol Mol Biol Rev 60:609-640

Conrad R (2007) Microbial ecology of methanogens and methanotrophs Advances in agronomy 96:1-63

Danilova OV, Kulichevskaya IS, Rozova ON, Detkova EN, Bodelier PL, Trotsenko YA, Dedysh SN (2013) Methylomonas paludis sp. nov., the first acid-tolerant member of the genus Methylomonas, from an acidic wetland International journal of systematic and evolutionary microbiology 63:2282-2289

Dianou D, Ueno C, Ogiso T, Kimura M, Asakawa S (2009) Diversity of cultivable methane-oxidizing bacteria in microsites of a rice paddy field: investigation by cultivation method and fluorescence in situ hybridization (FISH) Microbes and environments:1203160366-1203160366

Ferrando L, Tarlera S (2009) Activity and diversity of methanotrophs in the soil–water interface and rhizospheric soil from a flooded temperate rice field Journal of applied microbiology 106:306-316

Ganesan AL et al. (2017) Atmospheric observations show accurate reporting and little growth in India's methane emissions Nature Communications 8:836

Hoefman S, Heylen K, De Vos P (2014) Methylomonas lenta sp. nov., a methanotroph isolated from manure and a denitrification tank International journal of systematic and evolutionary microbiology 64:1210-1217

Hoefman S, Van Hoorde K, Boon N, Vandamme P, De Vos P, Heylen K (2012) Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria PLoS One 7

Houghton KM, Carere CR, Stott MB, McDonald IR (2019) Thermophilic methanotrophs: in hot pursuit FEMS Microbiol Ecol 95 doi:10.1093/femsec/fiz125

Kalyuzhnaya MG, Khmelenina VN, Kotelnikova S, Holmquist L, Pedersen K, Trotsenko YA (1999) Methylomonas scandinavica sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden Systematic and applied microbiology 22:565-572

Kalyuzhnaya MG, Lamb AE, McTaggart TL, Oshkin IY, Shapiro N, Woyke T, Chistoserdova L (2015) Draft genome sequences of gammaproteobacterial methanotrophs isolated from Lake Washington sediment Genome Announc 3:e00103-00115

Khatri K, Mohite JA, Pandit PS, Bahulikar R, Rahalkar MC (2020) Description of 'Ca. Methylobacter oryzae' KRF1, a novel species from the environmentally important Methylobacter clade 2 Antonie van Leeuwenhoek 113:729-735 doi:10.1007/s10482-019-01369-2

Kim M, Oh H-S, Park S-C, Chun J (2014) Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes International journal of systematic and evolutionary microbiology 64:346-351

Kits KD, Klotz MG, Stein LY (2015) Methane oxidation coupled to nitrate reduction under hypoxia by the G ammaproteobacterium M ethylomonas denitrificans, sp. nov. type strain FJG1 Environmental microbiology 17:3219-3232

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms Molecular Biology and Evolution 35:1547-1549 doi:10.1093/molbev/msy096

Lee HJ, Jeong SE, Kim PJ, Madsen EL, Jeon CO (2015) High resolution depth distribution of Bacteria, Archaea, methanotrophs, and methanogens in the bulk and rhizosphere soils of a flooded rice paddy Frontiers in microbiology 6:639

Lee I, Kim YO, Park S-C, Chun J (2016) OrthoANI: an improved algorithm and software for calculating average nucleotide identity International journal of systematic and evolutionary microbiology 66:1100-1103

Lüke C et al. (2014) Macroecology of methane-oxidizing bacteria: the β-diversity of pmoA genotypes in tropical and subtropical rice paddies Environmental microbiology 16:72-83

Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions BMC bioinformatics 14:60

Ogiso T, Ueno C, Dianou D, Van Huy T, Katayama A, Kimura M, Asakawa S (2012) Methylomonas koyamae sp. nov., a type I methane-oxidizing bacterium from floodwater of a rice paddy field International journal of systematic and evolutionary microbiology 62:1832-1837

Orata FD, Meier-Kolthoff JP, Sauvageau D, Stein LY (2018) Phylogenomic analysis of the gammaproteobacterial methanotrophs (Order Methylococcales) calls for the reclassification of members at the genus and species levels Frontiers in microbiology 9:3162

Pandit PS, Hoppert M, Rahalkar MC (2018) Description of 'Candidatus Methylocucumis oryzae', a novel Type I methanotroph with large cells and pale pink colour, isolated from an Indian rice field Antonie Van Leeuwenhoek 111:2473-2484

Pandit PS, Rahalkar MC (2019) Renaming of 'Candidatus Methylocucumis oryzae'as Methylocucumis oryzae gen. nov., sp. nov., a novel Type I methanotroph isolated from India Antonie van Leeuwenhoek 112:955-959

Pandit PS, Rahalkar MC, Dhakephalkar PK, Ranade DR, Pore S, Arora P, Kapse N (2016) Deciphering community structure of methanotrophs dwelling in rice rhizospheres of an Indian rice field using cultivation and cultivation-independent approaches Microbial ecology 71:634-644

Parks DH, Chuvochina M, Waite DW, Rinke C, Skarshewski A, Chaumeil P-A, Hugenholtz P (2018) A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life Nature Biotechnology 36:996-1004 doi:10.1038/nbt.4229

Rahalkar MC, Khatri K, Mohite J, Pandit PS, Bahulikar RA (2020) A novel Type I methanotroph Methylolobus aquaticus gen. nov. sp. nov. isolated from a tropical wetland Antonie van Leeuwenhoek 113:959-971

Rahalkar MC, Pandit P (2018) Genome-based insights into a putative novel Methylomonas species (strain Kb3), isolated from an Indian rice field Gene Reports 13:9-13

Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition Proceedings of the National Academy of Sciences 106:19126-19131

Sangal V, Goodfellow M, Jones AL, Schwalbe EC, Blom J, Hoskisson PA, Sutcliffe IC (2016) Next-generation systematics: an innovative approach to resolve the structure of complex prokaryotic taxa Scientific reports 6:1-12

Saunois M et al. (2016) The global methane budget 2000-2012 Earth System Science Data 8:697

Shrestha M, Shrestha PM, Frenzel P, Conrad R (2010) Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere The ISME journal 4:1545-1556

 doi:10.20944/preprints202207.0157.v1

Sieburth JN, Johnson PW, Eberhardt MA, Sieracki ME, Lidstrom M, Laux D (1987) The first methane-oxidizing bacterium from the upper mixing layer of the deep ocean: Methylomonas pelagica sp. nov Current Microbiology 14:285-293

Whittenbury R aNRK (1984) Family IV. Methylococcaceae, In N. R. Krieg and J. G. Holt (ed.) Bergey's manual of systematic bacteriology edn. edn. The Williams & Wilkins Co.: Baltimore,

Xu L et al. (2019) OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species Nucleic Acids Res 47:W52-w58 doi:10.1093/nar/gkz333

Yoon S-H, Ha S-m, Lim J, Kwon S, Chun J (2017) A large-scale evaluation of algorithms to calculate average nucleotide identity Antonie van Leeuwenhoek 110:1281-1286

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1

List of Figures

Figure 1

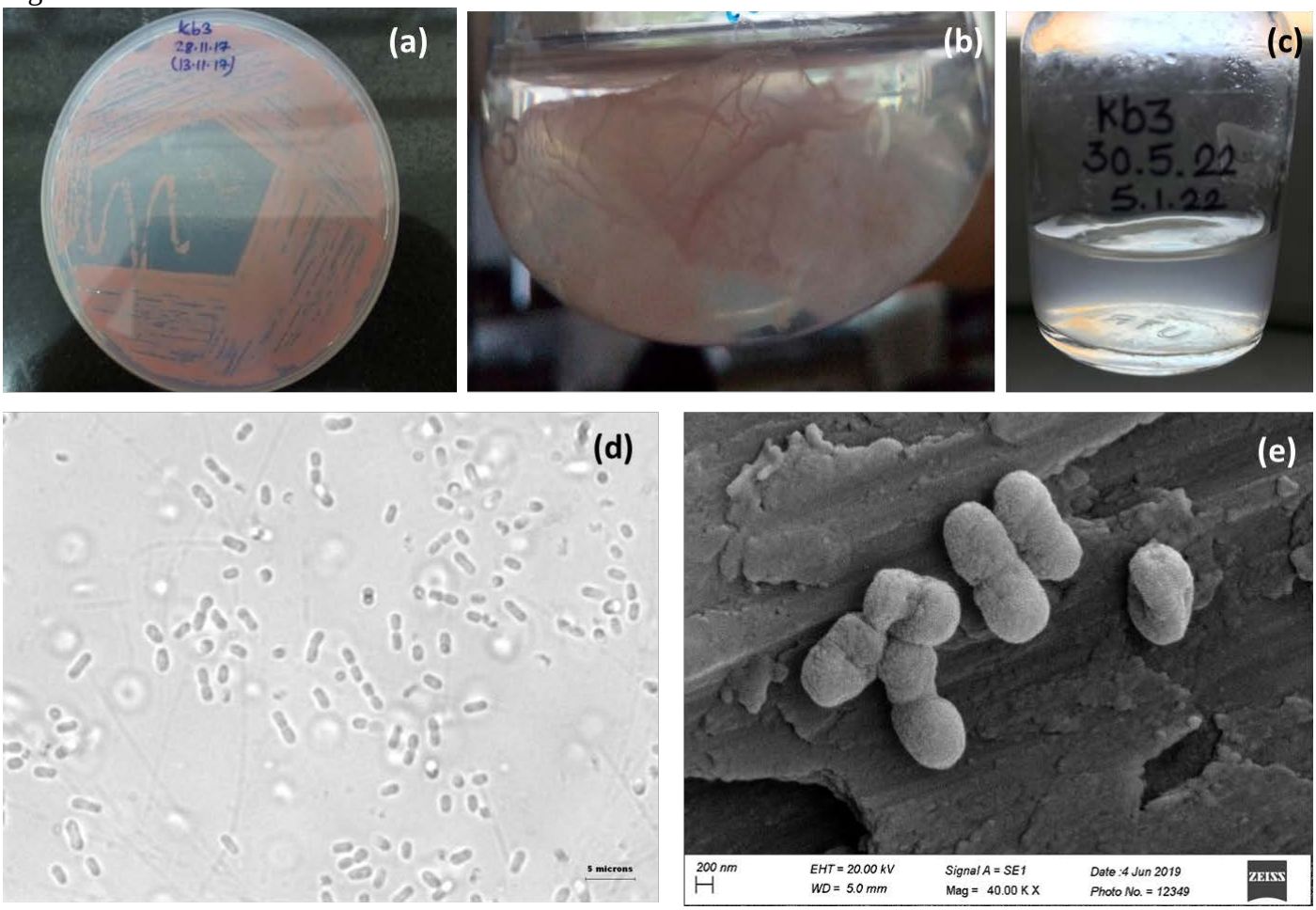
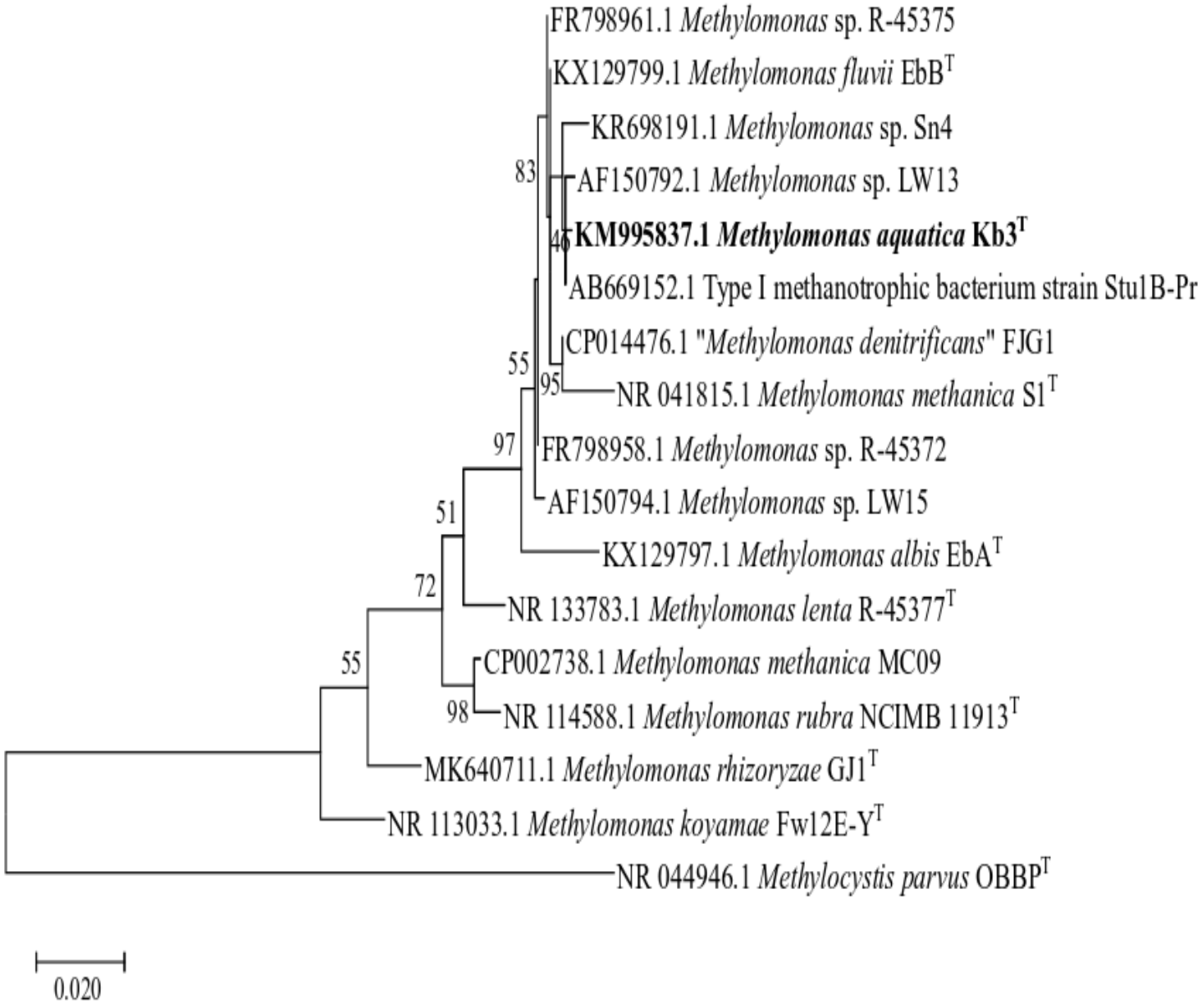


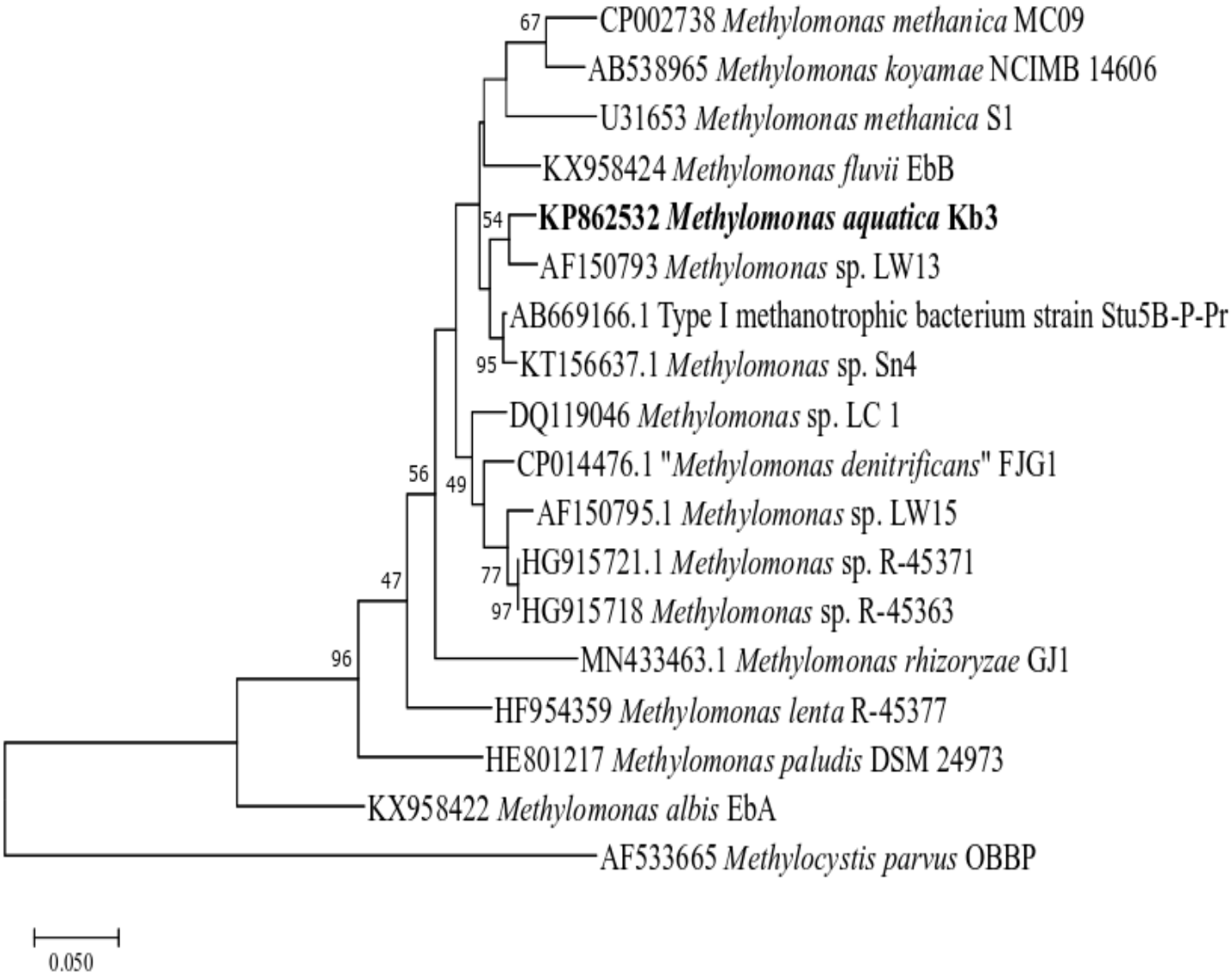
Figure 2

 doi:10.20944/preprints202207.0157.v1



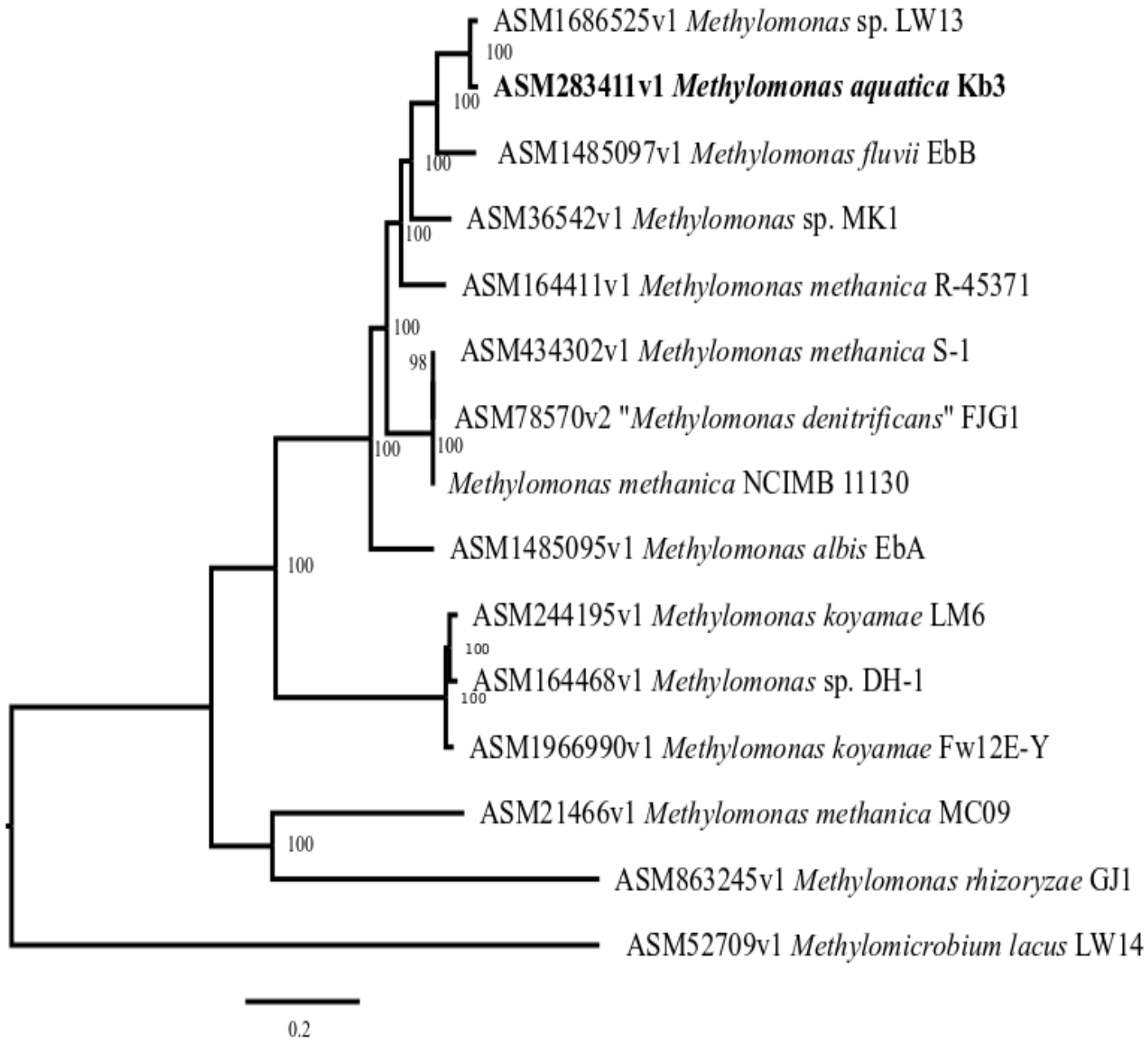
 doi:10.20944/preprints202207.0157.v1

Figure 3



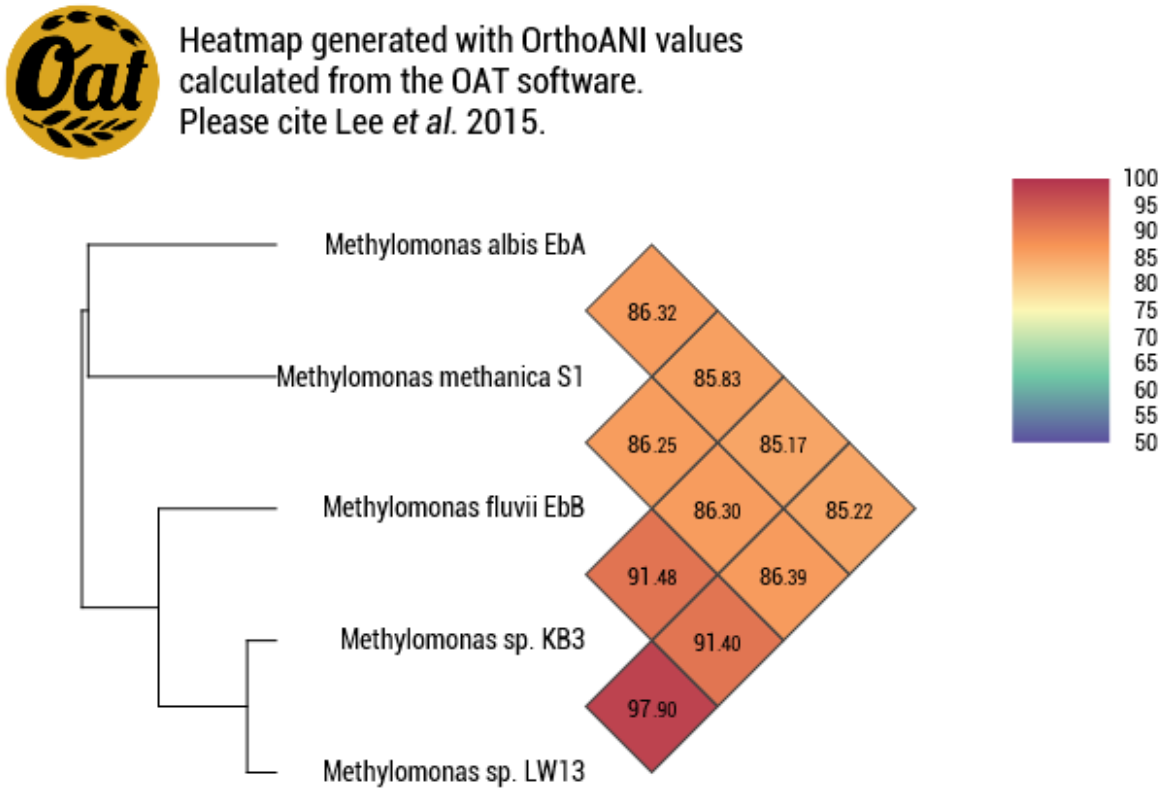
 doi:10.20944/preprints202207.0157.v1

Supplementary Figure 1



 doi:10.20944/preprints202207.0157.v1

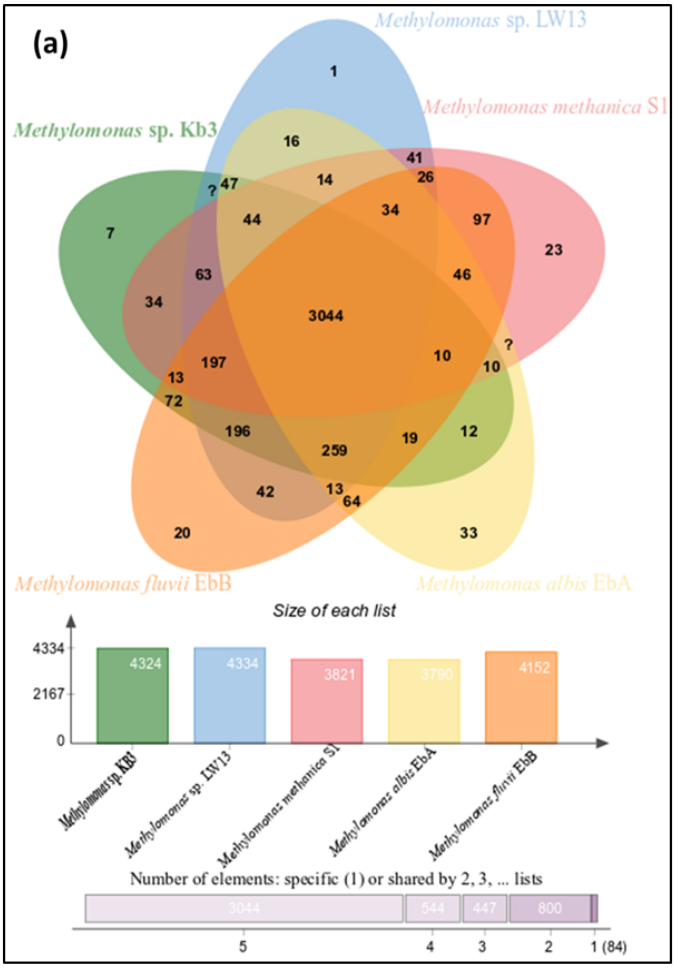
Supplementary Figure 2

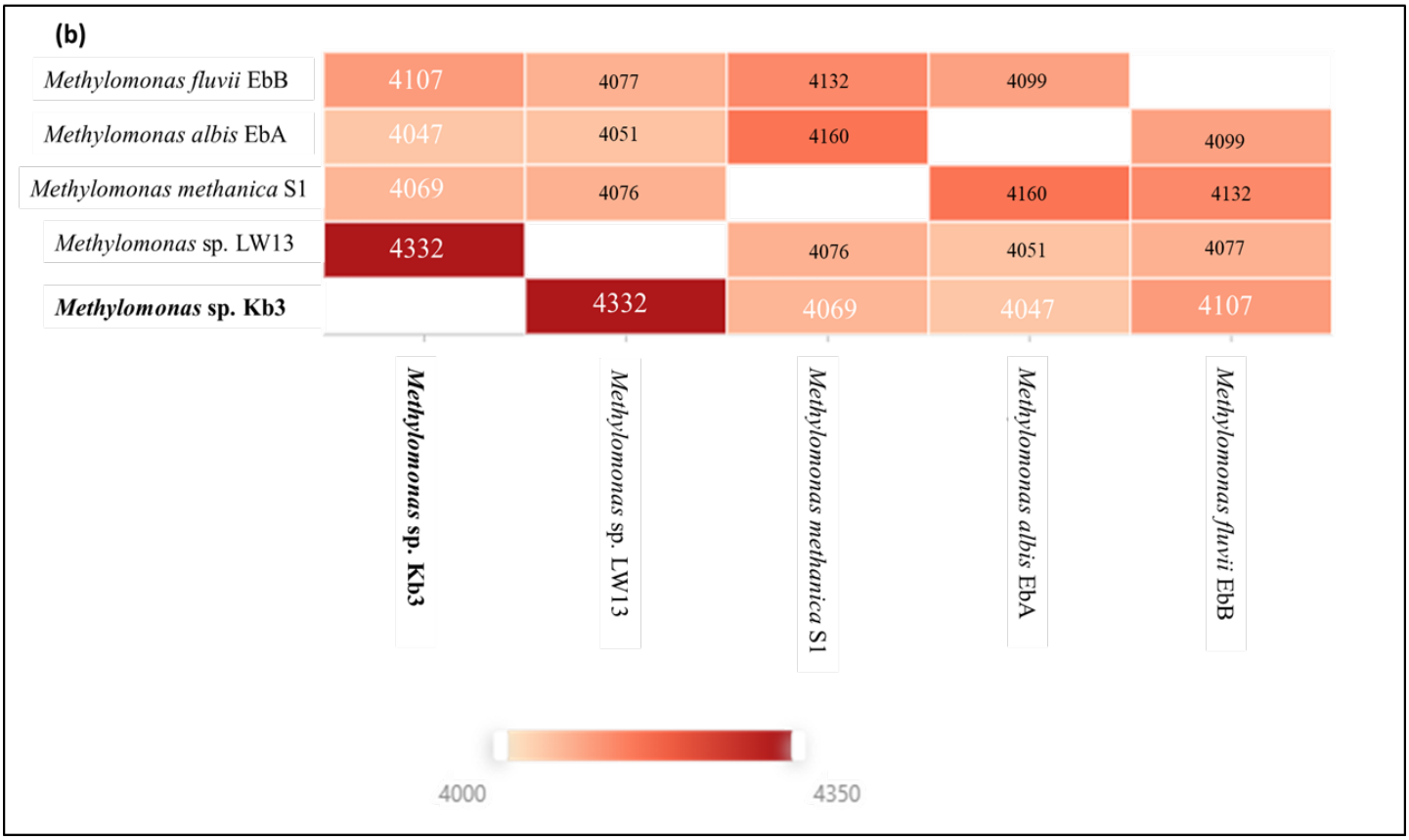


 doi:10.20944/preprints202207.0157.v1

Supplementary Figure 3

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 11 July 2022 doi:10.20944/preprints202207.0157.v1





 doi:10.20944/preprints202207.0157.v1