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Candidatus Nanopusillus

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5. AUTHORS NAMES AND INSTITUTIONS:

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6. ETYMOLOGY:

Na.no.pu.sil'lus. Gr. masc. n. *nânos,* a dwarf; L. masc. adj. *pusillus,* very small; N.L. masc. n. *Nanopusillus,* a very small member of the *Nanoarchaeota*

7. ABSTRACT:

The genus *Candidatus* Nanopusillus is comprised of small coccoid cells (~100-400 nm) that live epibiotically on the surface of archaeal hosts. The first described species, *Candidatus* Nanopusillus acidilobi, is an anaerobic, hyperthermophilic acidophile whose best growth is observed at 82°C, pH 3.6, cultivated from a hot spring in Yellowstone National Park. *Ca.* Nanopusillus acidilobi cells associate with the *Crenarchaeota* host organism *Acidilobus* sp. 7A. Archaeal flagella (archaella) have been predicted from the genome sequence and shown to be expressed in the proteome. A second putative species, *Candidatus* Nanopusillus massiliensis, was recently reported from human dental plaque and associates with the methanogen *Methanobrevibacter oralis*. The genome consists of a single scaffold which is highly fragmented by spans of ambiguous nucleotides, with 16S rRNA gene fragments from *Bacteria*. Both species have small genomes (~0.6 Mbp) encoding few biosynthetic genes and no apparent ATP synthase complex genes, suggesting that the nanoarchaeotes rely on their host for production of major cellular precursors.

Type species: ***Candidatus* Nanopusillus acidilobi** Wurch et al. 2016

8. KEYWORDS:

hot spring, hyperthermophile, acidophile, symbiote, *Nanopusillus*

9. DESCRIPTION:

Ultra-small cocci (~100-400 nm in diameter) cultivated under **anaerobic** conditions. Species within *Candidatus* Nanopusillus live as **obligate epibionts** on the surface of specific archaeal

hosts, and likely **rely on their hosts for production of the major precursors for cellular biosynthesis**. The first described species, *Ca.* Nanopusillus acidilobi, was grown from Cistern Spring, a high-temperature acidic geothermal spring in Yellowstone National Park (YNP). *Ca.* N. acidilobi is **hyperthermophilic** and **acidophilic**, with optimal growth observed at 82°C and pH 3.6. Cells are glycosylated, and **archaeal flagella** (archaella) genes are predicted from the genome and expressed in proteomic data. *Acidilobus* sp. 7A functions as the specific host. A second putative species, *Candidatus* Nanopusillus massiliensis, was recently described from the human oral environment. While the culture is no longer available (Stéphane Alibar 2022, personal communication, 28 November), *Ca.* N. massiliensis cells are **neutrophilic** and **mesophilic**, with best growth observed at 37°C, pH 7, and they grow ectosymbiotically on the surface of *Methanobrevibacter oralis.*

*DNA G + C content (mol %)***:** 24 (genome analysis)

Type species: ***Candidatus*** **Nanopusillus acidilobi** Wurch et al. 2016

Number of *Candidatus* species: 2

Family classification: *Nanobdellaceae*

10. NUMBER OF CANDIDATUS SPECIES:

2

11. FURTHER DESCRIPTIVE INFORMATION:

11.1. CELL MORPHOLOGY AND ULTRASTRUCTURE

Ca. N. acidilobi, the first described species of the genus *Ca.* Nanopusillus, was cultivated from an acidic, high-temperature hot spring in YNP. Cells are coccoid in shape, approximately 100-300 nm in diameter (Table 1), and are glycosylated (Wurch et al., 2016). Although an S-layer protein was predicted from the sequence of the *Ca.* N. acidilobi genome, the protein was not detected in proteomic analysis. However, identification of the S-layer protein via trypsin-based proteomics may be hampered by size and a low quantity of proteolytic sites (Wurch et al., 2016). *Ca*. N. acidilobi cells survive on the surface of their crenarchaeotal host, *Acidilobus* sp. 7A (Figure 1), and electron microscopy has shown distension at the attachment point between host and symbiont (Wurch et al., 2016). This suggests that the YNP *Nanoarchaeota*–host system forms an intimate association, potentially similar to what has been described for the shallow marine lineage *Candidatus* Nanoarchaeum equitans and its host *Ignicoccus hospitalis*, which appear to form a bridge-like structure that connects the two organisms and allows for cytoplasmic contact (Heimerl et al., 2017). A second lineage, *Ca.* N. massiliensis (150-400 nm), was recently reported from the human oral environment and associates with a methanogenic *Euryarchaeota, Methanobrevibacter oralis*.

<Table 1 near here>

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11.2 NUTRITION AND GROWTH CONDITIONS

Optimal growth of *Ca.* N. acidilobi with its host has been observed at 82°C, pH 3.6. The *Ca.* N. acidilobi–*Acidilobus* sp. 7A co-culture can be cultivated anaerobically under a N_2/CO_2 headspace (80:20, v/v) in media containing yeast extract and peptone (Wurch et al., 2016). The newly

proposed species *Ca.* N. massiliensis can be grown with its host in modified SAB media under an H_2/CO_2 headspace (80:20, v/v), with optimal growth observed at 37°C, pH 7 (Hassani et al., 2022).

11.3 GENOME FEATURES

A genome sequence has been determined for *Ca.* N. acidilobi, the first cultivated representative of *Ca.* Nanopusillus. The genome is 605,887 bp in length, with 656 predicted protein-coding sequences and a single 5S, 16S and 23S rRNA gene sequence. Genome size is comparable to other terrestrial *Nanoarchaeota* (Table 1), but somewhat larger than the shallow marine taxon *Ca.* Nanoarchaeum equitans (see gbm01370). Like other nanoarchaeotes, the *Ca.* N. acidilobi genome points to highly reduced biosynthetic potential, with very minimal genes involved in the generation of amino acids, nucleotides, lipids or cofactors (Kato et al., 2022; St. John et al., 2019; Waters et al., 2003; Wurch et al., 2016). In contrast to *Ca.* Nanoarchaeum equitans, but like its relatives from New Zealand and Japan, the *Ca.* N. acidilobi genome encodes a suite of genes involved in gluconeogenesis and glycolysis, including several gluconeogenesis genes highly expressed in the proteome (Wurch et al., 2016). Additionally, the *Ca.* N. acidilobi genome does not contain any detectable *trans*-encoded tRNA genes or ATP synthase genes, which have been identified in *Ca.* Nanoarchaeum equitans (Randau, 2012; Waters et al., 2003), and no CRISPR-Cas cassettes have been identified. Like other described *Nanoarchaeota,* the *Ca.* Nanopusillus genome encodes several split protein-coding genes, which have been linked to genome reduction associated with a symbiotic lifestyle (Kato et al., 2022; St. John et al., 2019; Waters et al., 2003; Wurch et al., 2016).

By contrast, the *Ca.* N. massiliensis genome quality is poor and consists of a single scaffold which is highly fragmented by spans of ambiguous nucleotides that link the 593 individual contigs. Based on genome quality assessment with CheckM (Parks et al., 2015), 64 to 66 of the unique archaeal marker genes used to estimate genome completeness can be identified in the *Ca.* N. massiliensis genome, depending on whether the scaffold or contig version of the genome sequence is analyzed. In contrast, 117 to 119 archaeal marker genes are detectable in the complete genomes of *Ca.* N. acidilobi, *Ca.* Nanoarchaeum equitans and *Nanobdella aerobiophila*. Thus, it is likely that ambiguous bases in the *Ca.* N. massiliensis genome hamper gene identification and severely impair genome quality. Nonetheless, analysis of the *Ca.* N. massiliensis genome suggests the presence of genes associated with glycolysis and gluconeogenesis, a single archaeal flagellum gene and the apparent absence of the ATP synthase complex (Hassani et al., 2022).

11.4. ECOLOGY

The first described *Ca.* Nanopusillus species, *Ca.* N. acidilobi, was cultivated with its host from Cistern Spring, a hot spring in Norris Geyser Basin, YNP (pH 4.5, 82°C). A few years prior, a single-cell nanoarchaeote genome “Nst1” was also co-sorted with its putative *Sulfolobales* host “Acd1” from Obsidian Pool, YNP (pH 5.2-5.5, 82°C) (Podar et al., 2013). Although Nst1 was proposed to represent the unique genus *Candidatus* Nanobsidianus stetteri, reclassification with the Genome Taxonomy DataBase (GTDB) has since placed Nst1 within the *Ca.* Nanopusillus (Parks et al., 2020). Additional single-cell genomics, 16S rRNA gene diversity studies and metagenomic data have

suggested that relatives of Nst1 and *Ca.* Nanopusillus are widely distributed across YNP geothermal springs (Clingenpeel et al., 2013; Jarett et al., 2018; Munson-McGee et al., 2015).

The recent description of a putative novel *Ca.* Nanopusillus from human dental plaque (Hassani et al., 2022) raises questions regarding the distribution of the genus. To our knowledge, *Ca.*N. massiliensis represents the first description of a mesophilic, *Euryarchaeota*-dependent, human-associated nanoarchaeote to date. However, the 16S rRNA gene associated with *Ca.* N. massiliensis genome (GenBank/EMBL/DDBJ accession NZ_OV100765.1; locus tag LUA84_RS03675) uses ambiguous bases to link five small contigs, several of which show high similarity to bacterial sequences, suggesting that the 16S rRNA gene sequence is chimeric. Also, given the lack of evidence of *Ca.* Nanopusillus in human microbiome datasets, additional investigation will be crucial to determining the validity of this new species and how it contributes to our understanding of the distribution and range of host associations found in *Ca.* Nanopusillus.

12. ENRICHMENTS AND ISOLATION PROCEDURES

Enrichment cultures of *Ca.* N. acidilobi and its host *Acidilobus* sp. 7A can be grown in the following medium, containing (per liter of deionized water): NH_4Cl, 0.33 g; KH_2PO_4, 0.33 g; $MgSO_4$ x $7H_2O$, 0.33 g; $CaCl_2$, 0.33 g; KCl, 0.33 g; SL-10 trace metals, 1 ml; Wolfe's vitamin solution, 5 ml of 1000X solution; yeast extract, 0.3 g; and peptone, 0.5 g. The medium is filter sterilized, and anaerobic conditions are achieved by three 20-minute rounds of degassing with N_2/CO_2 (80:20, v/v). The final medium is then reduced overnight at 80°C using 100 µM cysteine. Cultivation can be performed at 82°C, pH 3.6. Dilution-to-extinction and optical tweezer selection are effective for isolation of *Ca.* N. acidilobi–*Acidilobus* sp. 7A co-cultures.

Cultivation of the *Ca.* N. massiliensis–*M. oralis* co-culture was done using SAB media prepared under an H_2/CO_2 headspace (80/20, v/v), followed by growth at 37°C, pH 7 in an SAB medium supplemented with D-fructose (0.1 g), vitamins, fatty acids (valeric, isovaleric, 2-methylbutyric and isobutyric acids) and 5% 0.22 µm-filtered bovine rumen.

13. MAINTENANCE PROCEDURES

For long-term storage, liquid co-cultures of *Ca.* N. acidilobi and *Acidilobus* sp. 7A can be frozen at -80°C with the addition of 10% dimethylsulfoxide.

14. DIFFERENTIATION OF THE GENUS *CA*. NANOPUSILLUS FROM OTHER GENERA

Features differentiating *Ca.* Nanopusillus from *Candidatus* Nanoclepta (see gbm02046), *Ca*. Nanoarchaeum (see gbm01370) and *Nanobdella* (Kato et al., 2022) are listed in Table 1. 16S rRNA gene sequence divergence and whole genome sequence identity also distinguish *Ca.* Nanopusillus from related lineages in the *Nanoarchaeota.*

15. TAXONOMIC COMMENTS

Based on phylogenetic reconstruction of 16S rRNA genes (Figure 2), *Ca.* N. acidilobi forms a distinct clade with clone and single-cell genome sequences from YNP (YLNA023, OP-9, SCGC AB-777_F03, Nst1). The YNP-specific branch forms part of a larger clade comprised of sequences from other terrestrial hot springs, including locations in Japan (*Nanobdella aerobiophila*), Kamchatka, Russia (CU-1), China (A2, A39) and New Zealand (*Ca.* Nanoclepta minutus), which

branches out from marine hydrothermal vent-associated lineages (*Ca*. Nanoarchaeum equitans, MC-1). Due to the high proportion of ambiguous bases (~17%) and similarity to bacterial 16S rRNA gene sequences, the *Ca.* N. massiliensis 16S rRNA gene sequence is not included in the phylogenetic tree. A concatenated protein tree generated with the GTDB Toolkit (Chaumeil et al., 2020) based on 53 archaeal marker genes also shows a similar overall topology compared to the 16S rRNA gene tree (Figure 3), with the exception that the deep-sea hydrothermal vent associated lineage MC-1 clusters with a terrestrial hot spring sequence from Nevada, USA (SpSt-4), and *Ca.* N. massiliensis forms a small clade with *Ca.* N. acidilobi.

Currently, the genus *Ca.* Nanopusillus is classified by GTDB in phylum *Nanoarchaeota,* class *Nanoarchaeia,* order *Nanoarchaeales* and family *Nanopusillaceae* (Parks et al., 2020). However, a novel classification system was recently proposed and validly published for the entire *Nanoarchaeota* phylum, which places *Ca.* Nanopusillus within the class *Nanobdellia,* order *Nanobdellales* and family *Nanobdellaceae* (Kato et al., 2022).

<Figure 2 near here>

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16. LIST OF SPECIES OF THE GENUS

1. *Candidatus* Nanopusillus acidilobi Wurch et al. 2016.

a.ci.di.lo'bi. N.L. gen. masc. n. *acidilobi,* of acidolobus, growth dependent on the archaeal genus *Acidilobus*

Distinguishing features are shown in Table 1 and in the genus description. This taxon and its host were co-cultivated from Cistern Spring, YNP, USA (44.723°N, 110.704°W).

DNA G + C content (mol %): 24 (genome analysis)

Type strain: N7A

GenBank/EMBL/DDBJ accession (genome): CP010514.1

2. *Candidatus* Nanopusillus massiliensis Hassani et al. 2022.

mas.si.li.en'sis. L. fem. adj. *massiliensis,* referring to Massilia, the past Roman name of Marseille, France where this nano-organism has been discovered

Distinguishing features are listed in the genus description and Table 1. This nanoarchaeote and its host were co-cultivated from a human dental plaque.

DNA G + C content (mol %; reported in Hassani et al., 2022): 23.6 (genome analysis)

DNA G + C content (mol %; re-calculated with BBmap (https://sourceforge.net/projects/bbmap/): 24.0 (genome analysis)

Type strain: Marseille-Q6268

GenBank/EMBL/DDBJ accession (genome, contigs): CAKLBW000000000.1

GenBank/EMBL/DDBJ accession (genome, scaffold): OV100765.1

RELATED ARTICLES

gbm01370

gbm02046

fbm00399

obm00129

cbm00090

pbm00038

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20. TABLES AND FIGURES

Table 1. Major characteristics differentiating members of the *Ca.* Nanopusillus from *Ca.* Nanoclepta minutus, *Nanobdella aerobiophila* and *Ca.* Nanoarchaeum equitans. Data from Genbank/EMBL/DDBJ records (*Ca.* Nanopusillus); Wurch et al., 2016 (*Ca.* Nanopusillus acidilobi); Hassani et al., 2022 (*Ca.* Nanopusillus massiliensis); St. John et al., 2019 (*Ca*. Nanoclepta minutus); Kato et al., 2022 (*Nanobdella aerobiophila*); Huber et al., 2002; Jahn et al., 2008; Randau, 2012; Randau et al., 2005; Waters et al., 2003 (*Ca.* Nanoarchaeum equitans).

Characteristic	***Ca.* Nanopusillus acidilobi**	***Ca.* Nanopusillus massiliensis**	***Ca.* Nanoclepta minutus**	***Nanobdella aerobiophila***	***Ca.* Nanoarchaeum equitans**
Isolation location	YNP	Human mouth	Tikitere, NZ	Oku-shiobara, Tochigi, Japan	Kolbeinsey Ridge
Optimal temperature (°C)	82	37	80–85	65–70	85–90
Cultivation pH	3.6	7.0	6.0	2.5	5.5
Relationship to oxygen	Anaerobe	Anaerobe	Anaerobe	Aerobe	Anaerobe
Cell size, nm	100-300	150-400	~200	200-500	~400
Genome size (bp)	605,887	607,503[b]	575,637	668,961	490,885
G + C content (mol%)	24	23.6[c]	32.2	24.9	31.6

Partial ATP synthase complex genes	-	-	-	-	+
CRISPR-Cas cassette	-	n.d.	+	+	+
Trans-spliced tRNA genes	-	n.d.	-	-	+
Host	*Acidilobus* sp. 7A	*Methanobrevibacter oralis*	*Zestosphaera tikiterensis*	*Metallosphaera sedula*	*Ignicoccus hospitalis*

[a]n.d., no data

[b]Based on Genbank/ GenBank/EMBL/DDBJ accession OV100765.1

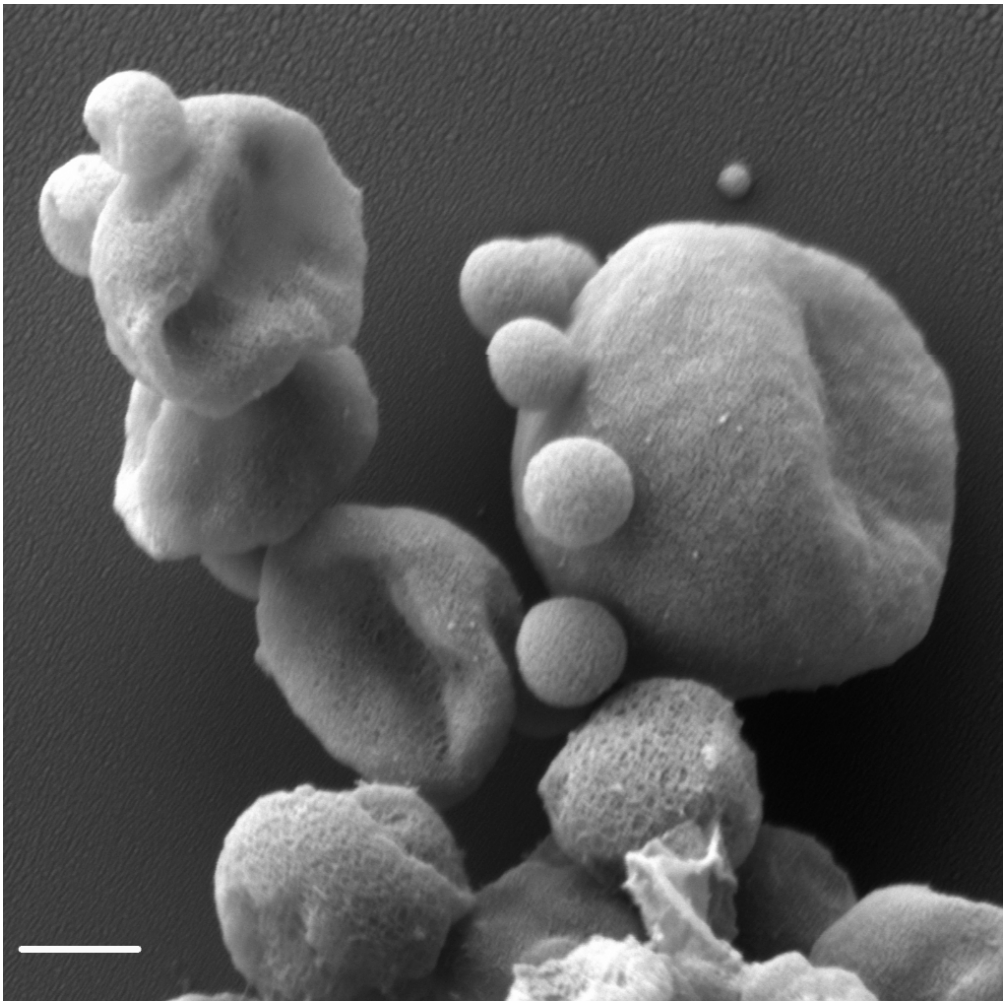
[c]Reported in Hassani et al., 2022

21. FIGURE CAPTIONS

Figure 1. Scanning electron micrograph of *Ca.* Nanopusillus acidilobi N7A cells attached to the surface of *Acidilobus* sp. 7A cells. Scale bar, 300 nm.

Figure 2. Phylogenetic reconstruction of 16S rRNA genes, showing the position of *Ca.* Nanopusillus acidilobi in relation to closely related genera *Ca*. Nanoarchaeum*, Nanobdella* and *Ca.* Nanoclepta, with additional clone, single-cell and metagenome derived sequences. The outgroup (not shown) consists of archaeon GW2011_AR15 (CP010425.1), archaeon GW2011_AR20 (CP010426.1), *Candidatus* Tiddalikarchaeum anstoanum LFW-252_1 (CABMEV000000000.1) and *Candidatus* Parvarchaeum acidiphilum ARMAN-4 (ADCE00000000.1). 80-100% bootstrap support based on 1000 rapid bootstraps is indicated with filled circles. Scale bar, 0.1 substitutions per nucleotide.

Figure 3. Unrooted concatenated protein phylogenetic tree based on 53 marker genes, showing the relative position of *Ca.* Nanopusillus compared to closely related taxa. The tree was constructed using the default GTDB Toolkit release 207_v2 database for archaeal tree building, with the addition of *Ca.* Nanopusillus massiliensis, *Nanobdella aerobiophila* and MC-1. Taxa assigned to *Nanobdellales* (GTDB order *Nanoarchaeales*) are shown. Filled circles indicate 80-100% local support values calculated with the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999). Scale bar, 0.1 substitutions per amino acid.



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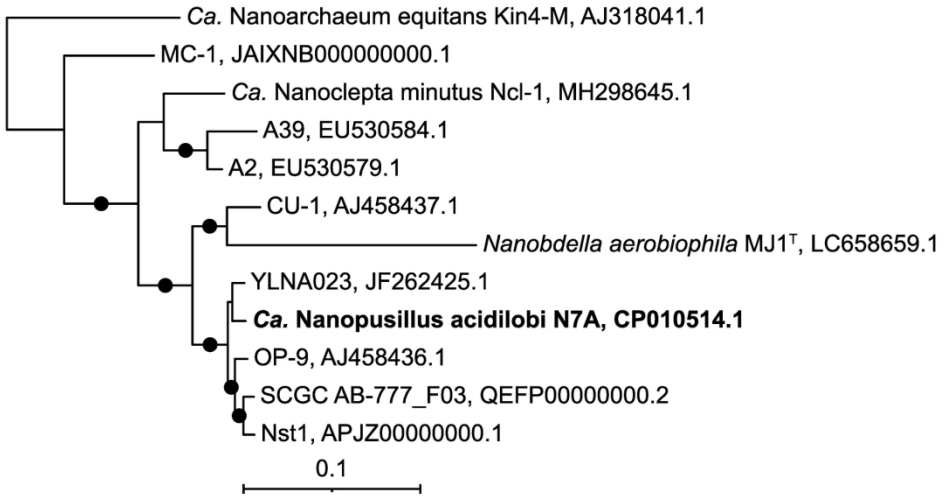
86x85mm (300 x 300 DPI)



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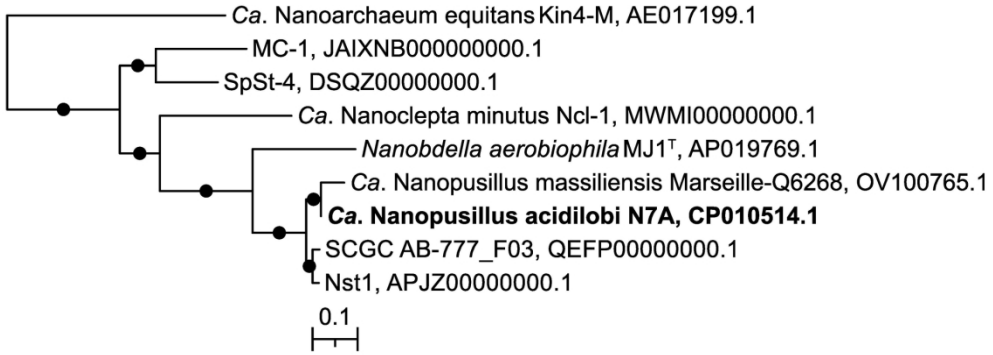

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