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2 ***Thiomicrohabdus immobilis* sp. nov., a mesophilic sulfur-**  
3 **oxidizing bacterium isolated from sediment of a brackish lake**  
4 **in northern Japan**

5

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19

20

21 **Abstract**

22           A novel sulfur-oxidizing bacterium, strain Am19<sup>T</sup>, was isolated from sediment  
23 of a brackish lake. Strain Am19<sup>T</sup> grew chemolithoautotrophically on inorganic sulfur  
24 compounds, and heterotrophic growth was not observed. Cells were rod-shaped with  
25 length of 1.1–3.0 μm and diameter of 0.5–0.8 μm. Growth was observed at 5–37°C with  
26 an optimum growth temperature of 30°C. The pH range for growth was 5.6–8.5 with an  
27 optimum pH of 6.6–7.0. Major fatty acids were summed feature 3 (C<sub>16:1ω7c</sub> and/or  
28 C<sub>16:1ω6c</sub>), summed feature 8 (C<sub>18:1ω7c</sub> and/or C<sub>18:1ω6c</sub>) and C<sub>16:0</sub>. The sole  
29 respiratory quinone was ubiquinone-8. The complete genome of strain Am19<sup>T</sup> is  
30 composed of a circular chromosome with length of 2.5 Mbp and G + C content of 42.7  
31 mol%. Phylogenetic analysis based on genomic data indicated that strain Am19<sup>T</sup>  
32 belongs to the genus *Thiomicrohabdus* but is distinct from any existing species.  
33 Analysis of the 16S rRNA gene supported creation of a new species to accommodate  
34 strain Am19<sup>T</sup>. On the basis of genomic and phenotypic characteristics, strain Am19<sup>T</sup> (=   
35 NBRC 114602<sup>T</sup> = BCRC 81336<sup>T</sup>) is proposed as the type strain of a new species, with  
36 name of *Thiomicrohabdus immobilis* sp. nov.

37

## 38 **Introduction**

39 The genus *Thiomicrohabdus* was established in 2017, within the family  
40 *Piscirickettsiaceae* (Boden et al., 2017a). According to the List of Prokaryotic Names  
41 with Standing in Nomenclature (LPSN) (Parte et al., 2020), there are 11 species with  
42 validly published names in this genus, at the time of writing (15 August 2022). They are  
43 all obligate chemolithoautotrophs which oxidize sulfur compounds. They require oxygen  
44 as electron acceptor for growth, and rely on sulfur compounds as electron donor. As  
45 exceptional cases, anaerobic growth with nitrite was reported in *T. sediminis* (Liu et al.,  
46 2021), and hydrogen-dependent growth was observed in *T. hydrogeniphila* and *T.*  
47 *heinhorstiae* (Watsuji et al., 2016; Boden et al. 2017b; Updegraff et al. 2022). As common  
48 chemotaxonomic feature, all species examined share three major fatty acids, C<sub>16:1</sub>, C<sub>18:</sub>  
49 <sub>1</sub> and C<sub>16:0</sub>. It was suggested that fourth-dominant fatty acid may work as diagnostic  
50 character among species in this genus (Boden et al. 2017b).

51 In this study, a novel aerobic and mesophilic autotroph was isolated and characterized,  
52 as a representative of a new species in the genus *Thiomicrohabdus*.

53

54

## 55 **Materials and methods**

56

57 Enrichment and isolation

58 Strains Am19<sup>T</sup> was isolated from a sediment collected at a site (43.05N, 144.89E), in  
59 Lake Akkeshi, a brackish lake in Japan. The sediment was inoculated into a bicarbonate-  
60 buffered enrichment medium. To prepare the medium, the following constituents (g l<sup>-1</sup>)  
61 were dissolved in distilled water and sterilized by autoclaving: Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> · 5H<sub>2</sub>O (2.5),  
62 NaCl (20), MgCl<sub>2</sub> · 6H<sub>2</sub>O (0.2), CaCl<sub>2</sub> · 2H<sub>2</sub>O (0.1), NH<sub>4</sub>Cl (0.1), KH<sub>2</sub>PO<sub>4</sub> (0.1), KCl  
63 (0.1). After cooling down to room temperature, the following solutions were aseptically  
64 added to the main body of medium (ml/l): trace element solution (1), selenite-tungstate  
65 solution (1), vitamin mixture solution (1) and 1M NaHCO<sub>3</sub> solution (30). The trace  
66 element solution contained 0.3% HCl and following constituents (mg l<sup>-1</sup>): FeSO<sub>4</sub> · 7H<sub>2</sub>O  
67 (2100), H<sub>3</sub>BO<sub>3</sub> (30), MnCl<sub>2</sub> · 4H<sub>2</sub>O (100), CoCl<sub>2</sub> · 6H<sub>2</sub>O (190), NiCl<sub>2</sub> · 6H<sub>2</sub>O (24), CuCl<sub>2</sub> ·  
68 2H<sub>2</sub>O (2), ZnSO<sub>4</sub> · 7H<sub>2</sub>O (144) and Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O (36). The selenite-tungstate solution  
69 consists of the followings (mg l<sup>-1</sup>): NaOH (400), Na<sub>2</sub>SeO<sub>3</sub> · 5H<sub>2</sub>O (6) and Na<sub>2</sub>WO<sub>4</sub> · 2H<sub>2</sub>O  
70 (8). The vitamin mixture solution contained the followings (mg l<sup>-1</sup>): biotin (20), folic acid  
71 (20), pyridoxine hydrochloride (100), thiamine hydrochloride (50), riboflavin (50), niacin  
72 (50), hemicalcium D-(+)-pantothenate (50), *p*-aminobenzoic acid (50),  $\alpha$ -lipoic acid (50)  
73 and cyanocobalamin (1). The complete medium was adjusted to pH 7.0-7.2 using  
74 hydrochloric acid. After some serial subcultures into the same enrichment medium,

75 cultures were switched to a medium hereafter termed 'basal medium', which was prepared  
76 in the same manner but with the following modifications. It contained  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.3  
77  $\text{g l}^{-1}$ ), and concentrations of  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  were increased to 5 and 3  
78  $\text{g l}^{-1}$ , respectively. From the enrichment culture grown on the basal medium, strain Am19<sup>T</sup>  
79 was isolated with the dilution-to-extinction approach. A grown culture was subjected to  
80 serial 10-fold dilutions in the basal medium. The resulting positive culture of the highest  
81 dilution was subjected to the same procedure again, to obtain pure culture of strain Am19<sup>T</sup>.  
82 The enrichment and isolation were performed at 22°C. Purity of culture was routinely  
83 checked by microscopy and sequencing of the 16S rRNA gene fragments.

84

#### 85 Phenotypic characterization

86 In experiments for phenotypic characterizations, strain Am19<sup>T</sup> was cultured at 30°C  
87 in the basal medium unless otherwise specified. Cell morphology was observed with  
88 phase-contrast light microscopy. Gram staining was performed using the Gram-Color kit  
89 (Merck). Activities of catalase and oxidase were assessed as described previously  
90 (Kojima & Fukui 2016).

91 Effect of temperature on growth was examined by attempting cultivation at 0, 5, 8, 10,  
92 13, 15, 18, 22, 25, 28, 30, 32, 37 and 45°C in the basal medium. Effect of salt

93 concentration on growth was examined with modified versions of the basal medium,  
94 which contained no MgCl<sub>2</sub> and varying concentrations of NaCl, ranging from 0 to 1,400  
95 mM at 100 mM intervals.

96 For test of pH effect on growth, media of various pH prepared as below. From the basal  
97 medium, MgCl<sub>2</sub> was excluded and bicarbonate concentration was reduced to 1 g l<sup>-1</sup>.  
98 Depending on the pH to be tested, the modified media were buffered with 20 mM of MES,  
99 MOPS or TAPS. All ingredients were mixed and then sterilized by filtration after pH  
100 adjustment with NaOH or HCl. The pH tested and buffering reagents were as follows;  
101 pH 3.3, 3.7, 4.2, 5.0, 5.1, 5.6, 6.1, 6.4, 6.6, 6.8, 7.0 and 7.2 with MES; pH 6.4, 7.0, 7.2,  
102 7.4, 7.6, 7.8, 7.9, 8.5 and 8.8 with MOPS; pH 8.1, 8.2, 8.5, 8.7 and 9.1 with TAPS.

103 Utilization of growth substrates was tested with the basal medium, by replacing  
104 thiosulfate with one of the followings (mM); tetrathionate (20), sulfite (5), sulfide (2),  
105 acetate (5), formate (5), succinate (2.5), fumarate (5), *iso*-butyrate (2.5), methanol (5),  
106 ethanol (2.5), D-(+)-glucose (2.5), D-(-)-fructose (5), D-(+)-xylose (2.5), D-(+)-maltose  
107 (5), L-(+)-arabinose (5), D-(+)-cellobiose (1). Utilization of elemental sulfur was tested  
108 by adding flowers of sulfur (approximately 0.5 g l<sup>-1</sup>, which is equivalent to 1.9 mmol  
109 *cyclo*-octasulfur, the major component of flowers-of-sulfur) to the thiosulfate-free basal  
110 medium. The sulfur was moistened and sterilized as described previously (Widdel &

111 Pfennig, 1992). Chemolithoautotrophic growth on hydrogen was tested by filling the  
112 headspace of the culturing containers with 1:1 mixture of air and H<sub>2</sub> gas, at a total pressure  
113 of 2 atm. Heterotrophic growth was also tested in complex liquid media listed below;  
114 Reasoner's 2A broth (R2A) broth neat and at 1:10 dilution; nutrient broth; lysogeny broth  
115 and tryptic soy broth. Utilization of electron acceptor was tested under anoxia, with the  
116 basal medium supplemented with nitrate (20 mM) or nitrite (5 mM).

117

#### 118 Chemotaxonomic characterization

119 For analyses of cellular fatty acids and respiratory quinone, the basal medium  
120 was simplified by omitting MgCl<sub>2</sub>. Strain Am19<sup>T</sup> was grown in the simplified basal  
121 medium at 30°C. The fatty acid profile was obtained with the Sherlock Microbial  
122 Identification System (MIDI) version 6.0 (database; TSBA6). Respiratory quinones were  
123 extracted as described previously (Bligh & Dyer, 1959), and then analyzed with HPLC.

124

#### 125 Genomic characterization

126 Whole genome sequencing was performed using the platforms of Illumina NextSeq  
127 and Nanopore GridION, to obtain short and long reads respectively. The resulting reads  
128 from the platforms were assembled by using Unicycler (Ver 0.4.7). The reconstructed  
129 genome sequence was annotated with DFAST (Tanizawa et al., 2018).

130 A genome-based taxonomic classification was made with reference to the  
131 Genome Taxonomy Database (GTDB) (Parks et al., 2018). Taxonomic position in GTDB  
132 release 95 was determined by analyzing the genome of strain Am19<sup>T</sup> with GTDB-Tk  
133 (Chaumeil et al., 2020).

134 To estimate genome relatedness between Am19<sup>T</sup> and its close relatives, values of  
135 average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) were  
136 calculated, by using tools provided by Kostas lab (<http://enve-omics.ce.gatech.edu/>) and  
137 DSMZ, respectively. In the calculation of dDDH with Genome-to-Genome Distance  
138 Calculator (GGDC), the formula 2 was applied (Meier-Kolthoff et al., 2013).

139

140 Phylogenetic analysis based on the 16S rRNA gene

141 Phylogenetic analysis of the 16S rRNA gene was conducted using MEGA version 11  
142 (Tamura et al., 2021). The gene sequence of strain Am19<sup>T</sup> was aligned with reference  
143 sequences, using the MUSCLE algorithm. The reference sequences were obtained from  
144 LPSN (accessed on 15 August 2022), as those of type strains representing species with  
145 validly published names. They include all species in the genera *Thiomicrohabdus*,  
146 *Thiomicrospira*, *Hydrogenovibrio*, *Thiosulfativibrio*, *Thiosulfatimonas*, *Galenea* and  
147 *Sulfurivirga* (as an outgroup). The sequences collected from LPSN were checked for

148 quality, and those including ambiguous bases were replaced with the 16S rRNA gene  
149 sequences extracted from genomes, when they were publicly available. With the resulting  
150 alignment, the best substitution model was selected by the model selection tool in MEGA.  
151 The selection was conducted with default settings, to identify the best model with the  
152 lowest Bayesian Information Criterion (BIC) score. Phylogenetic tree was constructed with  
153 the selected model by excluding positions with gaps. An alternative phylogenetic tree was  
154 constructed with a model with the lowest corrected Akaike Information Criterion (AIC<sub>C</sub>)  
155 value, to confirm that resulting tree topology is identical to that of the model selected by  
156 BIC.

157

## 158 **Results**

159

### 160 **Physiological and chemotaxonomic characteristics**

161       Cells of strain Am19<sup>T</sup> were Gram-stain-negative, rod-shaped, non-motile, 0.5–  
162 0.8 µm in diameter and 1.1–3.0 µm in length. They were catalase-negative and oxidase-  
163 positive. Strain Am19<sup>T</sup> grew at 5–37°C with optimum growth at 30°C. Its growth was  
164 observed at pH range of 5.6–8.5, with optimum growth at pH 6.6–7.0 (Table 1). In the  
165 test of salt concentration, growth was observed in the presence of 1,300 mM or lower  
166 concentrations of NaCl. Strain Am19<sup>T</sup> did not require NaCl for growth.

167 Strain Am19<sup>T</sup> grew chemolithotrophically on thiosulfate, tetrathionate, sulfide  
168 and elemental sulfur. Hydrogen gas did not serve as electron donor for  
169 chemolithotrophic growth. None of the tested organic substrate supported growth of the  
170 strain. No heterotrophic growth was observed in the complex media tested. In the  
171 presence of thiosulfate, anaerobic growth was not observed in the medium containing  
172 nitrate or nitrate as sole electron acceptor.

173 The full fatty acid profile of strain Am19<sup>T</sup> is shown in Table S1. Major  
174 components in the profile were summed feature 3 (C<sub>16:1</sub>ω7c and/or C<sub>16:1</sub>ω6c; 46%),  
175 summed feature 8 (C<sub>18:1</sub>ω7c and/or C<sub>18:1</sub>ω6c; 33%) and C<sub>16:0</sub> (14%). The fourth-  
176 dominant fatty acid, next to the major fatty acids mentioned above, was previously  
177 suggested to be a diagnostic character among *Thiomicrothabodus* species (Boden et al.,  
178 2017b). In strain Am19<sup>T</sup>, it was C<sub>17:1</sub> (C<sub>17:1</sub>ω6c; 1.3% and C<sub>17:1</sub>ω8c; 0.7%), unlike  
179 known *Thiomicrothabodus* species (Table 1).

180 In the analysis of respiratory quinones, only ubiquinone-8 was detected.

181

182 Genomic characteristics and phylogenetic position

183 The complete genome of strain Am19<sup>T</sup> was reconstructed from reads of

184 DNBSEQ and GridION. The sequencing coverage was 825.8-fold. The genome consists  
185 of a single circular chromosome with size of 2,539,444 bp and G+C content of 42.7  
186 mol%. In the genome, 2251 protein-coding sequences, 9 rRNA genes and 46 tRNA  
187 genes were predicted.

188           The reconstructed genome was analyzed with GTDB-tk, for phylogenetic  
189 analysis based on 120 conserved proteins. As a result, strain Am19<sup>T</sup> was classified into  
190 the genus *Thiomicrohabdus*. On the other hand, the analysis also indicated that strain  
191 Am19<sup>T</sup> does not belong to any existing species with validly published name. The  
192 genome-based phylogenetic tree obtained with GTDB-tk is shown in Fig S1.

193           Three copies of the 16S rRNA gene were identified in the genome, and they  
194 have an identical sequence. The sequence indicated high identities to the 16S rRNA  
195 gene sequences of *Thiomicrohabdus* species. Among species with validly published  
196 names, *T. frisia* indicated the highest identity of 97.7% (Table 1). Phylogenetic tree  
197 based on the 16S rRNA gene sequences is shown in Fig. 1. In the tree, monophyly of  
198 the genus *Thiomicrohabdus* was not supported. However, strain Am19<sup>T</sup> belonged to a  
199 clade exclusively consisting of *Thiomicrohabdus* species. This clade also included *T.*  
200 *frisia*, the type species of the genus. These results indicated that strain Am19<sup>T</sup> should be  
201 classified in the genus *Thiomicrohabdus*.

202           The values of dDDH and ANI between strain Am19<sup>T</sup> and genome-sequenced  
203 strains in the genus *Thiomicroorhabdus* are shown in Table 2. The values are lower than  
204 thresholds for species delineation, 70% and 95–96% for dDDH and ANI, respectively  
205 (Richter & Rosselló-Móra, 2009; Meier-Kolthoff et al., 2013).

206

## 207 **Conclusions**

208           The genomic characteristics of strain Am19<sup>T</sup>, including the 16S rRNA gene  
209 sequence, indicated that this novel isolate is close relative of the *Thiomicroorhabdus*  
210 species but should not be classified into any existing species. Between strain Am19<sup>T</sup> and  
211 type strains of existing species, dDDH and ANI values were lower than species  
212 delineation thresholds (Table 2). Within the genus, strain Am19<sup>T</sup> and existing species are  
213 differentiated by combinations of phenotypic characteristics (Table 1). On the basis of  
214 these genomic and phenotypic characteristics, strain Am19<sup>T</sup> is proposed to be assigned to  
215 a novel species of the genus *Thiomicroorhabdus*, with the name *Thiomicroorhabdus*  
216 *immobilis* sp. nov.

217

## 218 **Description of *Thiomicroorhabdus immobilis* sp. nov.**

219 *Thiomicroorhabdus immobilis* (im.mo'bi.lis. L. fem. adj. *immobilis*, immobile, immovable,

220 pertaining to a feature of the type strain).

221 Cells are rod-shaped, 1.1–3.0  $\mu\text{m}$  in length and 0.5–0.8  $\mu\text{m}$  in diameter. Strictly  
222 aerobic. Gram-stain-negative. Catalase-negative and oxidase-positive.  
223 Chemolithoautotrophic growth occurs with thiosulfate, tetrathionate, elemental sulfur and  
224 sulfide. Acetate, formate, succinate, fumarate, *iso*-butyrate, methanol, ethanol, D-(+)-  
225 glucose, D-(-)-fructose, D-(+)-xylose, D-(+)-maltose, L-(+)-arabinose and D-(+)-  
226 cellobiose are not utilized as growth substrates. Growth is observed at temperature range  
227 from 5°C to 37°C, with an optimum growth at 30°C. The pH range for growth is 5.6–8.5,  
228 and the optimum pH was 6.6–7.0. The G+C content of genomic DNA of type strain is  
229 42.7 mol% (genome).

230 The type strain Am19<sup>T</sup> (= NBRC 114602<sup>T</sup> = BCRC 81336<sup>T</sup>) was isolated from  
231 sediment of a brackish lake in Japan (Lake Akkeshi).

232 The GenBank/EMBL/DDBJ accession numbers for the complete of strain  
233 Am19<sup>T</sup> is AP024202.

234

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### 309 **Statements and Declarations**

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313

314 **Figure legend**

315 **Fig. 1** Phylogenetic tree of strain Am19<sup>T</sup> and related organisms, based on the 16S rRNA  
316 gene sequences. This tree was constructed with the maximum likelihood method based  
317 on Kimura 2-parameter model, and the tree displayed has the lowest log-likelihood (-  
318 5947.74). A discrete gamma distribution was used to model differences in evolutionary  
319 rates among sites (5 categories, parameter = 0.2510). The rate variation model allowed  
320 for some sites to be invariable (49.03% sites). All positions containing gaps and missing  
321 data were eliminated, leaving a total of 1,334 positions in the final dataset. Numbers on  
322 nodes represent percentage values of 1,000 bootstrap resampling (values lower than 70  
323 are not shown). Names of all 27 strains included in the analysis are shown in full version  
324 of the tree provided as Figure S2.

325

Table 1. Differential properties of strain Am19<sup>T</sup> and type strains of *Thiomicrothabodus* species. Strains: 1, Am19<sup>T</sup>; 2, *T. frisia* JB-A2<sup>T</sup> (Brinkhoff et al., 1999a); 3, *T. chilensis* Ch-1<sup>T</sup> (Brinkhoff et al., 1999b); 4, *T. arctica* SVAL-E<sup>T</sup> (Knittel et al., 2005); 5, *T. psychrophila* SVAL-D<sup>T</sup> (Knittel et al., 2005); 6, *T. hydrogeniphila* MAS2<sup>T</sup> (Watsuji et al., 2016); 7, *T. aquadulcis* HaS4<sup>T</sup> (Kojima & Fukui, 2019); 8, *T. indica* 13-15A<sup>T</sup> (Liu et al., 2020); 9, *T. sediminis* G1<sup>T</sup> (Liu et al., 2021); 10, *T. xiamenensis* G2<sup>T</sup> (Liu et al., 2021); 11, *T. heinhorstiae* HH1<sup>T</sup> (Updegraff et al., 2022); 12, *T. cannonii* HH3<sup>T</sup> (Updegraff et al., 2022). All strains are obligate chemolithoautotrophs which oxidize thiosulfate, tetrathionate and sulfur. NR, not reported.

	1	2	3	4	5	6	7	8	9	10	11	12
Optimum pH for growth	6.6-7.0	6.5	7	7.3-8.0	7.5-8.5	6	6.6-7.4	7	7.5	6.5	7.4	7.5
pH range for growth	5.6-8.5	4.2-8.5	5.3-8.5	6.5-9.0	6.5-9.0	5.0-8.0	6.2-8.8	5.0-9.0	6.0-9.0	5.5-8.0	6.5-7.5	6.0-8.0
Optimum temperature for growth (°C)	30	32-35	32-37	11.5-13.2	14.6-15.4	30	22	28	30	28	32.8	32.0
Temperature range for growth (°C)	5-37	3.5-39	3.5-42	-2.0-20.8	-2.0-20.8	2-40	0-25	10-45	10-40	4-45	15-35	15-35
Growth on H <sub>2</sub>	-	-	-	-	-	+	-	-	-	-	+	-
Anaerobic growth	-	-	-	-	-	-	-	-	+	-	-	-
Fourth most abundant fatty acid	C <sub>17:1</sub>	NR	C <sub>18:0</sub>	C <sub>14:1</sub>	C <sub>12:1</sub>	C <sub>12:0</sub>	C <sub>18:0</sub>	C <sub>10:0</sub> 3-OH	C <sub>18:0</sub>	C <sub>10:0</sub> 3-OH	C <sub>10:0</sub> 3-OH	C <sub>12:0</sub>
Percentage 16S rRNA gene identity to that of strain Am19 <sup>T</sup> (%)	100	97.7	96.5	97.3	96.7	97.1	95.3	94.9	96.2	94.7	93.4	94.6

Table 2. Values of dDDH and ANI between strain Am19<sup>T</sup> and genome-sequenced strains in the genus *Thiomicrothabodus*

Strain (accession number)	dDDH (%)	ANI (%)
<i>Thiomicrothabodus</i> sp. Kp2 (GCA_000478585.1)	21.1	80
<i>Thiomicrothabodus</i> sp. Milos-T2 (GCA_000702325.1)	20.9	80
<i>Thiomicrothabodus</i> sp. HH3 (GCA_013391695.1)	15.8	79
<i>Thiomicrothabodus</i> sp. HH1 (GCA_013391765.1)	16.2	78
<i>T. aquaedulcis</i> HaS4 <sup>T</sup> (GCA_004001325.1)	16.2	78
<i>T. indica</i> 13-15A <sup>T</sup> (GCA_004293625.1)	16.5	78
<i>T. sediminis</i> G1 <sup>T</sup> (GCA_005885815.1)	16.8	79
<i>T. xiamenensis</i> G2 <sup>T</sup> (GCA_013282625)	16.4	79
<i>T. arctica</i> DSM 13458 <sup>T</sup> (GCA_000381085.1)	16.4	78
<i>T. chilensis</i> DSM 12352 <sup>T</sup> (GCA_000483485.1)	16.3	79
<i>T. heinhorstiae</i> HH1 <sup>T</sup> (GCA_013391765.2)	20.6	78
<i>T. cannonii</i> HH3 <sup>T</sup> (GCA_013391695.1)	19.8	78

