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A novel *Thermotoga* strain TFO isolated from a Californian petroleum reservoir phylogenetically related to *Thermotoga petrophila* and *T. naphthophila*, two thermophilic anaerobic isolates from a Japanese reservoir: Taxonomic and genomic considerations

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1 **A novel *Thermotoga* strain TFO isolated from a Californian petroleum reservoir**
2 **phylogenetically related to *Thermotoga petrophila* and *T. naphthophila*, two**
3 **thermophilic anaerobic isolates from a Japanese reservoir: taxonomic and genomic**
4 **considerations**

5

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21

22 **Abstract**

23 Hot oil reservoirs harbor diverse microbial communities, with many of them inhabiting
24 thermophilic or hyperthermophilic fermentative *Thermotogae* species. A new *Thermotoga* sp.
25 strain TFO was isolated from an Californian offshore oil reservoir which is phylogenetically
26 related to thermophilic species *T. petrophila* RKU-1^T and *T. naphthophila* RKU-10^T, isolated
27 from the Kubiki oil reservoir in Japan. The average nucleotide identity and DNA-DNA
28 hybridization measures provide evidence that the novel strain TFO is closely related to *T.*
29 *naphthophila* RKU-10^T, *T. petrophila* RKU-1^T and can not be differentiated at the species
30 level. In the light of these results, the reclassification of *T. naphthophila* RKU-10 and strain
31 TFO as heterotypic synonyms of *T. petrophila* is proposed. A pangenomic survey of closely
32 related species revealed 55 TFO strain-specific proteins, many of which being linked to
33 glycosyltransferases and mobile genetic elements such as recombinases, transposases and
34 prophage, which can contribute to genome evolution and plasticity, promoting bacterial
35 diversification and adaptation to environmental changes. The discovery of a TFO-specific
36 transport system dctPQM, encoding a tripartite ATP-independent periplasmic transporter
37 (TRAP), has to be highlighted. The presence of this TRAP system assumes that it could assist
38 in anaerobic n-alkane degradation by addition of fumarate dicarboxylic acid, suggesting a
39 niche-specific gene pool which correlates with the oil reservoir that *T. petrophila* TFO
40 inhabits. Finally, *T. naphthophila* RKU-10, *T. petrophila* RKU-1^T, *T. petrophila* TFO form a
41 distinct phylogenetic lineage with different geographic origins, share the same type of
42 ecological niche including the burial history of fields. Theses findings might support the
43 indigenous character of this species in oil reservoirs.

44 **Keywords:** *Thermotoga*, Taxonomy, Classification, Genomics , Oil reservoirs, Anaerobes

45 **Introduction**

46 A wide range of anaerobic microorganisms with diverse physiological and metabolic abilities
47 are known to inhabit oil reservoirs where they are subjected to drastic *in situ* physicochemical
48 conditions (e.g. high temperature and/or high hydrostatic/lithostatic pressure) [45]. Most of
49 these inhabitants are methanogenic archaea, sulfate-, nitrate- and iron-reducing prokaryotes,
50 homoacetogens, as well as fermentative bacteria [45]. Among the latter, anaerobic
51 fermentative members of the phylum *Thermotogae* are recognized to be widespread in both
52 marine and terrestrial subsurface ecosystems [38,39,48,56].

53 The phylum *Thermotogae*, which forms an early branching lineage within the *Bacteria*
54 domain, consists of 65 cultivated species disseminated among 13 genera [25]. The originality
55 of the phylum *Thermotogae* stems from the physiological features of its bacterial
56 representatives, which are mostly thermophilic ($T_{opt} \geq 70$ °C) and hyper-thermophilic ($T_{opt} \geq$
57 80 °C) [14], with the exception of some mesophiles (T_{opt} 37-40 °C) pertaining to the genus
58 *Mesotoga* [17,43] and moderate thermo-acidophiles (optimal growth at pH 3-5, $T_{opt} \geq 55$ °C)
59 of the genera *Athalassotoga* and *Mesoaciditoga* [24,50]. Advances in species isolation
60 combined with the use modern taxonomy practices (i.e genomic signatures, Overall Genome
61 Relatedness Indices) continue to reshape the classification of the phylum *Thermotogae* [2,3].
62 The phylum is currently divided into four orders (*Thermotogales*, *Kosmotogales*,
63 *Petrotogales*, *Mesoaciditogales*) and five families (*Thermotogaceae*, *Fervidobacteriaceae*,
64 *Kosmotogaceae*, *Petrotogaceae*, *Mesoaciditogaceae*).

65 Only members of the families *Thermotogaceae*, *Kosmotogaceae*, and *Petrotogaceae* have
66 thus far been observed by either culture-dependent or independent approaches from oilfield
67 environments. Within the family *Thermotogaceae*, they include two representatives of the
68 genus *Thermotoga*, *T. petrophila* RKU-1 [56] and *T. naphthophila* RKU-10 [56], which have
69 been isolated from the Japanese Kubiki oil reservoir.

70 Herein, we report the phenotypic and genomic characterization of a new *Thermotoga* strain
71 (TFO), isolated from an offshore Californian oil reservoir, which is phylogenetically and
72 genomically related to *Thermotoga petrophila* RKU-1 and *Thermotoga naphthophila* RKU-
73 10.

74 **Methods**

75 **Isolation**

76 Pescado Monterey reservoirs have temperature ranges of (85-104 °C). Pescado Monterey
77 depth averages 2,225 m (300 m-700 m water depth, 1500 m overburden), resulting in an
78 average pressure of 22.25 MPa (~3200 psi). The produced fluids consisting of liquid
79 hydrocarbons and production water were collected from a production well into sterile and
80 anaerobic 156 mL serum bottles, and were chilled in the dark with ice and transported to
81 laboratory within 48 hours of collection. Enrichment of thermophiles was performed by
82 inoculating the collected production fluids from the Pescado offshore oil reservoir. Within 7
83 days, turbidity due to cell growth was observed. Initially, the enrichments consisted of a
84 mixed community, and the sheathed “toga” like hyperthermophilic bacterial strain was
85 purified by inoculating gelrite solidified medium in the bottom of an anaerobic 156 mL
86 serum bottle, and incubating them at 65 °C. After about 7 days of incubation, a few singles
87 colonies (<1 mm in diameter) were observed in the solid medium and were picked with
88 sterile glass Pasteur pipettes in an anaerobic chamber and transferred into liquid medium, and
89 the isolation was repeated three times to ensure the purity of the isolates. The isolated strains
90 were then further purified using dilution to extinction cultivation in a series of tubes
91 containing liquid medium. The isolated strain we discuss in this manuscript is an obligatory
92 anaerobic heterotrophic rod-shaped bacterium with a “toga”, a sheath like structure with
93 ballooning at both ends, which is characteristic of members of the phylum *Thermotogae*. The
94 isolated strain TFO have been deposited for conformity at the Leibniz Institute DSMZ-

95 German Collection of Microorganisms and Cell Cultures GmbH. The collection number
96 assigned to strain TFO is DSM 111359.

97 **Culture medium composition**

98 Strain TFO was cultivated in medium containing (per liter): 10 g NaCl, 1 g KCl, 0.2 g CaCl₂-
99 2H₂O, 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1 g NH₄Cl, 3 g Na₂S₂O₃, 1 g yeast extract, cysteine-HCl
100 0.5 g, resazurin (0.1 %) 1 ml and trace minerals [1] 10 ml. The pH was adjusted to 7 with a
101 10 M KOH solution. The medium was boiled under a stream of O₂-free N₂ gas and cooled at
102 room temperature. Aliquots of 5 ml were dispensed into Hungate tubes, stoppered with butyl-
103 rubber stoppers, and degassed under N₂-CO₂ (80/20, vol/vol) and subsequently sterilized by
104 autoclaving at 120 °C for 20 min. Prior to culture inoculation, 0.2 ml of 10 % (w/vol)
105 NaHCO₃, 0.1 ml of 150 g L⁻¹ MgCl₂-6H₂O, 0.1 ml of 2 % Na₂S and 20 mM of glucose from
106 sterile stock solutions were injected into the tubes. The Hungate technique was used
107 throughout the study [20]. A 0.5 ml aliquot of the sample was inoculated into the Hungate
108 tubes that were subsequently incubated at 70 °C. Growth experiments were performed in
109 duplicate.

110 **Determination of the optimal temperature, NaCl and pH conditions for growth**

111 The temperature, NaCl, and pH ranges for growth were determined using the above culture
112 medium. To study the NaCl tolerance, the required quantity of NaCl was added to the tubes
113 (from 0 g/L to 80 g/L). The pH of the medium was adjusted by injecting into the Hungate
114 tubes aliquots of anaerobic stock solutions of 10% NaHCO₃ or 8% Na₂CO₃ (to test pH
115 between 5 and 9.5) and checked after autoclaving. The strain was sub-cultured at least twice
116 under the same experimental conditions before growth rates were determined.

117 **Substrate utilization assays**

118 The various substrates were added at 20 mM concentration to the culture medium without
119 glucose, and in the presence or absence of sodium thiosulfate as potential electron acceptor to

120 be used by members of the phylum *Thermotogae* [10]. Complex organic substrates (casamino
121 acids, yeast extract, tryptone, and peptone) were tested at a final concentration of 2 g/L, in the
122 minimal culture medium described previously without yeast extract. To test electron acceptor
123 utilization, beside sodium thiosulfate (20 mM), sodium sulfate (20 mM), elemental sulfur (10
124 g/L), nitrate (20 mM), or nitrite (2 mM) were added at 20 mM final concentration in the
125 culture medium in the absence of thiosulfate. Growth was determined by OD_{600 nm}
126 measurements and microscopy observations, after 24 h of culture incubation at 70 °C.
127 Experiments were performed in duplicate. Substrate consumption and organic acid
128 production were measured by HPLC (using the UltiMate 3000, Thermo Scientific).
129 Thiosulfate consumption was measured by ion chromatography, using the Metrohm 761
130 Compact IC. H₂S production was measured using the Cord-Ruwisch method [7].

131 **Lipid analysis**

132 Cultures of the three strains were grown-up and harvested at the end of exponential phase and
133 sent to DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,
134 Braunschweig, Germany) for fatty acids and polar acids analyses.

135 **Light and scanning electron microscopy**

136 Cells of isolate TFO were analyzed by using the Scanning Electron Microscope (SEM)
137 protocol as described by Murtey et al. [42] and modified as follows. Cells on glass slides
138 were briefly rinsed three times in cacodylate buffer and transferred to freshly prepared alcian
139 blue-lysine added in glutaraldehyde/paraformaldehyde fixative for 2 hours at 4 °C. The glass
140 slides were then transferred to cacodylate buffered glutaraldehyde fixative for 24 hours. The
141 glass slides were then dehydrated in a series of ethanol concentrations for 20 min in each of
142 the following concentrations: 25%, 50%, 70%, 75%, 85%, 90%, 95% and two (20 min)
143 exchanges in anhydrous 100% ethanol followed by chemical fixation in hexamthylsilazane.
144 Samples were then air dried for more than 3 hours with desiccant before visualizing under
145 Zeiss Crossbeam 540 SEM.

146 **Spectroscopic DNA–DNA hybridization**

147 DNA-DNA Hybridization (DDH) studies were performed using isolate TFO (DSM 111359),
148 *Thermotoga petrophila* RKU-1 (DSM 13995) and *Thermotoga naphthophila* RKU-10 (DSM
149 13996). The DDH studies were carried out by the DSMZ. Cells were disrupted using a
150 Constant Systems TS 0.75 KW (IUL Instruments, Germany). DNA in the crude lysate was
151 purified by chromatography on hydroxyapatite as described by Cashion and colleagues [5].
152 DNA-DNA hybridization was carried out as described by De Ley et al. [33] under
153 consideration of the modifications described by Huss et al. [22] using a model Cary 100 Bio
154 UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and
155 a temperature controller with in-situ temperature probe (Varian).

156 **Genome sequencing**

157 Genomic DNA of the isolate TFO was extracted and purified following manufacturer's
158 instructions (Masterpure DNA isolation kit, Epicenter). Ten mL of cell culture was spun
159 down at 10,000 g for extraction and purification. The Genomic DNA of isolate TFO was
160 sequenced at Synthetic Genomics Inc. (La Jolla, CA, USA) on two SMRT cells on a PacBio
161 RS II (Pacific Biosciences, Menlo Park, CA, USA). The genome was assembled from
162 373,255,583 post-filtered bases comprising 36,575 reads with an average read length of
163 10,205 bases.

164 **Core genome-based phylogeny**

165 A core genome phylogeny was inferred using the genomes of strain TFO and its closely
166 related cultivated *Thermotoga* species, including *T. petrophila* RKU-1, *T. naphthophila*
167 RKU-10, *T. neapolitana* NS-E and *T. maritima* MSB8. Uncultivated *Thermotogae* spp. were
168 also included (complete and incomplete genomes). The genomes were retrieved from the
169 NCBI ftp (<ftp://ftp.ncbi.nlm.nih.gov/>), and the core genome identification was performed by
170 the Roary pipeline v3.10.2 [44]. The core gene alignment was generated by the Roary

171 pipeline with a 50% sequence identity cut-off. The phylogenetic tree was constructed from
172 the 492 core-genes with the BioNJ distance-based phylogeny reconstruction algorithm,
173 implemented in Seaview v4.7 [15]. The bootstrap branch supports were computed by the
174 'Transfer Bootstrap Expectation' method (TBE) with 1000 replicates [32].

175 **DOE-JGI genome submission and annotation of *Thermotoga* sp. strain TFO**

176 The genome sequence of *Thermotoga* sp. strain TFO was fully annotated using the DOE-JGI
177 Microbial Genome Annotation Pipeline [21] (MGAP v4.16.2). The MGAP pipeline uses
178 Prodigal V2.6.3 [23] for gene calling, the annotation algorithm is HMMER 3.1b2 [40] and
179 Rfam 12.0 is the database [26]. The functional annotation of genes was performed using
180 COG 2003 and Pfam v30 [12]. The annotated genome of *Thermotoga* sp. strain TFO (under
181 the recommended species name *Thermotoga petrophila* TFO) is available at the DOE-JGI
182 site <https://genome.jgi.doe.gov>) under the GOLD the project ID Gp0307586.

183 **Comparative genomics**

184 The genomes of *T. petrophila* RKU-1, *T. naphthophila* RKU-10, *T. neapolitana* NS-E, *T.*
185 *maritima* MSB8 and strain TFO were used for pan-genomic analysis. The gene prediction
186 and annotation were performed using the Prokka package [53]. The clustering of the
187 orthologs for pan-genomic analysis was carried out by Roary [44], applying a minimum of
188 sequence identity and coverage of 50%, as recommended by Tettelin and colleagues [57].
189 The singleton proteins (strain-specific proteins) did not belong to any orthologous cluster.
190 The specific COG (Cluster of Orthologous Gene) functional annotation was locally
191 performed by rpsblast with the predicted proteins against COG profiles included in the
192 Conserved Domain Database (CDD) at the NCBI (<ftp://ftp.ncbi.nlm.nih.gov/pub/mmdb/cdd>).
193 The COG annotation was selected for E-value below 10^{-5} . Pfam information for TFO-specific
194 proteins were retrieved (E-value $< 10^{-5}$) from local rpsblast using Pfam profiles of the CDD

195 database. The prophage prediction was investigated using PHAST
196 (<http://phast.wishartlab.com/>).
197 The Average Nucleotide Identity (ANI) values were calculated using JSpecies software [51]
198 and as described in [2]. Venn diagram was designed with the VennDiagram R package
199 version 1.7 and the “draw.quintuple.diagram” function. The Figure 6 was drawn by Circos
200 version 0.69 [31] and using the results of pairwise ortholog detection. The contigs of strain
201 TFO were reorder following the results provided by “move contig” function of MAUVE [9]
202 and using *T. petrophila* RKU-1 as reference genome.

203 **Results and discussion**

204 **Morphological and phenotypic properties of isolate TFO**

205 Strain TFO (DSM 111359) is a heterotrophic strict anaerobe and a Gram-negative rod-shaped
206 bacterium. The cell morphology of TFO isolate was 3 µm long and 0.5 µm wide, exhibiting
207 the characteristic sheath-like structure (called “toga”) of *Thermotoga* representatives
208 (**Figure 1**). The growth range temperature varied from 55 °C to 85 °C with an optimum at 75
209 °C. No growth was observed below 50 °C or above 90 °C (**Figure 2, A**). The isolate grew in
210 the absence of NaCl and tolerated it up to 60 g/L (**Figure 2, B**). This differs from both *T.*
211 *petrophila* RKU-1 and *T. naphthophila* RKU-10 as they require NaCl for growth [56] and
212 should therefore be considered as slight halophiles. In contrast, strain TFO should be
213 considered as halotolerant [56]. The optimum pH for growth was 7 and no growth was
214 observed at below pH 5.5 or or above pH 9 (**Figure 2, C**) similarly to other *Thermotoga*
215 representatives (**Table 1**).

216 Strain TFO was unable to grow autotrophically under H₂/CO₂ (80%/20%) atmosphere with or
217 without acetate (2 mM) as carbon source. The strain could grow heterotrophically on the
218 following sugars: glucose, sucrose, lactose, maltose, fructose, galactose, xylose, mannose,
219 ribose, arabinose, and raffinose with the exception of sorbose and cellulose. Yeast extract

220 (1g/L) was required for growth. No growth was observed on yeast extract, tryptone, peptone,
221 casamino acids or organic acids including propionate, butyrate, lactate, and acetate when
222 provided as sole energy source (**Table 1**). Contrary to its two closest phylogenetically
223 relatives *T. naphthophila* RKU-10 and *T. petrophila* RKU-1 (**Figure 3**) [56], strain TFO was
224 able to grow on xylose (**Table 1**). However, to be confident with this peculiar phenotypic
225 feature of strain TFO, the growth of *T. naphthophila* RKU-10 and *T. petrophila* RKU-1 on
226 xylose was tested as part of this study and we observed that both strains were also able to
227 grow on this substrate. Additionally, a survey of the gene content of all three strains revealed
228 the presence of shared xylose isomerase, the key enzyme required in the xylose catabolism
229 pathway [54]. Acetate, CO₂ and H₂ were produced by strain TFO when grown on sugars. It
230 was also observed that higher amounts of H₂ was produced when the cells were grown in the
231 absence of thiosulfate compared to growth in the presence of thiosulfate, suggesting that
232 thiosulfate can be used as electron acceptor as confirmed by sulfide production in the
233 presence of this sulfur compound in the culture medium. This is a common physiological trait
234 shared by several *Thermotoga* species [11,48]. The produced acetate/consumed glucose ratio
235 was similar in the presence or absence of thiosulfate. Beside thiosulfate, in the presence of 20
236 mM glucose, strain TFO was also able to reduce elemental sulfur to sulfide. Addition of
237 either thiosulfate or elemental sulfur in the culture medium enhanced the growth yields as
238 compared to control in the absence of them. On the contrary, the presence of these sulfur
239 compounds in the culture medium of *T. naphthophila* RKU-10 and *T. petrophila* RKU-1
240 decreases final growth yields and growth rates, even if both strains are recognized as
241 thiosulfate and elemental sulfur reducers [56]. When cultivated under elemental sulfur, strain
242 TFO produced higher amounts of H₂S (4-times more) than when grown under comparable
243 thiosulfate reduction conditions. Sulfate was not used as electron acceptor by strain TFO, and
244 the addition of either nitrate (20 m) or nitrite (2 mM) completely inhibited growth.

245 **Polar lipids**

246 Glycolipids, phospholipids and phosphoglycolipids were identified as polar lipids present for
247 the three strains (data not shown). The profiles of fatty acids were similar (some variations
248 observed for C_{10:0}/C_{14:0}) for the three strains cultivated under same growth conditions (**Table**
249 **2**). The major fatty acids were C_{16:0} (> 70%), C_{14:0} (6.98 to 17.7%), C_{10:0} (3.34 to 13.66%),
250 and C_{18:0} (2.0 to 2.84%) for the three strains, as for other *Thermotoga* species [41].

251 **Phylogenetic placement of strain TFO**

252 The 16S rRNA genes do not provide a confident taxonomic identification since the
253 nucleotide sequence of the gene from the novel isolate TFO and those of representatives
254 within the genus *Thermotoga* shared more than 99% of sequence identity. Since the
255 phylogenetic inference relies on 16S rRNA genes, we cannot therefore resolve the species
256 relatedness within the genus *Thermotoga* as previously shown by Belahbib and colleagues
257 [2]. The relationships of strain TFO with the related *Thermotoga* species were thereby
258 explored by a core-genome based phylogeny (utilizing 492 shared genes) that resulted in a
259 well-supported clade (**Figure 3**). Strain TFO forms a lineage with *T. naphthophila* RKU-10
260 and *T. petrophila* RKU-1 and the uncultivated *T. naphthophila* 46_26.

261 **Species delineation by ANI and DDH measures**

262 In addition to the phylogenetic placement of strain TFO within the genus *Thermotoga*,
263 species delineation was determined by Overall Genome Relatedness Index (OGRI) [6],
264 particularly by the recommended ANI measure [30]. The delineation of inter-species to intra-
265 species relationships is signified by the ANI interval of 95-96% [29], and the ANI measures
266 of the strain TFO with *T. naphthophila* RKU-10 and *T. petrophila* RKU-1 reached the
267 boundary of intra-species relationship with ANI values of 95.80 % and 96.08%, respectively.
268 Strikingly, the ANI value between the species *T. naphthophila* RKU-10 and *T. petrophila*
269 RKU-1 was slightly over the boundary of intra-species relationship (ANI = 96.38%). Overall,

270 the ANI clustering highlighted species delineation ambiguities within the genus *Thermotoga*
271 (**Figure 4**), exemplified by intra-species relationships for the uncultivated *Thermotoga* sp.
272 Xyl 54, *Thermotoga*. sp Cell2, *Thermotoga*. sp. TBGT1765 and *Thermotoga* sp. TBGT1766
273 (ANI values > 98%).

274 Considering the ambiguous ANI results for species delineation, complementary DDH
275 experiments were carried out in duplicate with *T. petrophila* RKU-1, *T. naphthophila* RKU-
276 10 and the strain TFO. The results indicated DNA–DNA homology of 83.15% (81.3; 85.0)
277 between the strain TFO and *T. petrophila* RKU-1, and DNA-DNA homology of 74.7% (75.3;
278 74.1) with *T. naphthophila* RKU-10. Likewise, DNA reassociation between *T. petrophila*
279 RKU-1 and *T. naphthophila* RKU-10 was 77.65% (75.9; 79.4), which is in disagreement with
280 the average value (15%) reported in the original work of Takahata and colleagues [56]. All
281 DDH values of pairwise species comparisons were above 70%, providing evidence that *T.*
282 *naphthophila* RKU-10, *T. petrophila* RKU-1 and strain TFO could not be differentiated at the
283 species level and, therefore, should be considered as a single species. Based upon ANI and
284 DDH results, *T. petrophila* RKU-1, *T. naphthophila* RKU-10 and *Thermotoga* sp. TFO
285 should be considered as heterotypic synonyms. According to rules 38, 42 and 24b of the
286 bacteriological code [46] the name *Thermotoga petrophila* RKU-1 has priority and should be
287 used for the unified taxon. For that reason, *Thermotoga petrophila* TFO is proposed as
288 species name for the TFO isolate.

289 **Biogeography and geology**

290 Strain TFO forms a distinct lineage with *T. naphthophila* RKU-10 and *T. petrophila* RKU-1
291 and the uncultivated *T. naphthophila* 46_26 (**Figure 3**). While strain TFO originates from an
292 offshore Californian oil reservoir (Pescado Field), the strains RKU-10, RKU-1 and the
293 uncultivated strain 26_46 originate from Japan (Kubiki Field) and Alaska oil reservoirs,
294 respectively. The global presence of particular bacterial lineage in geographically distant oil

295 reservoirs could be an indicator of their indigenous nature [35]. Additionally, the Late
296 Miocene Monterey Formation, a siliceous mudstone, is both source and reservoir for the
297 Pescado Field oil [8] (TFO isolate). Reservoir depths are at ~2225 m with temperatures
298 ranging from ~85 to 104 °C. Low gravity, high sulfur crude oil was generated locally. The
299 reservoirs of the Kubiki Field oils are primarily in the Late Miocene Teradomari Formation
300 [27] at shallower depths (of ~1100 m) and lower temperatures (~50-58 °C) [56]. The source
301 of the lighter, low sulfur crude oil in the Kubiki Field (*T. petrophila* and *T. naphthophila*) is
302 not certain, but is also believed to be of Miocene age [58]. It suggests that the burial history
303 of both fields is similar in that the source strata were rapidly buried at similar geologic times
304 and that the reservoir rocks are currently at their maximum temperatures.

305 **Genomic features of *T. petrophila* TFO**

306 The genome properties of strain TFO and its closely related cultivated species are
307 summarized in **Table 3**. The genome was assembled in eight scaffolds with a total count of
308 1,885,773 base pairs (bp), slightly larger than the complete *Thermotoga* genomes (*T.*
309 *neapolitana* NS-E, *T. maritima* MSB8, *T. petrophila* RKU-1, *T. naphthophila* RKU-10). The
310 GC content of the *Thermotoga* representatives ranges 46-47%, which is in agreement with
311 the 46.09% of strain TFO. The genome contained a total of 1966 predicted genes consisting
312 of 52 RNA genes and 1914 proteins. The functional annotation indicated that 76.80% of the
313 proteins were assigned to functional COG categories, with the major designations being
314 general function (8.88%), carbohydrates (8.78%) and amino acid transport and metabolism
315 (8.10%).

316 **Specificities of *T. petrophila* TFO**

317 The pan-genome of cultivated *Thermotoga* species included a core-genome (essential genes
318 for shared across all species) of 1583 orthologous proteins (**Figure 5**). The *Thermotoga* core-
319 genome represents a large fraction of all predicted proteins of each species including 83%,

320 87%, 88%, 83% and 86% for strain TFO, *T. naphthophila* RKU-10, *T. petrophila* RKU-1, *T.*
321 *neapolitana* NS-E and *T. maritima* MSB8, respectively. Considering only the phylogenetic
322 closest neighbors, the orfome of *T. petrophila* and *T. naphthophila* shared 94% and 92% of
323 orthologs with that of TFO strain, respectively. These orthologs were highly conserved at the
324 amino acid identity level since almost all exhibited a score above 90% (**Figure 6**). These
325 three closely related species also displayed conservation of genome synteny and high genome
326 compactness (**Figure 6**). Finally, the number of species/strain-specific proteins (non-essential
327 genes) for strain TFO, *T. petrophila* RKU-1, *T. naphthophila* RKU-10, *T. neapolitana* NS-E
328 and *T. maritima* MSB8 was 55, 37, 56, 131, and 54, respectively. Most of species/strain-
329 specific genes are often acquired through horizontal gene transfer and are assumed to encode
330 adaptive function [36].

331 In this study, among the 55 strain-specific proteins of *T. petrophila*. TFO (**Table 4, Figure**
332 **6**), eight were glycosyltransferases (mainly in the same cluster of genes), which are known to
333 be involved in the biosynthesis of di- oligo- and polysaccharides, protein glycosylation [52],
334 as well as cell wall peptidoglycan synthesis [37]. Likewise, this gene cluster harbors an
335 oligosaccharide-flippase transporter that can promote translocation of lipid-linked
336 oligosaccharides for polysaccharide synthesis and cell wall construction [18].

337 Mobile genetic elements including insertion elements, transposases or bacteriophages are of
338 particular importance for genome diversification. In this way, an incomplete 38-kb prophage
339 (44.37% GC content) was predicted in the TFO genome and localized in the region 1.357.190
340 bp (attL site) to 1.395.092 bp (attR site). Although this prophage region was not detected in
341 the other genomes, only 14 (of the 23 predicted proteins in this region) were TFO-specific
342 proteins, of which nine were prophage-related and five were bacteria-related proteins.

343 Moreover, two transposases of the ISCc3 family (integrase and OrfB) and two
344 recombinases/resolvases were identified solely in *T. petrophila* TFO. The transposases were

345 localized next to homologous proteins to DEAD/DEAH helicases which are important
346 players of processes involving RNA molecules including ribosome biogenesis, transcription,
347 RNA turnover and RNA-protein folding [49].

348 In addition, among the 55 strain-specific proteins of *T. petrophila* TFO, a cluster of eleven
349 proteins (**Table 4**, from NJCICCOL_00759 to NJCICCOL_00770) were mostly associated to
350 bacterial transport systems, including an ABC type transporter of sugar (substrate specificity
351 unknown), and the dctPQM system which encodes a tripartite ATP-independent periplasmic
352 transporter (TRAP) [13]. The TRAP proteins of the strain TFO were found to be
353 phylogenetically related (not shown) to the uncultivated *Thermotoga naphthophila* 46_26
354 (Biosample: SAMN03445142) which originates from the metagenome of the Kuparuk oil
355 field in the Alaskan Arctic North Slope [19]. TRAP systems can transport a variety of
356 substrates under different contexts, including the transport of solutes that could play an
357 important role in the piezophilic lifestyle [47], and particularly in the import of C4-
358 dicarboxylic including succinate, fumarate, butyrate [13]. These organic acids can be the
359 result of the transformation of alkanes, as recently shown by members of candidate phylum
360 *Atribacteria* that ferment short-chain n-alkanes into carboxylic acids [34]. Furthermore, the
361 extracellular uptake of C4-dicarboxylic fumarate could play a key role in the activation of the
362 anaerobic degradation of alkanes, naphthalene and toluene from oil-rich environments [4,28].
363 The homologues of enzymes for the fumarate addition mechanism in anaerobic degradation
364 of alkanes [4] were not unambiguously identified in the genome of strain TFO. Indeed, the
365 key enzyme alkylsuccinate synthase AssA, MassD and pflD genes [28,55] shared at best 32%
366 of sequence identity with a predicted hypothetical protein from the genome of strain TFO,
367 which is an ambiguous score for homolog definition [57]. One has to keep in mind that genes
368 could be missing due to the incompleteness of the genome of *T. petrophila* TFO (eight

369 scaffolds). As a consequence, the potential alkylsuccinate synthase activity as well as TRAP
370 functional specificity/activity of strain TFO should be experimentally evaluated.

371 **Conclusions**

372 Herein, a novel *Thermotoga* strain (TFO) isolated from a Californian petroleum reservoir was
373 phenotypically and genomically characterized. In the light of modern taxonomy practice with
374 the use of core genome phylogeny, whole genome relatedness indices and DDH measures,
375 our results recommend considering *T. petrophila* RKU-1, *T. naphthophila* RKU-10 and
376 *Thermotoga* TFO as heterotypic synonyms. Based upon Takahata and colleagues' work [56],
377 the name *Thermotoga petrophila* has priority and should be used for the unified taxon. For
378 that reason, *Thermotoga petrophila* TFO is proposed as species name for this oil reservoir
379 isolate.

380 These three strains accompanied by the uncultivated *Thermotoga naphthophila* 26_46 belong
381 to the same phylogenetic lineage and share the same type of ecological niche with similar
382 burial history (oil field of cultivated species) from distinct geographic regions (Japan,
383 California and Alaska). These considerations could be in favor of the indigenous character of
384 such species in oil reservoirs [35]. The conservation of the genome synteny (**Figure 6**) of the
385 three oilfield strains and the very high level of homologous proteins suggest that they are
386 slow evolving organisms. Nonetheless, the TFO-specific proteins, including mobile genetic
387 elements, could promote bacterial diversification or specialization. These genes can have
388 clear relationships to the local environment and tagged as “niche-specific gene pool” that
389 could encode for degradation of new type of nutrient source [16]. The hypothetical proteins,
390 orphans (protein with no hits), sugar and TRAP transporters that belong to the TFO-specific
391 proteins could thereby bring new metabolic functions associated to its niche which requires
392 further study.

393

394 **Figure legend**

395 **Figure 1: Scanning electron microscopy of the isolate TFO**

396 Morphological features of *T. petrophila* TFO revealed by electron microscopy. Arrows indicate
397 the toga (tg) and cell (c). Bar, 1 μ m.

398 **Figure 2: Effects of temperature (A), NaCl concentration (B) and pH (C) on the growth**
399 **rates of *T. petrophila* TFO**

400 Strain TFO was grown as described in the Methods section and the growth rates were
401 calculated from a linear regression along the logarithmic part of the growth curves.

402 **Figure 3: Core genome-based phylogeny of the *T. petrophila* TFO within the**
403 ***Thermotogaceae* family**

404 Phylogenetic tree of 17 *Thermotoga* species deduced from 492 core-genes with the BioNJ
405 distance-based phylogeny reconstruction algorithm. The bootstrap branch supports were
406 computed by the 'Transfer Bootstrap Expectation' method (TBE) with 1000 replicates. The
407 tree is rooted with *Pseudothermotoga elfii* and *P. lettingae*. Species names in bold are
408 cultivated type species. Asterisk (*) means species isolated from oil reservoir.

409 **Figure 4: Whole-genome relatedness within the *Thermotogaceae* family**

410 Hierarchical clustering from Average Nucleotide Identity measures using 22 *Thermotogaceae*
411 genomes including the TFO isolate. Value scale is depicted from red to blue colors for ANI
412 genome pairwise comparison. The value scale ranges from 0 to 100% according to sequence
413 identity level. The vertical red line at the value 95-96 indicates the ANI species demarcation
414 threshold.

415 **Figure 5: Pangenome of *Thermotoga* cultivated species**

416 Five-set Venn diagram of the pangenome of five cultivated *Thermotoga* species based on the
417 prediction of orthologous proteins. Values on diagram represent the numbers of orthologs
418 shared between the species or unique proteins for one species. The core-genome (black bold

419 value, center), variable-genome (black values, small size) and strain-specific (colored values)
420 sizes are represented.

421 **Figure 6. Ortholog relationships and strain TFO-specific proteins**

422 Visualization of the ortholog relationships between strain TFO and *T. petrophila* RKU-1 and
423 *T. naphthophila* RKU-10. From outside to inside. Size labels is 10^4 base pairs. Genomes are
424 labelled and correspond to circle segments. The ORF positions for strain TFO, *T.*
425 *naphthophila*, *T. petrophila* are colored light blue, deep red and light green inside the circle
426 segments, respectively. The ORF distribution shows the compactness of genomes since
427 spaces between ORFs are sparsely present (white color). TFO-specific ORFs are colored in
428 black inside the circle segment, and mainly organized in short clusters. Red links, orthologs
429 between strain TFO and *T. naphthophila* sharing more than 90% of sequence identity. Blue
430 links, orthologs between strain TFO and *T. petrophila* sharing more than 90% of sequence
431 identity. Yellow links are orthologs sharing less than 90% of sequence identity. The link
432 directions (uncrossed) highlight the conservation of genome synteny between the species.
433

Table 1. Characteristics of strain TFO with its closely related species within the genus***Thermotoga***

Characteristic	<i>Strain TFO</i>	<i>Thermotoga naphthophila</i> <i>RKU-10</i> (Takahata et al, 2001)	<i>Thermotoga petrophila</i> <i>RKU-1</i> (Takahata et al, 2001)	<i>Thermotoga neapolitana</i> <i>DMS4359</i> (Jannasch et al, 1988)	<i>Thermotoga mariitima</i> MSB8 (Huber et al, 1986)
Origin	Californian produced water	Oil reservoir, Japan	Oil reservoir, Japan	Geothermal hot spring, Italy	Geothermal hot spring, Italy

Temperature for growth (°C)

Range	55-85	48-86	47-88	55-90	55-90
Optimum	75	80	80	80	80

pH for growth

Range	6.0-8.5	5.4-9.0	5.2-9.0	5.5-9.0	5.5-9
Optimum	7.0	7.0	7.0	7.0	6.5

Salinity (%)

Range	0-6	0.1-6	0.1-5.5	0.25-6	0.25-3.75
Optimum	0	1	1	2	2.7

Optimal doubling time (min)	45 min	59 min	54 min	45 min	75 min
Oxygen tolerance (%)	<0	<0	<0	<15	<0
Electron acceptors	Thiosulfate, Elemental sulfur	Thiosulfate Elemental sulfur	Thiosulfate Elemental sulfur	Thiosulfate, Elemental sulfur (Ravot et al, 1995)	Thiosulfate, Elemental sulfur (Ravot et al, 1995)

Utilization of

Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
Galactose	+	+	+	+	+
Lactose	+	+	+	+	+
Fructose	+	+	+	+	+
Arabinose	+	+	+	ND	ND
Sorbose	-	ND	ND	ND	ND
Raffinose	+	ND	ND	ND	ND
Mannose	+	ND	ND	ND	ND
Maltose	+	+	+	+	+
Ribose	+	+	+	+	+
Cellulose	-	-	+	ND	ND
Xylose	+	-	-	+	+
Casamino acids	-	-	-	-	-
Tryptone	-	ND	ND	+	+
Peptone	-	+	+	ND	ND
Yeast Extract	-	-	-	ND	+
Glycerol	+	+	+	-	+
Mannitol	-	+	-	-	-
Acetate	-	-	-	-	-
Lactate	-	-	-	-	-
Propionate	-	-	-	-	-
H ₂ CO ₂	-	-	-	-	-

G+C DNA (mol %)	46.0	46.1	46.6	41	46
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+, growth; - no growth; ND: not determined.

Table 2. Cellular fatty acid contents of strains TFO, *T. petrophila* RKU-1, and *T. naphthophila* RKU-10.

Fatty acid	Strain TFO	<i>T. petrophila</i> RKU-1	<i>T. naphthophila</i> RKU-10
C _{10:0}	7.30	13.66	3.34
C _{12:0}	0.77	0.48	0.53
C _{14:0}	15.29	6.98	17.70
C _{15:0}	0.38	-	0.16
C _{16:0}	73.40	75.67	74.58
C _{16:0} N alcohol	-	-	0.42
C _{16:0} 3OH	0.46	0.37	0.43
C _{17:0}	0.40	-	-
C _{18:0}	2.00	2.54	2.84
C _{18:1} w9c	-	0.29	-

Values correspond to percentage (%).

Table 3. Genome features of *T. petrophila* TFO and closely cultivated species

Attribute	Isolate TFO	<i>T. naphthophila</i>	<i>T. petrophila</i>	<i>T. neapolitana</i>	<i>T. maritima</i>
Genome size (bp)	1,885,773	1,809,823	1,823,511	1,884,562	1,869,644
DNA coding (bp)	1,791,612	1,731,557	1,744,574	1809617	1,790,184
GC%	46.09	46.14	46.10	46.94	46.25
Total genes	1966	1867	1865	1988	1909
Protein coding	1914	1815	1810	1937	1858
RNA genes	52	52	55	51	51
Pseudo genes	–	47	25	–	–
Genes in internal clusters	340	117	124	126	144
Genes with function prediction	1604	1447	1456	1580	1553
Genes assigned to COGs	1451	1420	1426	1439	1504
Genes with Pfam domains	1674	1370	1605	1662	1657
Genes with signal peptides	36	29	36	27	37
Transmembrane helices	460	442	432	497	461
CRISPR repeats	6	8	7	7	6

Table 4. The TFO-specific proteins

Gene name	Prokka Annotation	SI %	cover %	CDD complementary annotation	PHAST annotation
NJCICCOL_00401	Hypothetical protein	100	100	EpsG superfamily	
NJCICCOL_00402	Glycosyl transferase	100	100	GT4_MtfB-like	
NJCICCOL_00403	Glycosyltransferase	100	100	Glyco_trans_1_2	
NJCICCOL_00404	Glycosyltransferase	100	98	Glyco_transf_4	
NJCICCOL_00405	Glycosyltransferase	99	97	stp1 superfamily	
NJCICCOL_00407	Glycosyltransferase	53	100	Glycosyltransferase family A	
NJCICCOL_00408	4Fe-4S dicluster domain-containing protein	63	48	Glycosyltransferase_GTB-type	
NJCICCOL_00409	Oligosaccharide flippase	76	100	MATE_like superfamily	
NJCICCOL_00410	DNA recombinase RMUC	92	100		
NJCICCOL_00411	HP	71	88		
NJCICCOL_00425	Glycosyltransferase family 2	97	100	Glyco_transf_GTA_type	
NJCICCOL_00426	HP	99	100	Glycosyltransferase_GTB-type	
NJCICCOL_00427	Oligosaccharide flippase	99	100	MATE_like superfamily	
NJCICCOL_00593	Integrase	100	100	HTH superfamily regulator	
NJCICCOL_00594	Transposase repeat family ISCc3	100	100	putative transposase OrfB	
NJCICCOL_00759	ABC transporter permease	86	99	AraH	
NJCICCOL_00760	Sugar ABC transporter ATPase	86	99	MglA	
NJCICCOL_0076	ABC transporter substrat binding	75	100	PBP1_ABC_sugar_binding_like_2	
NJCICCOL_00762	Hypothetical protein	60	100	Ureidogly_lyase superfamily	
NJCICCOL_00764	TRAP transporter large permease	93	100	DctQ	
NJCICCOL_00765*	DctQ component; TRAP transporter small permease	100	100		
NJCICCOL_00766*	TRAP transporter solute receptor, DctP family	100	100	Periplasmic_Binding_Protein_Type_2 superfamily	
NJCICCOL_00767*	TRAP transporter solute receptor, DctP family	100	100		
NJCICCOL_00768	4-hydroxy-tetrahydrodipicolinate synthase	79	100	DRE_TIM_metallolyase superfamily	
NJCICCOL_00769	4-hydroxy-tetrahydrodipicolinate synthase	76	100	DapA superfamily	
NJCICCOL_00770	IcIR transcriptional regulator	78	98	DNA-binding transcriptional regulator, IcIR family	
NJCICCOL_00839	Transposase repeat family ISCc3	100	100	Putative transposase OrfB	
NJCICCOL_00840	Integrase	100	100	transcription regulator	
NJCICCOL_00943	No significant hit	<50	100		
NJCICCOL_01065	Beta-glucosidase B	100	84	beta-D-glucoside glucohydrolase	
NJCICCOL_01406	Recombinase family	50	92	Resolvase Recombinase	Phage

NJCICCOL_01407	DEAD/DEAH box helicase	63	99	Predicted helicase	Phage Helicase
NJCICCOL_01408	HP	58	87	Nuclease homologue	DNAse/Rnase endonuclease Phage
NJCICCOL_01409	No hit				
NJCICCOL_01410	Putative HTH-type transcriptional regulator	65	77	HTH_XRE	Phage transcriptional regulator
NJCICCOL_01411	No significant hit			HTH_XRE	Phage repressor
NJCICCOL_01412	No hit				
NJCICCOL_01413	No significant hit	<50	<40		
NJCICCOL_01414	Hypothetical protein	77	100	MethyltransfD12 superfamily	
NJCICCOL_01415	No significant hit	<50	<50		
NJCICCOL_01416	Phage recombinase RecT	80	99		Phage RecT-like
NJCICCOL_01417	HP	76	100		Phage replication
NJCICCOL_01418	HP	87	100	YdjM,SOS response, DNA damage	Phage hydrolase
NJCICCOL_01419	HP	92	100		
NJCICCOL_01420	HP	80	100	Outer membrane efflux protein	
NJCICCOL_01421	HP	80	100	YjgR/ P-loop_NTPase superfamily	
NJCICCOL_01422	Carbopeptidase regulatory-like domain	80	100	CarboxypepD_reg	
NJCICCOL_01423	no hit				
NJCICCOL_01424	Hypothetical protein	74	95	_	
NJCICCOL_01493					
NJCICCOL_01949	DEAD/DEAH box helicase	63	99	Predicted helicase	
NJCICCOL_01950	recombinase	49	92	Resolvase/recombinase	
NJCICCOL_01951	HP	79	84	_	
NJCICCOL_01952	No hit				
NJCICCOL_01953	carbopeptidase regulatory-like domain	86	97	_	

HP means hypothetical protein. No hit means orphan. No significant hit means that best blast hit is below the threshold 50% of sequence identity. SI corresponds to sequence identity in %. Cover is query sequence cover in %. Asterisk (*) means protein phylogenetically related to *T. naphthophila* 26_46.

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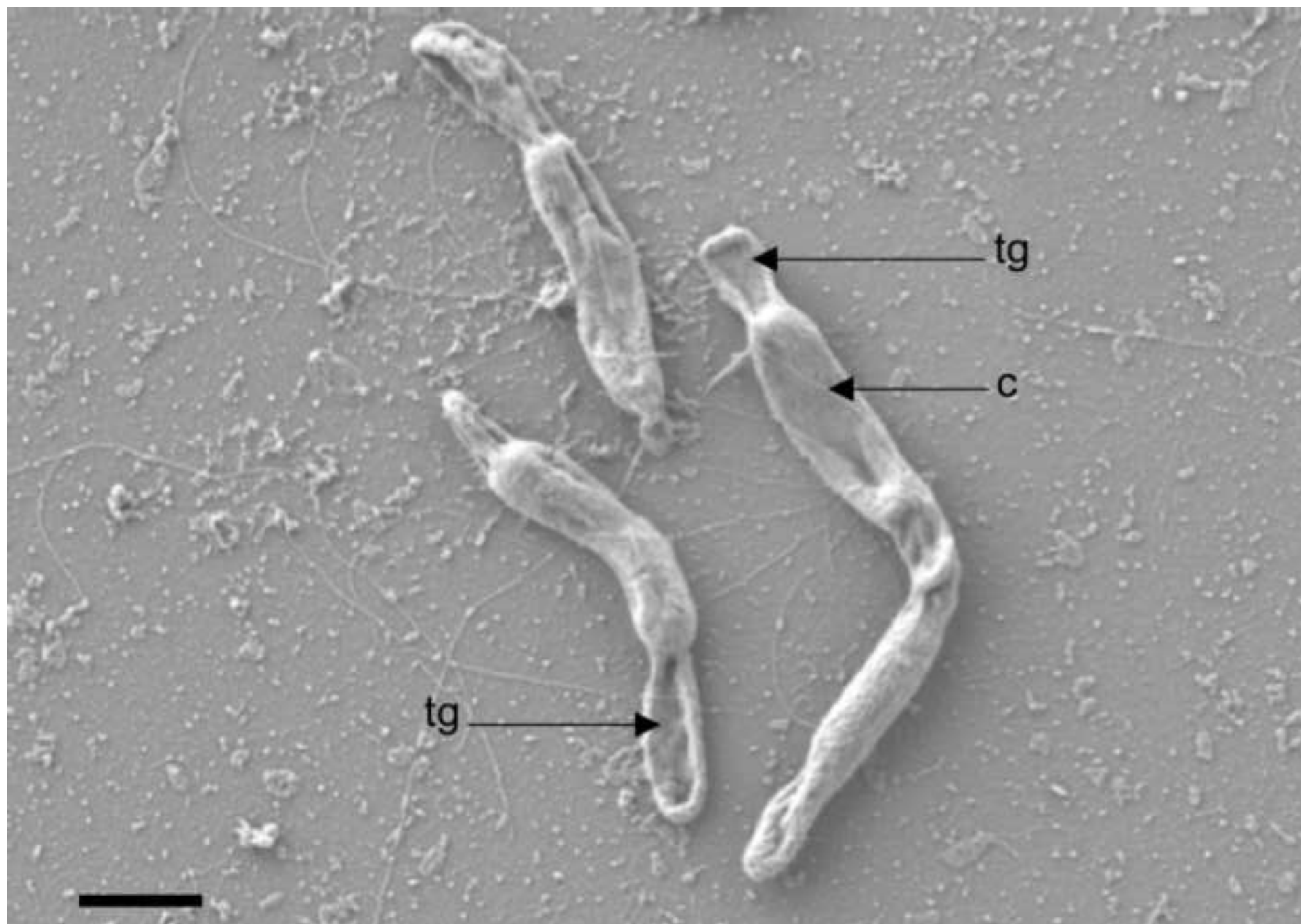


Figure2

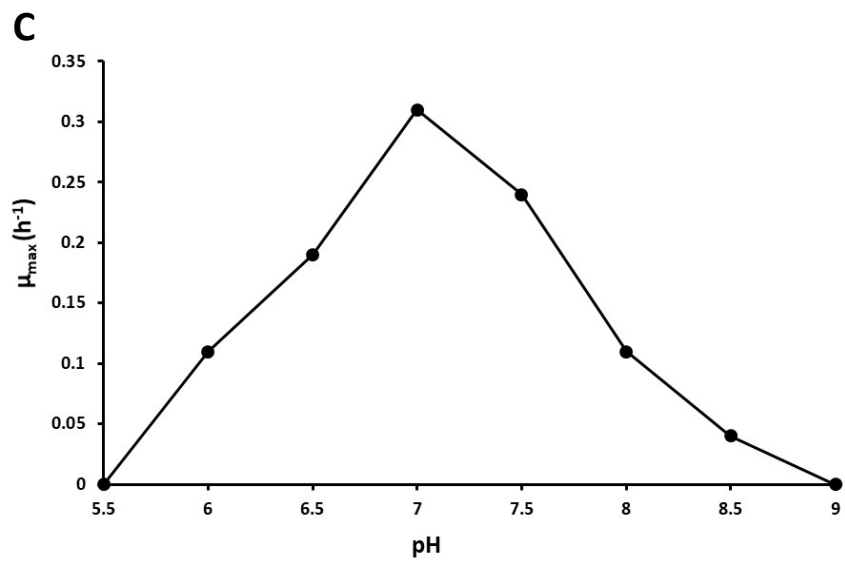
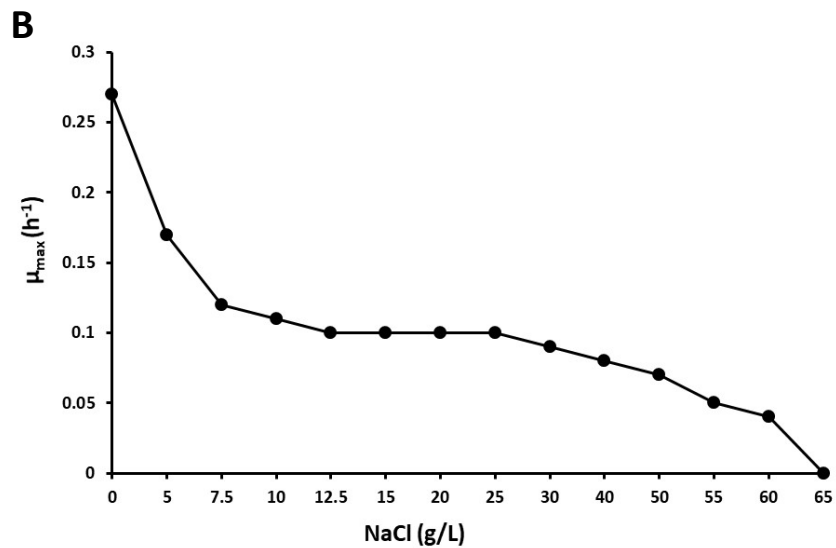
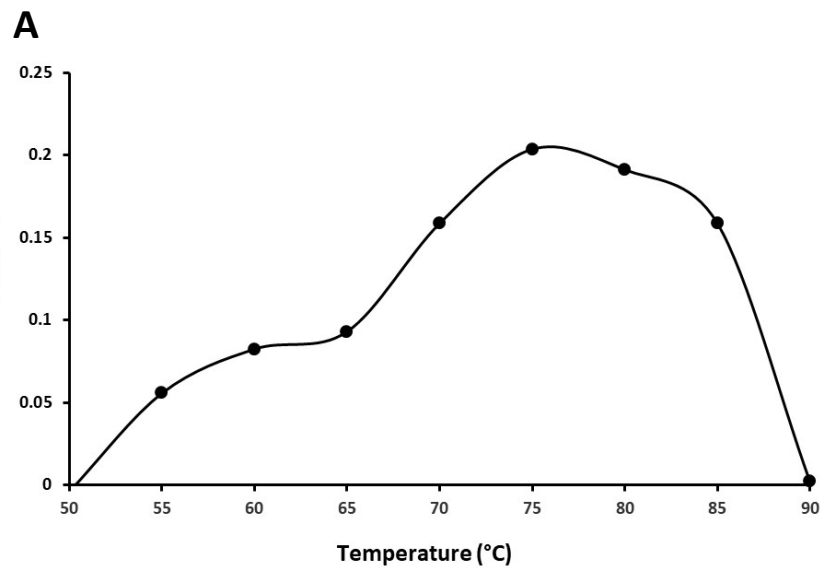


Figure3

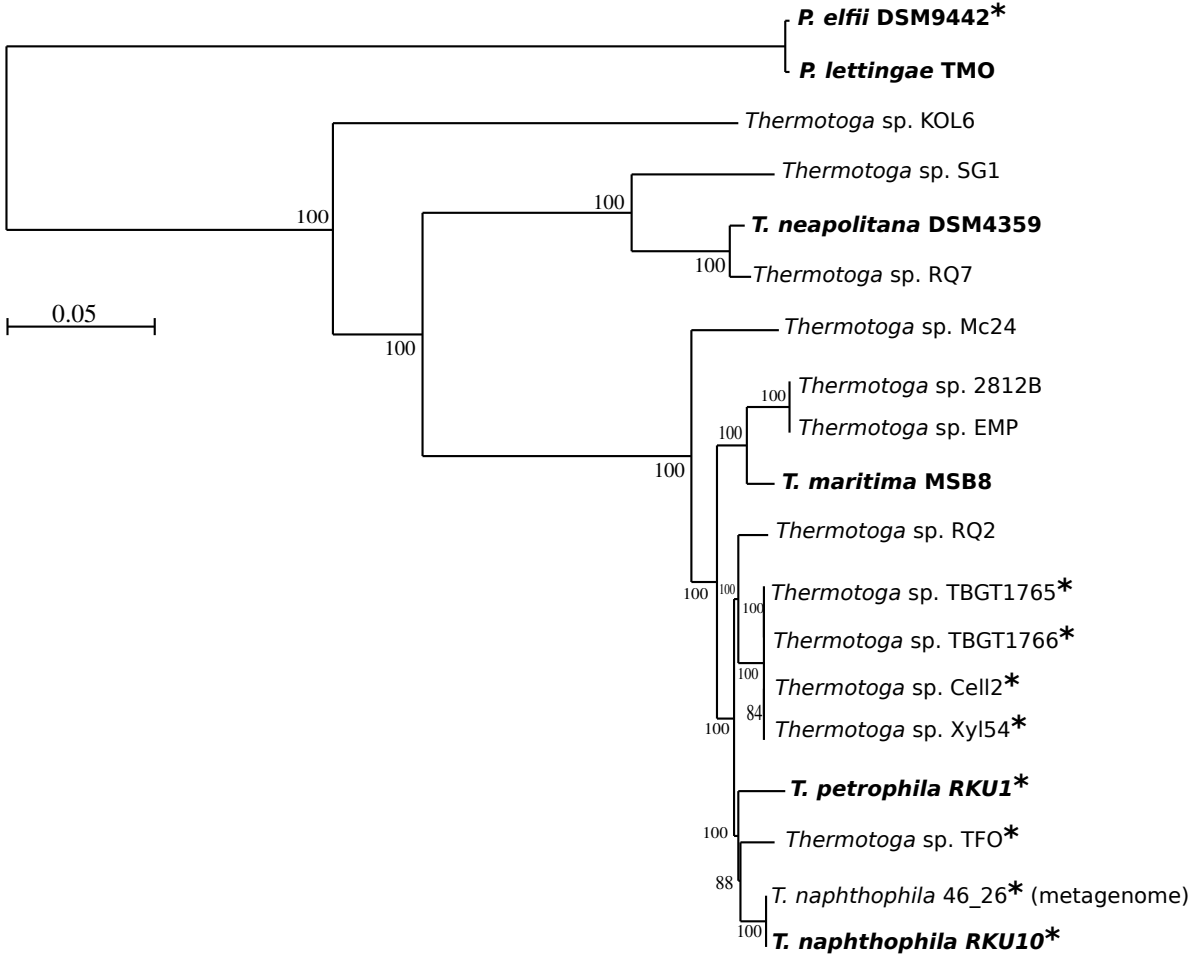
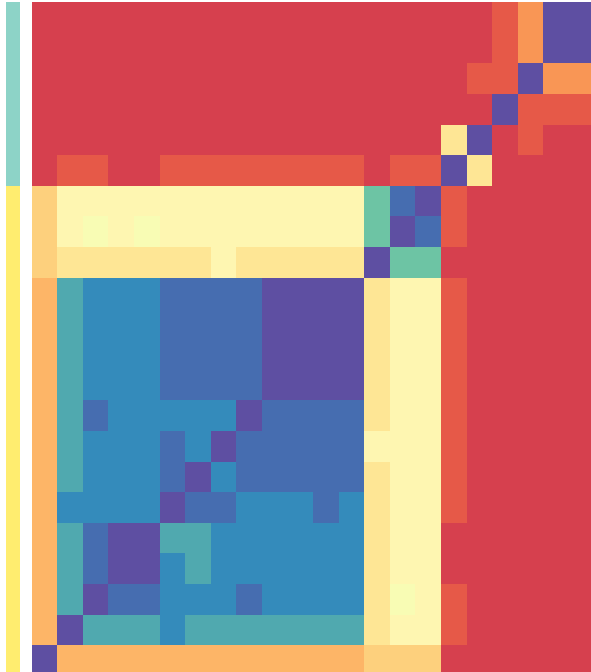
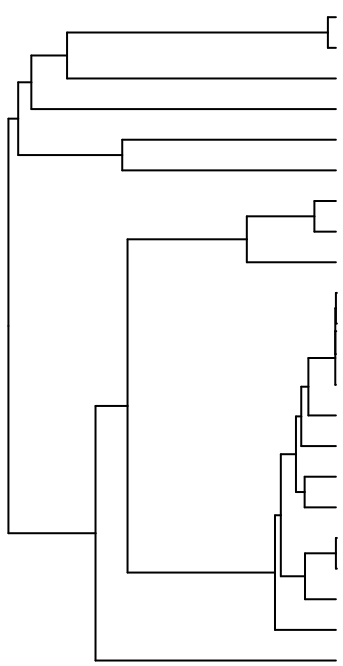
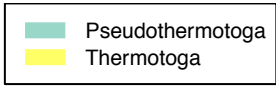
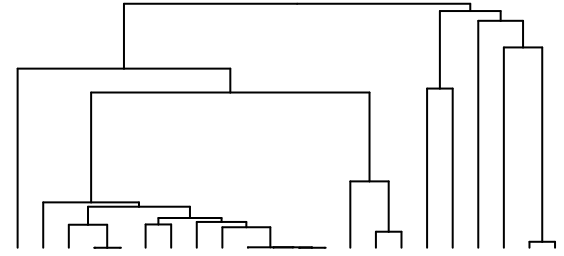
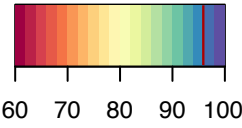


Figure 4 **Color Key**



- P. elfii* DSM 9442
- P. lettingae* TMO
- P. profunda* AZM34c06
- P. thermarum* DSM 5069
- P. hypogea* DSM 11164
- T. caldifonctis* AZM44c09
- Thermotoga* sp. RQ7
- T. neapolitana* NS-E
- Thermotoga* SG1
- Thermotoga* sp. TBGT1765
- Thermotoga* sp. TBGT1766
- Thermotoga* sp. Xyl54
- Thermotoga* sp. Cell2
- Thermotoga* sp. RQ2
- Thermotoga* sp. TFO**
- T. naphthophila* RKU-10
- T. petrophila* RKU-1
- Thermotoga* sp. EMP
- Thermotoga* sp. 2812B
- T. martima* MSB8
- Thermotoga* sp. Mc24
- Thermotoga* sp. TKOL6

- Thermotoga* sp. TKOL6
- Thermotoga* sp. Mc24
- T. martima* MSB8
- Thermotoga* sp. 2812B
- Thermotoga* sp. EMP
- T. petrophila* RKU-1
- T. naphthophila* RKU-10
- Thermotoga* sp. TFO**
- Thermotoga* sp. RQ2
- Thermotoga* sp. Cell2
- Thermotoga* sp. Xyl54
- Thermotoga* sp. TBGT1766
- Thermotoga* sp. TBGT1765
- Thermotoga* SG1
- T. neapolitana* NS-E
- Thermotoga* sp. RQ7
- T. caldifonctis* AZM44c09
- P. hypogea* DSM 11164
- P. thermarum* DSM 5069
- P. profunda* AZM34c06
- P. lettingae* TMO
- P. elfii* DSM 9442

Figure5

Thermotoga sp. TFO

Thermotoga maritima

Thermotoga petrophila

54

55

37

1583

131

56

Thermotoga neapolitana

Thermotoga naphthophila

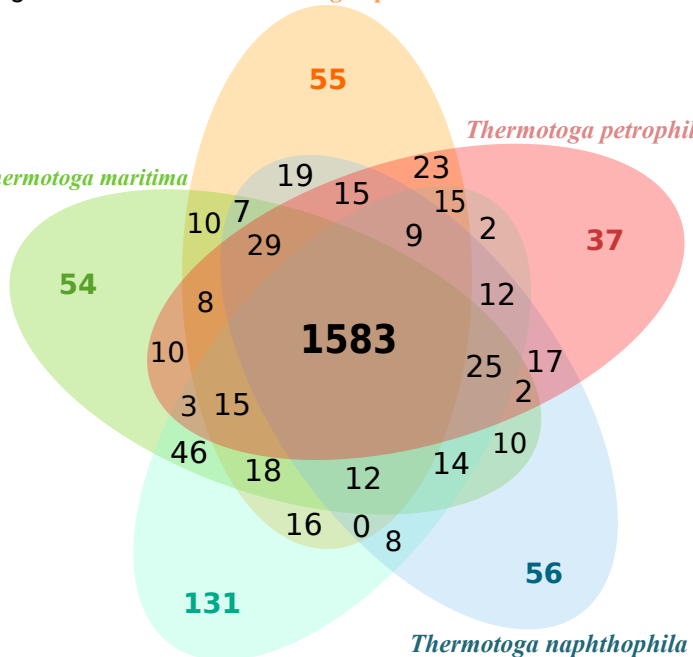
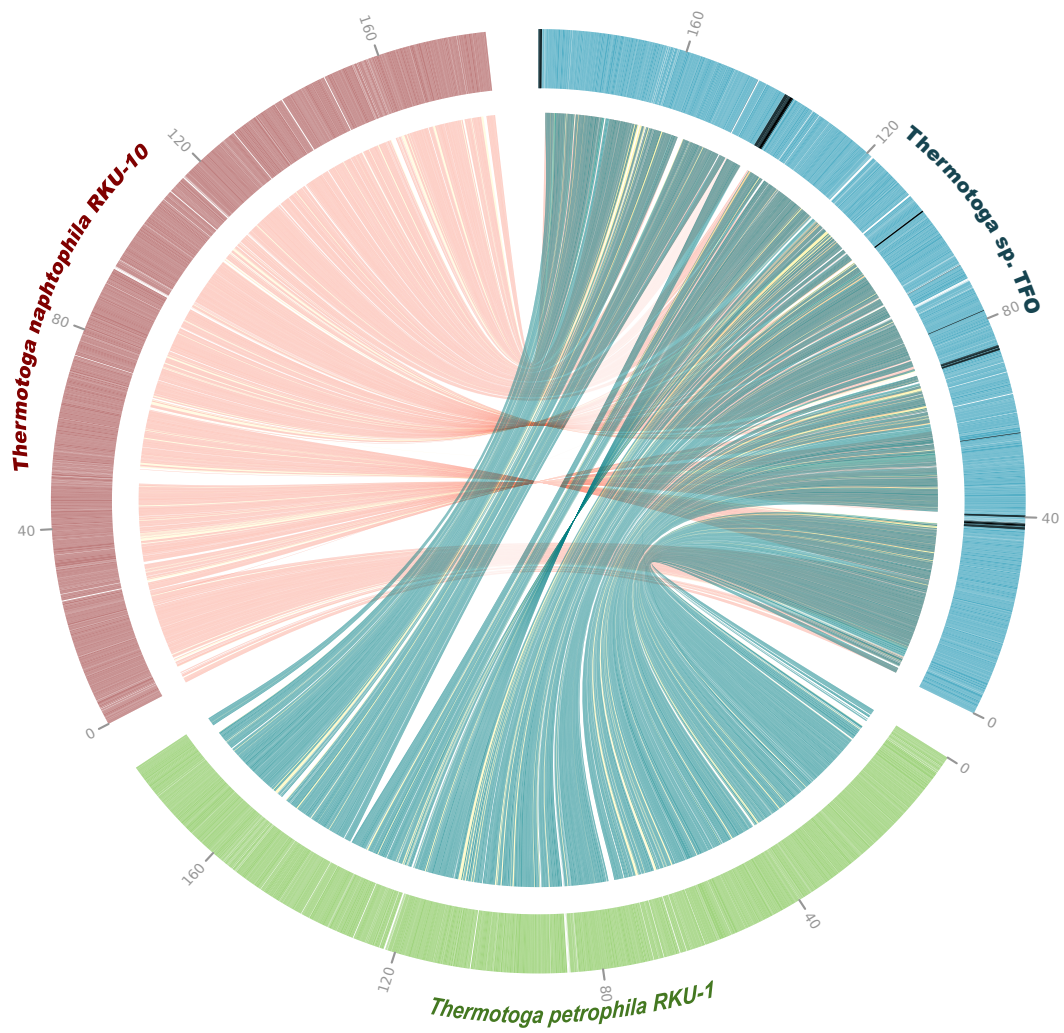


Figure6





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