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# **Thermospira aquatica gen. nov., sp. nov., a novel thermophilic spirochete isolated from a Tunisian hot spring, and description of the novel family**

## **Thermospiraceae**

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1 ***Thermospira aquatica* gen. nov., sp. nov., a novel thermophilic spirochete**  
2 **isolated from a Tunisian hot spring, and description of the novel family**  
3 ***Thermospiraceae*.**

4  
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7  
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17 Running title: ***Thermospira aquatica***

18 New taxa – Spirochaetota.

19 The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of F1F22<sup>T</sup> is  
20 KT767982 and the one for the whole genome sequence is CP073355.

21

22 The type strain is F1F22<sup>T</sup> deposit in DSMZ (= DSM 101182<sup>T</sup>) and in JCM (= JCM 31314<sup>T</sup>).

23

24 **Abstract**

25 **A novel thermophilic, anaerobic bacterium, strain F1F22<sup>T</sup>, was isolated from a hot spring**  
26 **water located north of Tunisia. The cells were non-motile, Gram-negative, and helical**  
27 **with hooked ends, 0.5 x 10-32 μm in size. Growth of the strain was observed at 45-70°C**  
28 **(optimum 55°C), in 0.0 - 1.0% (w/v) NaCl (optimum without NaCl) and at pH 6.5 - 8.5**  
29 **(optimum pH 7.5). Yeast extract was required for growth, and the strain grew on glucose,**  
30 **sucrose, and maltose. The major fatty acids for the strain were C<sub>16:0</sub> (40.2%), iso-C<sub>16:0</sub>**  
31 **(30.2%) and C<sub>16:0</sub> DMA (14.5%). The genome consists of a circular chromosome (2.5 Mb)**  
32 **containing 2672 predicted protein-encoding genes with a G+C content of 43.15 mol%.**  
33 **Based on a comparative 16S rRNA gene sequence analysis, strain F1F22<sup>T</sup> formed a deeply**  
34 **branching lineage within the phylum *Spirochaetota*, class *Spirochaetia*, order**  
35 ***Brevinematales*, and had only low sequence similarity with other species of the phylum**  
36 **(lower than 83%). Genome-based analysis of average nucleotide identity and digital**  
37 **DNA–DNA hybridization of strain F1F22<sup>T</sup> with *Treponema caldarium*<sup>T</sup>, *Brevinema***  
38 ***andersonii*<sup>T</sup>, and *Spirochaeta thermophila*<sup>T</sup> showed respectively values between 63.26%**  
39 **and 63.52%, and between 20% and 25%. Hence, we propose strain F1F22<sup>T</sup> as a novel**  
40 **family (*Thermospiraceae* fam. nov.), genus and species of *Brevinematales* : *Thermospira***  
41 ***aquatica* gen. nov., sp. nov. (Type strain F1F22<sup>T</sup> =JCM 31314<sup>T</sup> =DSM 101182<sup>T</sup>).**

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46 Members of the phylum *Spirochaetota* are mainly helical, gram-negative bacteria, with free-  
47 living or host-associated lifestyles, some of which are highly prevalent disease-causing agents  
48 [1,2]. They are widespread, either pathogenic in the human body or animals, or saprophytic in  
49 diverse natural environments, including extreme ones like the deep-sea [3,4].

50 In 2013, a large taxonomic rearrangement of the phylum *Spirochaetota* was proposed. The  
51 families *Brachyspiraceae*, *Brevinemataceae* and *Leptospiraceae* were transferred respectively  
52 to the novel orders *Brachyspiriales*, *Brevinematales* and *Leptospiriales* all three belonging to  
53 the class *Spirochaetia* and the genera *Borrelia* and *Cristispira* were transferred to the novel  
54 family *Borreliaceae* within the order *Spirochaetales* [5]. The phylum *Spirochaetota* still  
55 comprises a single class *Spirochaetia*, but currently contains four orders (*Brachyspiriales*,  
56 *Brevinematales*, *Leptospiriales* and *Spirochaetales*) and seven families (*Brachyspiraceae*,  
57 *Brevinemataceae*, *Borreliaceae*, *Leptospiraceae*, *Treponemataceae*, *Sphaerochaetaceae* and  
58 *Spirochaetaceae*) [6].

59 Today, the phylum *Spirochaetota* counts 20 genera with various lifestyle and phenotypic or  
60 genomic characteristics [2,7]. Previously, the presence of periplasmic endoflagella was  
61 considered the only reliable phenotypic feature characteristic of this phylum [5]. However  
62 today, even this feature is not a strict criterion as novel species have been described which do  
63 not possess an endoflagellum such as the motile species *Rectinema subterraneum* [8] or partly  
64 non-motile species within the genus *Sphaerochaeta* [6] or even the non-motile species  
65 *Rectinema cohabitans* [9]. Therefore, the main characteristics of each family belonging to this  
66 phylum have been detailed in [5, 6].

67 Only a few thermophilic species belonging to the phylum *Spirochaetota* have been isolated  
68 from various aquatic springs, including not fully characterized strains from thermal springs  
69 [10]. Within the order *Spirochaetales*, only two thermophiles have been previously  
70 characterized: family *Spirochaetaceae*, with *Spirochaeta thermophila* isolated from a marine

71 hot spring [11], and family *Treponemataceae*, with *Treponema caldarium* initially proposed as  
72 *Spirochaeta caldaria*, isolated from a freshwater hot spring [12]. A third thermophilic species  
73 has been then described as a new genus but without an assigned family, *Exilispira thermophila*,  
74 an anaerobic bacterium isolated from a deep-sea hydrothermal vent chimney [13]. This isolate  
75 grew up to 60°C with an optimum of 50°C. Moreover, from the two known species of the genus  
76 *Rectinema*, both described as mesophilic and isolated respectively in 2017 from a naphthalene-  
77 degrading enrichment culture [9] and in 2020 from a deep terrestrial subsurface [8], only the  
78 second ones can grow up to 50°C. Recently in 2020, two not sporulating thermophilic strains  
79 (optimum 50-55°C), representative of a novel phylogenetically distinct group of thermophilic  
80 spirochetes belonging to the order of *Brevinematales* was described with a proposed family  
81 “*Longinemaceae*” and the species name “*Longinema margulisiae*” [14] but with unvalidly  
82 published names of genus and family and with the whole genome sequence which remains  
83 currently unavailable (accession number CP053071).

84 In this study, we have successfully isolated an anaerobic, thermophilic spirochete, designated  
85 strain F1F22<sup>T</sup>, from the water of a Tunisian hot spring. We describe the detailed morphological,  
86 physiological, and genomic characteristics of strain F1F22<sup>T</sup> and propose a novel species of a  
87 new genus for this strain.

88 Thermal water has been collected in a hot spring (36° 48' 59" North, 10° 34' 07" East) in  
89 Korbous city, north Tunisia. The temperature, pH, and salinity of  
90 the fluid *in situ* was 60 °C, 7.8 and 8 g/l NaCl respectively. Strain F1F22<sup>T</sup> was isolated using  
91 enrichment techniques described by Mechichi *et al.* [15]. A basal medium used for enrichment,  
92 isolation, and cultivation was prepared using the anaerobic techniques [16-18]. The basal  
93 medium contained (per liter of distilled water): KH<sub>2</sub>PO<sub>4</sub>, 0.20 g; NH<sub>4</sub>Cl, 0.30 g; NaCl, 1 g;  
94 KCl, 0.50 g; MgCl<sub>2</sub> · 6·H<sub>2</sub>O, 0.40 g; cysteine · HCl, 0.50 g; CaCl<sub>2</sub> · 2·H<sub>2</sub>O, 0.15 g; yeast  
95 extract, 0.50 g (Difco); trace-element solution [19], 1 ml; and 1 ml of 0.1% (w/v) resazurin. The

96 pH was adjusted to 7.0 with 10 M KOH solution, then the medium was boiled under a stream  
97 of O<sub>2</sub>-free N<sub>2</sub> gas and cooled at room temperature; 5 ml aliquots were dispensed into Hungate  
98 tubes under N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) and subsequently sterilized by autoclaving at 121°C for 20  
99 min. For enrichment purposes, prior to use, 0.2 ml of 10% (w/v) NaHCO<sub>3</sub>, 0.2 ml of 2% (w/v)  
100 Na<sub>2</sub>S · 9·H<sub>2</sub>O and 0.1 ml glucose (1M) were injected from sterile stock solutions into pre-  
101 sterilized basal medium [15] and incubated at 50°C. For isolation purposes, positive enrichment  
102 cultures were serially diluted into Hungate tubes containing 5 ml of culture medium [16-19]  
103 and incubated at 50°C. The culture grown at the higher dilution was used as an inoculum for a  
104 novel dilution series. The process was repeated several times until isolates were deemed axenic.  
105 Purity was checked by microscopic examination of cultures grown in the basal medium. The  
106 last dilution culture showing only one cell morphology was considered for further studies.

107 Enrichment cultures incubated for three days showed substantial bacterial growth. Several  
108 isolates were obtained after three successive serially diluted sub-cultures, and single isolated  
109 colonies were selected from different enrichments obtained from water samples of the hot  
110 spring. Three were inventoried and finally named F1F22, F1F22p, and F1F22t. Strain F1F22  
111 was chosen for further studies.

112 Physiological optimal growth conditions were determined in duplicate experiments conducted  
113 in basal medium where glucose (20 mM) was added, except when substrates utilization were  
114 determined. For testing pH on growth, the culture medium was adjusted to the desired pH using  
115 anaerobically prepared stock solutions of NaHCO<sub>3</sub> (10 %) or Na<sub>2</sub>CO<sub>3</sub> (2 %). The temperature  
116 range for growth was determined using the culture medium adjusted to the optimum growth  
117 pH. For studies of NaCl requirements, NaCl was weighed directly into the tubes at  
118 concentrations ranging from 0 to 40 g l<sup>-1</sup> before dispensing basal medium without NaCl,  
119 followed by autoclaving. The tubes were incubated at 55 °C. Growth was measured by inserting

120 tubes directly into a model Cary 50 Scan spectrophotometer (Varian) and measuring the OD580  
121 (nm).

122 Substrate-utilization tests were carried out at 55 °C and an initial pH of 7.5 using the basal  
123 medium without glucose, as described by Ben Ali Gam *et al.* [20]. Substrates utilization was  
124 determined using a chromatograph equipped with a UV Detector (HP 1100, Hewlett Packard,  
125 Les-Ulis, France). Separation of metabolites was achieved using C<sub>18</sub>-Symmetry (4.6x100 mm,  
126 3.5 µm particle size) column (Waters Chromatography, Saint-Quentin-en-Yvelines, France).  
127 The tested substrates were added at a final concentration of 20 mM unless indicated : casamino  
128 acids (0.1%), tryptone (0.1%), peptone (0.1%), crotonate, H<sub>2</sub>/CO<sub>2</sub> (80/20 v/v; ~100 kPa in the  
129 headspace) plus acetate (1 mM), betaine (10 mM), arabinose, fructose, galactose, xylose,  
130 cellobiose, lactate, formate plus acetate (1 mM), acetate, propionate, butyrate, malate, fumarate,  
131 succinate, pyruvate, benzoate (5 mM), ethanol (10 mM), trimethylamine (10 mM), glycerol (5  
132 mM). In addition, the following inorganic substances were tested as electron acceptors in the  
133 presence of 0.5 g/l yeast extract: nitrate (10 mM), nitrite (2 mM), sulphate, sulphite (2 mM),  
134 thiosulphate (20 mM), and elemental sulphur (1 % w/v).

135 Genomic DNA was extracted using a phenol-chloroform-isoamyl alcohol method as previously  
136 described by Quéméneur *et al.* [21]. 16S rRNA genes were amplified by using primers 27F (5'-  
137 AGAGTTTGATCCTGGCTCAG-3') and 1531R (5'-AAGGAGGTGATCCAGCC-3'), then  
138 sequenced by GATC-Biotech (Konstanz, Germany). The 16S rRNA sequence was edited in  
139 BioEdit [22], a sequence alignment Editor software, and was manually corrected for errors. The  
140 16S rRNA gene sequences were aligned with closest relative sequences from the NCBI nr  
141 database using the MUSCLE tool [23] implemented in MEGA7 package [24]. Pairwise  
142 evolutionary distances based on 1306 unambiguous nucleotides were computed by the Jukes  
143 and Cantor [25] method. Evolutionary analyses were conducted from 24 nucleotide sequences

144 using three different methods: maximum-likelihood [26], maximum-parsimony, [27]  
145 and neighbour-joining [28].

146 Whole-genome sequencing was performed at the GeT-PlaGe platform (INRAe Toulouse,  
147 France) by combining long reads (Oxford Nanopore) and short reads (Illumina MiSeq)  
148 technologies, as described by Quéméneur *et al.* [21]. Genome assembly was performed with  
149 Unicycler v0.4.6 [29]. The genome sequence was deposited in the NCBI database under the  
150 accession number CP073355. Genome annotation was carried out online using bioinformatic  
151 tools available on three web platforms: MaGe (Microscope Genome Annotation) [30],  
152 Integrated Microbial Genomes [31], and Galaxy protologger [32]. Value of orthologous average  
153 nucleotide identity (OrthoANI) and Genome-to-Genome Distance was calculated with  
154 Orthologous Average Nucleotide Identity Tool (OAT) software  
155 (<https://www.ezbiocloud.net/tools/orthoani>) [33, 34].

156 The fatty acid composition of the strain F1F22<sup>T</sup> was determined at the Identification Service of  
157 DSMZ [35]. The cellular fatty acid analysis was performed on a culture grown in basal medium  
158 containing 0.5 g/l (w/v) yeast extract and stopped in the exponential growth phase. Fatty acids  
159 were extracted using Miller's method [36] with modifications of Kuykendall *et al.* [37]. Then  
160 the cellular fatty acids profile was analysed using the MIDI Microbial Identification system  
161 (e.g. Method: TSBA40).

162 Cells F1F22<sup>T</sup> from active cultures observed under phase-contrast microscopy never showed any  
163 motility. F1F22<sup>T</sup> cells were helical revealed the presence of hooked ends and spherical bodies  
164 (Fig. 1a) while negatively stained cells showed an helicoidal cell shape and a protoplasmic  
165 cylinder (Fig. 1b), the whole being typical of *Spirochaetota* morphology. The F1F22<sup>T</sup> cells  
166 stained Gram-reaction negative and electron microscopy revealed a Gram negative-type cell



167 wall structure (Fig. 1c). Electron microscopy revealed also that the strain possessed a  
168 periplasmic space, but periplasmic flagella could not be observed in any case.

169 Strain F1F22<sup>T</sup> grew between 45 and 70 °C with optimal growth at 55 °C. The pH range for  
170 growth of the strain was pH 6.5–8.5, with an optimum at pH 7.5. The range of NaCl  
171 concentration for growth was 0–1 % (w/v), with the highest growth rate in the culture medium  
172 without NaCl. No growth was observed in a yeast extract-free medium. The strain was strictly  
173 anaerobic; growth was completely inhibited in the presence of trace quantities of oxygen (0.1 %  
174 v/v O<sub>2</sub>). Strain F1F22<sup>T</sup> used glucose, sucrose, and lactose with 0.5 g/l yeast extract in the culture  
175 medium. However, the following substrates did not support growth: Casamino acids, tryptone,  
176 peptone, crotonate, H<sub>2</sub>/CO<sub>2</sub> (80:20) plus acetate, betaine, arabinose, fructose, galactose, xylose,  
177 cellobiose, lactate, formate plus acetate, acetate, propionate, butyrate, malate, fumarate,  
178 succinate, pyruvate, benzoate, ethanol, trimethylamine, glycerol. In addition, the following  
179 inorganic substances were not reduced in the presence of 0.5 g/l yeast extract: nitrate, nitrite,  
180 sulphate, sulphite, thiosulphate and elemental sulphur (Table 1).

181 The DNA G+C content of F1F22<sup>T</sup> is 43.15 mol%. Fatty acid methyl ester analysis (Table 2)  
182 showed that the strain F1F22<sup>T</sup> contained C<sub>16:0</sub> (40.2%), iso-C<sub>16:0</sub> (30.2%) and C<sub>16:0</sub> DMA  
183 (14.5%) as the major fatty acids. Minor fatty acids upper than 1% were C<sub>16:1</sub> DMA (4.2%),  
184 C<sub>16:0</sub> 3-OH (3.7%), C<sub>18:0</sub> (1.8%), anteiso-C<sub>17:0</sub> (1.7%) and C<sub>14:0</sub> (1.2%).

185 The species *Brevinema andersonii*<sup>T</sup> affiliated to the family *Brevinemataceae*, order  
186 *Brevinematales*, class *Spirochaetia*, phylum *Spirochaetota* [38] was identified as the closest  
187 type species of strain F1F22<sup>T</sup> with 83% 16S rRNA gene sequence similarity (Fig. 2). The  
188 phylogenetic analysis based on its 16S rRNA gene sequence (Fig. 2 and Fig. S1) and the whole-  
189 proteome-based phylogeny (Fig. S2) show that strain F1F22<sup>T</sup> should be affiliated to a new  
190 genus and novel family belonging to the order *Brevinematales*, class *Spirochaetia*, phylum

191 *Spirochaetota*. Moreover, POCP values (obtained from Protologger) was 15.64% between  
192 strain F1F22<sup>T</sup> and *Spirochaeta thermophila* GCF\_000184345.1, and 13.76% with *Spirochaeta*  
193 *africana* GCF\_000242595.2 and 9.61% with *Brevinema\_andersonii* GCF\_900112165.1, though  
194 far below the threshold of 50% delineating genera [39]. The proposed name for the new genus  
195 is *Thermospira* and for the novel family is *Thermospiraceae* that are both described below.

196 The complete genome (one single contig) of strain F1F22<sup>T</sup> consists of a circular chromosome  
197 of 2.515.453 bp, with 43.15 % G+C content (Table 3). A circular view of the genome is shown  
198 in Fig. S3. The size of the strain F1F22<sup>T</sup> genome is larger than that of *Brevinema andersonii*<sup>T</sup>  
199 (ATCC 43811), 1.501.400 bp, smaller than that of *Treponema caldarium*<sup>T</sup> (DSM 7334),  
200 3.239.340 bp, and in the range of *Spirochaeta thermophila*<sup>T</sup> (DSM 6578), 2.560.222 bp,  
201 genome. The G+C content of F1F22<sup>T</sup> genome is close the one of *Treponema caldarium*<sup>T</sup>, but  
202 higher than that of *Brevinema andersonii*<sup>T</sup> and lower than that of *Spirochaeta thermophila*<sup>T</sup>  
203 (Table 3). The ANI and dDDH values between F1F22<sup>T</sup> and *Treponema caldarium*<sup>T</sup>, *Brevinema*  
204 *andersonii*<sup>T</sup> and *Spirochaeta thermophila*<sup>T</sup> were respectively between 63.26% and 63.52% and  
205 between 20% and 25% (Table 4), i.e., far below the species boundaries of 95~96 and 70 %,  
206 respectively, recommended by Chun *et al.* [40].

207 The genome contains two copies of a 16S-23S-5S rRNA operon, 46 tRNA genes 2672 protein-  
208 coding genes (CDS) (Table 3). A group of genes encoding a bacterial flagellar machinery and  
209 chemotaxis was identified, consistent with the typical spirochete morphological characteristics.  
210 In agreement with the culture-based conclusions on the metabolism, analysis of the potential  
211 metabolic pathways showed that strain F1F22<sup>T</sup> metabolism is based on sugars utilization.  
212 Searches against the carbohydrates-active enzymes (CAZYmes) database [41] identified 26  
213 CAZymes including 6 alpha-amylases genes encoding both cytoplasmic and secreted extra-  
214 cellular enzymes. This abundance and diversity of alpha-amylases suggest that the F1F22<sup>T</sup>

215 strain is mostly uses on starch or similar polysaccharides (glycogen, dextrin). Besides, genes  
216 for converting of pyruvate to lactate (lactate dehydrogenase) or acetate (phosphate acetyl  
217 transferase and acetate kinase) were found, representing main fermentation pathways in F1F22<sup>T</sup>  
218 strain grown on sugars. Genes encoding typical membrane electron transporter (cytochromes,  
219 quinones...) involved in aerobic or anaerobic respirations were not detected, while genes  
220 encoding the complete Embden-Meyerhof pathway (glycolysis) are present in F1F22<sup>T</sup> genome.  
221 The presence of genes for gluconeogenesis, and the pathways for glycogen and trehalose  
222 biosynthesis and degradation, suggest that strain F1F22<sup>T</sup> utilizes these compounds either for  
223 energy storage or as osmoprotectants or a protection against desiccation. A partial pentose-  
224 phosphate pathway is also detected. The tricarboxylic acids cycle (TCA) pathway is atypical,  
225 resembling *Helicobacter pylori* TCA but missing the genes for the acetoacetate CoA-  
226 transferase, the fumarate reductase, and the malate dehydrogenase [42]. Like in *Helicobacter*  
227 *pylori*, F1F22<sup>T</sup> possess an operon encoding a 2-oxo-glutarate oxidoreductase (OOR; EC  
228 1.2.7.3), instead of the classical  $\alpha$ -ketoglutarate dehydrogenase [43]. This OOR uses ferredoxin  
229 (Fd) as electron acceptor cofactor, instead of NAD(P) for the dehydrogenase. We hypothesize  
230 that the oxidative branch of this TCA pathway is crucial in energy conservation in F1F22<sup>T</sup>  
231 strain. Indeed, in anaerobic bacteria, ferredoxin, which has a very low redox potential (-420 to  
232 -500 mV) and thus is a very potent reductant for electron transport chains, can be utilized to  
233 power several processes, including hydrogen evolution and a transmembrane ion gradient [44].  
234 The F1F22<sup>T</sup> genome encodes a RnF complex, a respiratory enzyme that catalyzes the oxidation  
235 of reduced ferredoxin to the reduction of NAD<sup>+</sup>, and the negative free energy change of this  
236 reaction is used to generate a transmembrane ion gradient (either Na<sup>+</sup> or H<sup>+</sup>). Moreover, an  
237 operon for a cytoplasmic [FeFe] hydrogenase (*hndACD*) was found. Hnd is a flavin-based  
238 electron-bifurcating (FBEB) hydrogenase, coupling an exergonic redox reaction to an  
239 endergonic one allowing energy conservation in anaerobic microorganisms. Hnd hydrogenase  
240 catalyze the simultaneous exergonic oxidation of ferredoxin (Fd) and the endergonic oxidation

241 of NADH to produce H<sub>2</sub> (electron confurcation) [45] or the reverse reaction in which the  
242 exergonic reduction of NAD<sup>+</sup> is coupled to the endergonic reduction of the Fd in the presence  
243 of dihydrogen (electron bifurcation). The genome of strain NST lacked. The genome of strain  
244 F1F22T does not encode typical F1F0-type ATP synthase that uses transmembrane proton  
245 gradient for ATP production; only genes for a V-type ATPase were found. V-ATPases are  
246 typically used in anaerobic bacteria to generate a transmembrane ion gradient at the expense of  
247 ATP. However, it has been demonstrated that the reverse reaction, c.a., synthesis of ATP by V-  
248 type ATPase is possible, although energetically less favorable, depending on the  
249 electrochemical proton gradient ( $\Delta\mu\text{H}$ ) [46]. Therefore, we assume strain F1F22T uses the  
250 proton-motive force generated by the RnF complex to produce ATP by the V-Type ATPase,  
251 contributing to energy conservation in addition to the substrate-level phosphorylation for ATP  
252 synthesis.

253 As a strict anaerobic bacterium, the strain F1F22<sup>T</sup> genome also encodes a protection system to  
254 oxidative stress: a superoxide reductase an enzyme that catalyzes the conversion of highly  
255 reactive and toxic superoxide (O<sub>2</sub><sup>-</sup>) into less toxic hydrogen peroxide which in turn is reduced  
256 to water by rubrerythrin.

257 The morphology of strain F1F22<sup>T</sup> cells is typical of spirochete species. For example, strain  
258 F1F22<sup>T</sup> cells had an outer envelope, a protoplasmic cylinder, and hook-shaped ends. Similar  
259 cell structures have also been found for members of the genus *Leptospira* [47,48] and  
260 *Brevinema* [38], both exclusively mesophilic genera. Moreover, strain F1F22<sup>T</sup> cells showed the  
261 formation of spherical bodies in late-exponential phase cultures, a characteristic observed in  
262 members of three different genera *Exilispira*, *Salinispira* and *Treponema*, where two of them  
263 *Exilispira* and *Treponema* included thermophilic species [13, 49, 50, 51]. Contrarily to its  
264 closest type species *Brevinema andersonii* (strain CT11616<sup>T</sup>), possessing two wave-shaped cell  
265 structures of irregular length [38], strain F1F22<sup>T</sup> harbors more than two wave-shaped cell

266 structures of regular length. Additionally, *Brevinema andersonii* type strain was mesophilic and  
267 pathogenic for humans, contrary to strain F1F22<sup>T</sup>. However, contrasting with most spirochetes,  
268 cells of strain F1F22<sup>T</sup> are always non-motile and never exhibit any flexing, rotational or  
269 translational movements or periplasmic flagella.

270 Strain F1F22<sup>T</sup> was isolated from a water sample of a Tunisian hot spring and we showed it is  
271 thermophilic like only three species among the phylum *Spirochaetota*: *Spirochaeta thermophila*  
272 (strain Z-1203<sup>T</sup>), *Treponema caldarium* (strain H1<sup>T</sup>), and *Exilispira thermophila* (strain  
273 RASEN<sup>T</sup>). All strains are non-pathogenic for humans and phylogenetically distant to strain  
274 F1F22<sup>T</sup> (< 79%). These three thermophilic strains were described as helical, highly motile, and  
275 with an outer membrane enclosing a periplasmic flagellum, at the difference of strain F1F22<sup>T</sup>.

276 Moreover, various physiological features of strain F1F22<sup>T</sup> differ from those of other members  
277 of the phylum *Spirochaetota*, including substrate utilization, optimum growth temperature, and  
278 NaCl growth requirement (Table 1). Regarding the lifestyle of strain F1F22<sup>T</sup>, we consider that  
279 the strain is derived from a free-living microbial population in the freshwater of a hot spring.  
280 The water sample for isolation contained no animal individuals and no specific physical  
281 association with animals. Strain F1F22<sup>T</sup> also differs from the closest relatives in many  
282 physiological features: first, the range of substrate utilized by strain F1F22<sup>T</sup> for growth is  
283 limited to glucose and maltose with a slight growth when sucrose was utilized. In contrast,  
284 *Exilispira thermophila* (strain RASEN<sup>T</sup>) grew only with yeast extract while *Treponema*  
285 *caldarium* (strain H1<sup>T</sup>) and *Spirochaeta thermophila* (strain Z-1203<sup>T</sup>) utilized a larger range of  
286 sugar including glucose, sucrose, arabinose, xylose and cellobiose (although substrate  
287 utilization patterns have not been determined for the species belonging to the genus *Brevinema*).  
288 In addition, the range of temperature of growth exhibited by strain F1F22<sup>T</sup> is rare amongst  
289 members of the phylum *Spirochaetota*, starting not lower than 45°C and up to 70°C. Indeed,  
290 both strains (RASEN<sup>T</sup> and H1<sup>T</sup>) grow only up to 60°C (not 70°C) but also tolerate lower

291 temperatures than strain F1F22<sup>T</sup> as they can grow at 25°C and 37°C, respectively. Only strain  
292 Z-1203<sup>T</sup> can grow up to 73°C, but with an optimum higher than strain F1F22<sup>T</sup>, at 66-68°C.  
293 Moreover, inside the phylum *Spirochaetota*, the NaCl requirement of *Thermospira aquatica*  
294 (strain F1F22<sup>T</sup>) differs from the three other validly named type species of thermophilic species  
295 (strains RASEN<sup>T</sup>, Z-1203<sup>T</sup> and H1<sup>T</sup>). NaCl is not required for strain F1F22<sup>T</sup>, and this is shared  
296 partly by *Exilispira thermophila* strain RASEN<sup>T</sup> but only when it grows under optimal  
297 conditions. Contrarily, the other two thermophilic species (strains H1<sup>T</sup> and Z-1203<sup>T</sup>), required  
298 less than 1.5% and 2.5-3% of NaCl respectively.

299 Regarding the G+C% content, strain F1F22<sup>T</sup> (43.15% mol) differs markedly from the  
300 thermophilic species *Exilispira thermophila* strain RASEN<sup>T</sup> (27%) and *Spirochaeta*  
301 *thermophila* strain H1<sup>T</sup> (60.92%), but it was closer to the other thermophilic *Treponema*  
302 *caldarium* strain Z-1203<sup>T</sup> (45.58%).

303 In addition to the physiological and biochemical characteristics, the phylogenetic analysis  
304 concluded that strain F1F22<sup>T</sup> is distinct from other members of the phylum *Spirochaetota*.  
305 Indeed, the 16S rRNA gene sequence-based phylogenetic analysis showed that strain F1F22<sup>T</sup> is  
306 only distantly related to other members of the phylum *Spirochaetota* (Fig. 2) and should  
307 represent a new genus of a novel family. This assumption is further supported by genome-based  
308 analyses (genomic indexes and phylogenomic trees) (Fig. 2 and Fig. S2).

309 Based on its overall phylogenetic, genomic, morphologic, physiologic, and metabolic features,  
310 distinct from any previously described species of the phylum *Spirochaetota*, we propose strain  
311 F1F22<sup>T</sup> represents the first species of a novel family, namely *Thermospiraceae* of a new genus,  
312 namely *Thermospira aquatica* gen. nov. sp. nov.

313

314 **Description of *Thermospiraceae* fam. nov.**

315 *Thermospiraceae* (Ther.mo.spi'ra.ce'ae N.L. fem. n. *Thermospira* type genus of the family; L.  
316 suff. –aceae ending to denote a family; N.L. fem. pl. n. *Thermospiraceae* the family of the genus  
317 *Thermospira*).

318 The family is defined by phylogenetic analysis based on 16S rRNA gene sequences obtained  
319 currently from not sporulating thermophilic cultured strains, which come from hot  
320 environments (spring water and deep biosphere).

321 The type genus is *Thermospira*.

322

323 **Description of *Thermospira* gen. nov.**

324 *Thermospira* (Ther.mo.spi'ra. Gr. masc. adj. thermos, hot; L. fem. n. *spira* coil, spire; N.L. fem.  
325 n. *Thermospira*, a hot spiral)

326 Gram-stain negative. Strictly anaerobic and thermophilic organisms. Cells are helical with a  
327 cell structure composed of an outer membrane without periplasmic flagella.  
328 Chemoorganoheterotrophic. Sugars are the preferred growth substrates. The G+C content of  
329 the genomic DNA is 43.15 mol%. Its phylogenetic position is in a deeply branched lineage of  
330 the phylum *Spirochaetota*. The type species is *Thermospira aquatica* and the genus description  
331 is based on the type species, currently the only validly published name of species ascribed to  
332 this genus.

333 **Description of *Thermospira aquatica* sp. nov.**

334 *Thermospira aquatica* (a.qua'ti.ca. L. fem. adj. *aquatica* aquatic).

335 Displays the following characteristics in addition to those listed in the genus description. Strain  
336 F1F22<sup>T</sup> cells are helical and 0.5×10–32 μm in size, often with hooked ends. Cells are non-  
337 motile. Growth occurs between 45 and 70 °C, with optimum growth at 55 °C. The pH range for  
338 strain growth was estimated at pH 6.5–8.5, with optimum growth at pH 7.5. The range of NaCl  
339 concentration for growth was 0–1% (w/v) but the optimum growth was without NaCl added in  
340 the culture medium. Uses glucose, maltose, and sucrose. Yeast extract is necessary for growth.  
341 Growth was not observed on casamino acids, tryptone, peptone, crotonate, H<sub>2</sub>/CO<sub>2</sub> (80:20) plus  
342 acetate, betaine, arabinose, fructose, galactose, xylose, cellobiose, lactate, formate plus acetate,  
343 acetate, propionate, butyrate, malate, fumarate, succinate, pyruvate, benzoate, ethanol,  
344 trimethylamine and glycerol. The major cellular fatty acids were C<sub>16:0</sub>, iso-C<sub>16:0</sub> and C<sub>16:0</sub>  
345 DMA; minor fatty acids were C<sub>16:1</sub> DMA, C<sub>16:0</sub> 3-OH, C<sub>18:0</sub>, anteiso-C<sub>17:0</sub> and C<sub>14:0</sub>.

346 The type strain is strain F1F22<sup>T</sup> (=JCM 31314<sup>T</sup> =DSM 101182<sup>T</sup>), isolated from hot spring  
347 water in the North of Tunisia.

348 The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of F1F22<sup>T</sup> is  
349 KT767982 and the one for the whole genome sequence is CP073355.

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### 355 **Conflicts of interest**

356 The authors declare that there are no conflicts of interest.

357



358 **References**

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494

495

496 **Table 1.** Comparison of the morphological and physiological properties of strain F1F22<sup>T</sup> and  
 497 other thermophilic spirochete species. Taxa: 1, F1F22<sup>T</sup> (data from this study); 2, *Exilispira*  
 498 *thermophila* [13]; 3, *Treponema caldarium* [12,49]; 4, *Spirochaeta thermophila* [11]; +,  
 499 Positive; -, Negative; NR, not reported.

Characteristics	1	2	3	4
Isolation source	Hot spring	Deep sea	Hot spring	Thermal site
Cell morphology	Helical	Helical	Helical	Helical
Cell size (µm)	0.5x10-32	0.23-0.28x15-27	0.2-0.3x15-45	0.2-0.25x16-50
Temperature Range (°C)	45-70	37-60	25-65	40-73
Optimum Temperature (°C)	55	50	48-52	66-68
pH range	6.5-8.5	6-7.5	5.8-8.5	5.9-7.7
Optimum pH	7.7	7.0	7.2-7.5	7.5
NaCl range (%)	0-1	2.5-3.5	0-0.4	0.5-4.5
Optimum NaCl (%)	0	2.5-3	NR	1.5
Substrate utilization (with 0.5 g/l yeast extract)				
Glucose	+	-	+	+
Sucrose	+	-	+	+
Arabinose	-	-	+	+
Fructose	-	-	+	NR
Galactose	-	-	+	NR
Xylose	-	-	+	+
Maltose	+	-	+	NR
Cellobiose	-	-	+	+
Butyrate	-	-	NR	-
Nitrate	-	NR	NR	-
Inorganic substance reduction (with 0.5 g/l yeast extract)				
Sulphate	-	-	NR	-
Sulphite	-	-	NR	-
Thiosulphate	-	-	NR	-
Elemental sulfur	-	-	NR	-
DNA G+C content (mol %)	43.15	27	45.58	60.92

500

501

502

503 **Table 2.** Comparison of the cellular fatty acid methyl ester content of strain F1F22<sup>T</sup> and  
 504 *Exilispira thermophila* strain RASEN<sup>T</sup> (Method TSBA40; Calcul method ANAER6). Strains:  
 505 1, F1F22<sup>T</sup> (data from this study); 2, *E. thermophila* [13]; 3, *B. andersonii* [38]; Values are  
 506 percentage (w/v) of total fatty acids; c, cis; NR, not reported.

Fatty acids	Percentage (w/v) of total fatty acids		
	1	2	3
C14:0	1.2	2	47.5
C15:0	0.4	1	2
C16:0	40.2	29	20.7
iso-C16:0	30.2	NR	NR
C16:0 DMA	14.5	NR	NR
C16:0 3-OH	3.7	NR	NR
C16:1 DMA	4.2	7	NR
iso-C17:0	0.3	3	NR
anteiso-C17:0	1.7	NR	NR
C18:0	1.8	14	5.3
iso-C18:0	0.5	2	NR
C18:1 ω9c	0.2	22	8.2

507  
 508  
 509 **Table 3.** Genomic features of strain F1F22<sup>T</sup> and the closest genomes of type species:  
 510 *Treponema caldarium*<sup>T</sup> DSM 7334 (CP002868.1); *Brevinema andersonii*<sup>T</sup> ATCC 43811  
 511 (FOKY00000000) and *Spirochaeta thermophila*<sup>T</sup> DSM 6578 (CP002903.1).

Characteristics	F1F22 <sup>T</sup>	<i>Treponema caldarium</i> <sup>T</sup> (DSM 7334)	<i>Brevinema andersonii</i> <sup>T</sup> (ATCC 43811)	<i>Spirochaeta thermophila</i> <sup>T</sup> (DSM 6578)
Size (base pair)	2.515.453	3.239.340	1.501.400	2.560.222
G+C content	43.15	45.58	35.24	60.92
Contig nb	1	1	39	1
Gene	2.678	2.928	1581	2.350
Protein-coding genes (CDS)	2.672	2.869	1442	2.296
rRNA genes (16S, 23S, 5S)	6 (2, 2, 2)	9 (3, 3, 3)	2 (1, 1, 0)	6 (2, 2, 2)
tRNA	46	48	48	46

512  
 513



514 **Table 4.** OrthoANI (lower left) and Genome-to-Genome Distance Calculator (GGDC) (upper  
515 right) values between the genome of strain F1F22<sup>T</sup> (CP073355, this study) and those of  
516 *Treponema caldarium*<sup>T</sup> DSM 7334 (CP002868.1); *Brevinema andersonii*<sup>T</sup> ATCC 43811  
517 (FOKY00000000) and *Spirochaeta thermophila*<sup>T</sup> DSM 6578 (CP002903.1). Orthologous  
518 Average Nucleotide Identity Tool (OAT) software (<https://www.ezbiocloud.net/tools/orthoani>)  
519 was used for calculations.

OrthoANI \ GGDC	Strain F1F22 <sup>T</sup>	<i>Treponema caldarium</i> <sup>T</sup>	<i>Brevinema andersonii</i> <sup>T</sup>	<i>Spirochaeta thermophila</i> <sup>T</sup>
Strain F1F22 <sup>T</sup>	\	0.25	0.20	0.24
<i>Treponema caldarium</i> <sup>T</sup>	63.36	\	0.22	0.19
<i>Brevinema andersonii</i> <sup>T</sup>	63.52	62.11	\	0.26
<i>Spirochaeta thermophila</i> <sup>T</sup>	63.26	64.03	61.51	\

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## 522 Legends of figures

523 **Fig. 1:** Micrographs of strain F1F22<sup>T</sup>. (a) Phase-contrast micrographs of cells with arrows  
524 indicating hooked ends (HE) and spherical bodies (SB). Bar, 10 μm. (b) Transmission electron  
525 micrograph of a negatively stained cell without any periplasmic flagella shows wave-shaped  
526 cell structures of regular length and the central axis. Bar, 200 nm. (c) Electron microscopy:  
527 cross-section of cells, showing the outer membrane typical of Gram-negative but no periplasmic  
528 flagella. Bar, 100 nm.

529 **Fig. 2.** Phylogenetic dendrogram based on 1193 unambiguous bp of 16S rRNA gene sequence  
530 data, showing the position of the new genus *Thermospira aquatica* F1F22<sup>T</sup> including the  
531 different families and orders of the class *Spirochaetia*. Bootstrap values (expressed as  
532 percentages of 2000 replications) greater than 40% are shown at branch points. Constructed

533 from the Maximum Likelihood method based on the Tamura-Nei model. The tree with the  
534 highest log likelihood (-16807.01) is shown. Initial tree(s) for the heuristic search were obtained  
535 automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise  
536 distances estimated using the Maximum Composite Likelihood (MCL) approach, and then  
537 selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch  
538 lengths measured in the number of substitutions per site. The analysis involved 59 nucleotide  
539 sequences. All positions containing gaps and missing data were eliminated. There were a total  
540 of 1087 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. the  
541 numbers in brackets are Genbank/EMBL/DDBJ accession numbers for the 16S rRNA gene  
542 sequences. Bar, 5% estimated sequence divergence.