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1 ***Thermococcus henrietii* sp. nov., a novel extreme thermophilic and piezophilic sulfur-**
2 **reducing archaeon isolated from a deep-sea hydrothermal chimney (EPR 9°N)**

3

4 Karine Alain,^{1,2} Erwann Vince,^{1,2} Damien Courtine,^{1,2,3} Lois Maignien,^{1,2} Xiang Zeng,^{4,2}
5 Zongze Shao^{4,2} & Mohamed Jebbar^{1,2}

6

7 ¹Univ Brest, CNRS, Ifremer, Laboratoire de Microbiologie des Environnements Extrêmes LM2E,
8 IUEM, Rue Dumont d'Urville, F-29280 Plouzané, France

9 ²IRP 1211 MicrobSea, Sino-French Laboratory of Deep-Sea Microbiology, LM2E (Plouzané, France)-
10 KLAMBR (Xiamen, China)

11 ³³Aix Marseille Univ, CNRS, INSERM, CIML, Turing Centre for Living Systems, Marseille, France –
12 Current address

13 ⁴Key Laboratory of Marine Genetic Resources, Third Institute of Oceanography, Ministry of Natural
14 Resources, Xiamen 361005, China

15

16 **Correspondence** Karine Alain Karine.Alain@univ-brest.fr

17 **Subject:** NEW TAXA Archaea

18 **Running title:** *Thermococcus henrietii* sp. nov.

19 **Abbreviations:** AlaAT, alanine aminotransferase; ANI, Average Nucleotide Identity; CAPSO, *N*-
20 cyclohexyl-3-aminopropanesulfonic acid; dDDH Digital DNA-DNA Hybridization; DMSO,
21 Dimethylsulfoxide; EPR, East Pacific Rise; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic
22 acid; GlkA, ADP-dependent glucokinase; HOMOPIPES, Homopiperazine-1,4-*bis*(2-ethanesulfonic
23 acid); MaGe, MicroScope Microbial Genome Annotation and Analysis Platform; MES, 2-(*N*-
24 Morpholino)EthaneSulfonic acid; PIPES, Piperazine-*N,N'*-*bis*(2-ethanesulfonic acid); UBOCC
25 University of Brest (UBO) Culture Collection.

26 The DDBJ/[GenBank/ENA] accession number for the genome sequence of *Thermococcus henrietii*
27 EXT12c^T sp. nov. is LT900021. The complete 16S rRNA gene sequence of *T. henrietii* sp. nov. strain
28 Ext12c^T is available at GenBank/EMBL/DDBJ/PIR under accession no. MT731286.

29

30 Two supplementary figures and one supplementary table are available with the online version of this
31 paper.

32

33 **Abstract:** A novel extreme thermophilic and piezophilic chemoorganoheterotrophic archaeon,
34 strain EXT12c^T, was isolated from a hydrothermal chimney sample collected at a depth of
35 2,496 m at the East Pacific Rise 9°N. Cells were strictly anaerobic, motile cocci. The strain
36 grew at NaCl concentrations ranging from 1 to 5% (w/v) (optimum 2.0%), from pH 6.0 to 7.5
37 (optimum 6.5-7.0), at temperatures between 60 and 95 °C (optimum 80-85 °C) and at pressures
38 from 0.1 to at least 50 MPa (optimum: 30 MPa). Strain EXT12c^T grew
39 chemoorganoheterotrophically on complex proteinaceous substrates. Its growth was highly
40 stimulated by the presence of elemental sulfur or L-cystine which were reduced to hydrogen
41 sulfide. The DNA G+C content was 54.58 mol%. Phylogenetic analyses based on 16S rRNA
42 gene sequences and concatenated ribosomal protein sequences showed that strain EXT12c^T
43 falls into the genus *Thermococcus*, and is most closely related to *Thermococcus nautili* strain
44 30-1^T. Overall genome relatedness index analyses (average nucleotide identity scores and *in*
45 *silico* DNA-DNA hybridizations) showed a sufficient genomic distance between the new
46 genome and the ones of the *Thermococcus* type strains, for the delineation of a new species.
47 On the basis of genotypic and phenotypic data, strain EXT12c^T is considered to represent a
48 novel species, for which the name *Thermococcus henrietii* sp. nov. is proposed, with the type
49 strain EXT12c^T (=UBOCC-M-2417^T =DSM 111004^T).

50

51 Archaea of the order *Thermococcales* are common inhabitants and key players of the hot areas
52 of deep-sea hydrothermal vents (Godfroy *et al.*, in press). They also populate various high-

53 temperature environments around the world ranging from hot terrestrial hot springs, to marine
54 subseafloor sediments and then to subterranean oil reservoirs, and so on (Godfroy *et al.*, in
55 press). Nevertheless, the vast majority of the *Thermococcales* strains isolated so far, which are
56 formerly described, originate from marine hydrothermal vents. At the time of writing, the order
57 *Thermococcales* (phylum *Euryarchaeota*) encompasses 3 genera: *Pyrococcus* (Fiala & Stetter,
58 1986) (6 validly published species + 2 not validly published ones), *Thermococcus* (Zillig *et al.*,
59 1983) (33 validly published species + 3 not validly published ones), and *Palaeococcus* (Takai
60 *et al.*, 2000) (3 species). With 33 formerly described species, the *Thermococcus* genus is the
61 most represented in public collections of microorganisms, and it is also the one that has been
62 the subject of the largest number of functional studies. *Thermococcus* species are mainly
63 obligate chemoorganoheterotrophs, growing in organic-rich peptide media, and very preferably
64 in the presence of inorganic sulfur compounds. Some species are able to grow by
65 carboxydrotrophy (i.e. Sokolova *et al.*, 2004; Vannier *et al.*, 2011; Kozhevnikova *et al.*, 2016),
66 few others have been isolated from oligotrophic or methanogenic-targeting culture media
67 (Byrne *et al.*, 2009; Dalmaso *et al.*, 2016), and others have been suggested to convey
68 exoelectrogenic abilities (Pillot *et al.*, 2018). These archaea often dominate enrichment cultures
69 grown anaerobically at 75-90°C, on rich media, with deep-sea hydrothermal vent samples.
70 They are therefore ubiquitous and metabolically versatile actors that are very important in the
71 high temperature zones of hydrothermal ecosystems. In this article, we describe a novel
72 extreme thermophilic organoheterotrophic sulfur-reducer, strain EXT12c^T, isolated from a

73 hydrothermal sulfide sample from the East-Pacific Rise. Genomic and phenotypic studies
74 indicate that it represents a novel species of the genus *Thermococcus*.

75 An outside part of a deep-sea sulfide chimney sample was collected at a depth of 2,496 m from
76 a hydrothermal vent at the East Pacific Rise 9°N (9°50'40.2''N, 104°17'37.798''W) in October
77 2001, during the EXTREME oceanographic cruise. Onboard, the sample was preserved
78 anaerobically in a sealed sterile vial and stored at 4 °C. Once in the lab, enrichment cultures
79 followed were performed at 85°C, in reduced TRM medium (pH 6.8), containing 5 g.L⁻¹
80 elemental sulfur, as described elsewhere (Zeng *et al.*, 2009). A collection of pure strains was
81 isolated by repeated streaking on plates (solidified with 8 g.L⁻¹ Gelrite gellan gum), and then
82 deposited in the UBOCC collection (<https://ent.univ-brest.fr/lm2e/home/#/>), at -80 °C with 5%
83 (v/v) DMSO. The strain EXT12c^T described here bears the accession number UBOCC-M-
84 2417^T. The purity of this isolate was confirmed routinely by microscopic examination, and by
85 sequencing of its genome.

86

87 The genome of strain EXT12c^T was sequenced using the Illumina MiSeq technology (2 × 300
88 bp paired-reads, V3 chemistry), as detailed elsewhere (Courtine *et al.*, 2017). It was analyzed
89 and annotated with the MicroScope Microbial Genome Annotation and Analysis Platform
90 (MaGe) (<https://mage.genoscope.cns.fr/microscope/home/index.php>) using KEGG and BioCyc
91 database (Vallenet *et al.*, 2020). It consists of a single chromosome of 2,155,760 base pairs in
92 size (with an average coverage of 350×) and has a G+C content of 54.58 mol%. The genome

93 consists of 2,375 encoding protein sequences, 46 tRNA genes, a single 16S-23S rRNA operon,
94 2 5S rRNA, and 18 miscellaneous RNA genes. This genome is available in
95 DDBJ/ENA/GenBank under the accession number LT900021.

96 Pairwise 16S rRNA gene sequence similarity was determined using the EzTaxon-e server
97 (<http://eztaxon-e.ezbiocloud.net/>; (Kim *et al.*, 2012)). 16S rRNA gene sequences were aligned
98 using MAFFT v7.427 (parameters -maxiterate 1000 -localpair) (Katoh and Stanley, 2013), and
99 the alignment was trimmed with BMGE v1.12 (default parameters) (Criscuolo and Gribaldo,
100 2010). Then, PhyML v. 3.3.20190909 was used to build the tree thanks to the web-server
101 <http://www.atgc-montpellier.fr/phyml/> (Guindon *et al.*, 2010). The evolutionary model was
102 selected with the SMS algorithm (Lefort *et al.*, 2017) and the branch support was computed
103 with the aLRT SH-like method. The tree was visualized with iTOL (Letunic and Bork, 2019)
104 and rooted between the *Thermococcus* and *Pyrococcus* genera. Since the genes encoding the
105 16S rRNA gene sequences are highly conserved and therefore not very discriminating between
106 *Thermococcales*, a phylogenetic tree based on ribosomal proteins was also calculated. This
107 phylogenomic tree was based on the concatenation of 49 ribosomal proteins shared by all ge-
108 nomes in the tree (Proteins associated with the large ribosomal subunit: L1, L2, L3, L4, L6,
109 L7AE, L11, P1 (=L12P), L13, L15, L15E, L18, L18A, L18E, L19E, L21E, L22, L24, L29, L30,
110 L30E, L31E, L32E, L37AE, L37E, L39E, L40E, L44E; Proteins associated with the small ri-
111 bosomal subunit
112 : S2, S3, S3AE, S4, S4E, S5, S6E, S7, S8, S8E, S9, S10, S12, S13, S15, S17, S17E, S19, S19E,

113 S27E, S28E). Each protein was aligned and trimmed separately, MAFFT (parameters -
114 globalpair -maxiterate 1000) and BMGE (default parameters), respectively. Then, each align-
115 ment block was concatenated into a single alignment, and was submitted to PhyML to build the
116 tree. The evolutionary model was selected with SMS and the branch support was computed
117 with the aLTR SH-like method. The tree was visualized with iTOL and rooted between the
118 *Thermococcus* and *Pyrococcus* branches. Average Nucleotide Identity scores (ANI)
119 (OrthoANIu values) were calculated using the ANI calculator tool provided by the EzBioCloud
120 web server (<https://www.ezbiocloud.net/tools/ani>) on genomes of strain EXT12c^T with those of
121 all type strains of the genus *Thermococcus* with 16S rRNA gene sequences with more than
122 98.7% identity to that of strain EXT12c^T (Table S1 and Table1) (Yoon *et al.*, 2017). *In silico*
123 DNA-DNA hybridization (DDH) estimate values were also determined using the genome-to-
124 genome distance calculator (GGDC 2.1), using the formula 2 (Meier-Kolthoff *et al.*, 2013)
125 (Table 1, Table S1).

126 Based on 16S rRNA gene and concatenated ribosomal protein phylogenetic analyses, the novel
127 isolate was affiliated with the order *Thermococcales*, in the archaeal domain, falling into the
128 genus *Thermococcus* (Fig. 1 and Supplementary Fig. 1). Strain EXT12c^T shared the highest
129 16S rRNA gene sequence similarity of 99.9% with *Thermococcus nautili* 30-1^T, followed by *T.*
130 *gammatolerans* EJ3^T with 99.7%. It also shared more than 98.7% 16S rRNA gene sequence
131 similarity with the sequences of 14 other type species of this genus (Table S1). The OrthoANIu
132 values between the genome of strain EXT12c^T and the genome of its 10 closest relatives were

133 all below 91.5%, which is far below the standard ANI criterion for species identity (95–96%)
134 (Richter & Rossello-Mora, 2009) (Table 1, Table S1). The digital DDH (dDDH) scores
135 between the genome of strain EXT12c^T and the ones of its 10 closest relatives whose genomes
136 are sequenced, were all between 20.30% and 43.60%, so well below the threshold level (70%)
137 for a new species delineation (Wayne *et al.*, 1987) (Table 1, Table S1). Strain EXT12c^T
138 therefore meets currently accepted criteria for describing a new genomic species (Rosselló-
139 Móra and Whitman, 2019).

140

141 Morphological characteristics of strain EXT12c^T were observed by using light microscopy
142 (Olympus BX60 and CX40) and scanning electron microscopy (FEI Quanta 200) (Fig. S2).
143 Cells were irregular motile cocci ($1.60 \pm 0.32 \mu\text{m}$ in diameter, $n= 30$) that occurred generally
144 singly.

145

146 Unless noted otherwise, physiological tests were carried out anaerobically (N₂ headspace) in
147 modified Ravot medium, at 80°C (pH 7.0, atmospheric pressure), in duplicates (+ negative
148 controls), using elemental sulfur as an external electron acceptor, as described elsewhere
149 (Dalmasso *et al.*, 2016). Growth tests were generally carried out as described previously
150 (Dalmasso *et al.*, 2016). Cells were routinely counted by direct cell counting by using a
151 modified Thoma chamber (depth 10 μm), and checked by flow cytometry (CyFlowSpace,
152 Sysmex Partec, GmbH, Görlitz, Germany). Cells were fixed with 2.5% (v/v) glutaraldehyde

153 (Sigma) and stored at -80°C , before counting by the two methods described above.

154 Determination of the temperature range for growth was carried out at 45, 50, 55, 60, 65, 70, 75,
155 80, 85, 90, 95 and 100°C . The isolate was hyperthermophilic and grew between 60 and 95°C
156 with an optimum at $80\text{-}85^{\circ}\text{C}$. Salt tolerance was tested at 80°C with various concentrations of
157 NaCl (0, 1, 2, 3, 4, 5, 6, 7 and 8 %, w/v). Strain EXT12c^T required NaCl for growth and grew
158 at NaCl concentrations between 1 and 5 % (optimum: 2%). The pH range for growth was
159 tested from pH 3.0 to pH 10.0 (initial pH at 20°C) with increments to 1 unit near the limits of
160 the pH range, and with increments of 0.5 unit around the optimum. For this experiment, we
161 used the following buffers (each at 20 mM, Sigma-Aldrich): for pH 3.0, no buffer; for pH 4.0
162 and 5.0, HOMOPIPES buffer; for pH 5.5 and 6.0, MES buffer; for pH 6.5 and 7.0, PIPES
163 buffer; for pH 7.5 and 8.0, HEPES buffer; for pH 9.0, 9.5 and 10.0, CAPSO buffer. Growth of
164 the isolate was observed in a narrow pH range, between pH 6.0 and 7.5, with an optimum
165 around 6.5-7.0. The pressure range for growth of strain EXT12c^T was tested into high-pressure
166 high-temperature reactors (Top Industrie, Vaux-le-Pénil, France), at 0.1, 10, 20, 30, 40 and 50
167 MPa, as described previously (Alain *et al.*, 2002). The novel isolate was piezophilic, growing
168 from atmospheric pressure (0.1 MPa), to at least 50 MPa, and showed optimal growth under 30
169 MPa. Under optimal growth conditions (80°C , pH 6.8, 2% NaCl, $5\text{ g}\cdot\text{L}^{-1}\text{ S}^{\circ}$, and 30 MPa), the
170 doubling time of the novel isolate was 103 minutes.

171 Utilization of various individual substrates for growth was tested in a basal medium
172 supplemented with 0.05 % (w/v) yeast extract (YE) as growth factor, and without this growth

173 factor, as described previously (Dalmasso *et al.*, 2016). The following substrates were tested, at
174 the final concentrations shown in brackets: tryptone (0.5% w/v), peptone (0.5% w/v), yeast
175 extract (0.5% w/v), beef extract (0.5% w/v), casamino acids (0.4% w/v), casein (0.5% w/v),
176 formate (20 mM), acetate (20 mM), pyruvate (20 mM), fumarate (20 mM), propionate (20
177 mM), succinate (20 mM), maltose (20 mM), fructose (20 mM), lactose (20 mM), ribose (20
178 mM), galactose (20 mM), glucose (20 mM), chitin (0.2% w/v) and starch (0.2% w/v).
179 Unsupplemented media were used as negative controls for all conditions. To examine the
180 ability of the strain to grow in the absence of elemental sulfur, cells were cultivated in modified
181 Ravot medium without sulfur. Alternative electron acceptors were also tested in a sulfur-
182 depleted medium, under a gas phase of N₂ (100%, 150 kPa): L-cystine (5 g.L⁻¹), polysulfides
183 (0.5 mM), thiosulfate (20 mM), sulfate (20 mM), sulfite (5 mM), nitrate (20 mM), nitrite (5 mM)
184 and dioxygen (0.5%, 5%, 20% v/v). Growth was monitored over 3 days of incubation. The
185 results were considered positive when growth was still observed after 2 successive subcultures
186 (1/100th subculturing), on the test medium. All tests were performed in duplicates and growth
187 was confirmed after microscopic observation. Hydrogen sulfide production was followed as
188 described previously (Cord-Ruwisch, 1985). Cations and anions produced from peptone and
189 yeast extract fermentation were identified by ionic chromatography on a Dionex ICS-900 Ion
190 Chromatography System (Dionex, Camberley UK) coupled with a CERS 500 4 mm suppressor
191 and a DS5 conductivity detector (40°C) and fitted with a RFC-10 Reagent-Free Controller™,
192 an ASDV autosampler, and an IonPac CS16 column maintained at 60°C in a UltiMate™ 3000

193 Thermostated Column Compartment (Thermo Scientific, Waltham, MA, USA).

194

195 Significant growth was observed under strict anaerobic conditions, on complex proteinaceous
196 substrates such as yeast extract, peptone, tryptone and beef extract as carbon and energy
197 sources, in the presence of elemental sulfur. Cations and anions produced by the fermentation
198 of peptone and yeast extract included formate, acetate, propionate, isobutyrate, succinate/ or
199 malate, isovalerate, ammonia and hydrogen sulfide. The strain was also able to grow on these
200 substrates by fermentation in the absence of sulfur-containing compounds, but in that case,
201 growth was very poor. Indeed, growth was very clearly stimulated by L-cystine and elemental
202 sulfur, that were both reduced to hydrogen sulfide. Sulfate, thiosulfate, sulfite, polysulfides,
203 nitrate, nitrite and oxygen (aerobic to microaerophilic conditions) were not used by the cells as
204 terminal electron acceptors. In the presence of elemental sulfur, slow and weak growth was
205 also observed on the few following substrates: casamino acids, casein and pyruvate (with 0.05%
206 (v/v) YE). No growth was observed in the presence of the other substrates tested. Growth by
207 carboxydutrophy was not tested as the gene encoding the carbon monoxide dehydrogenase
208 CooF, one central protein in the *Thermococcales*'s carbon monoxide metabolism, was absent
209 from the genome.

210 Metabolic predictions from the genome confirmed these *in vitro* growth results and
211 demonstrated that the strain has the genetic potential to degrade peptides and amino acids.
212 Indeed, its genome codes for peptidases/proteases and for the two key enzymes of amino acid

213 catabolism (the alanine aminotransferase (AlaAT; locus tag TEXT12C_1080) and the
214 glutamate dehydrogenase (GDH; TEXT12C_0879). In addition, it contains 6 complete
215 pathways of amino acid degradation (alanine, arginine, aspartate, asparagine, glutamine,
216 glycine) according to MicroCyc. Concerning the catabolism of sugars, this strain's genome
217 contains the complete Embden-Meyerhof-Parnas pathway (including genes of the ADP-
218 dependent glucokinase GlkA (locus tag TEXT12C_1056), an ADP-specific phosphofructo-
219 kinase (TEXT12C_2105) and a glyceraldehyde-3-phosphate:ferredoxin oxidoreductase
220 (TEXT12C_0008)), typically found within this archaeal order, and contains complete pathways
221 for D-mannose and glycerol degradation. While the genome of strain Ext12c^T has genes coding
222 for several enzymes of the chitin degradation pathway (namely, chitinases TEXT12C_2122-
223 2123; diacetylchitobiose deacetylase TEXT12C_0385; exo-beta-D-glucosaminidase
224 TEXT12C_0375; GlkA; glucosamine-6-phosphate deaminase TEXT12C_0376), we have not
225 demonstrated this property in our experimental conditions. Furthermore, we could not find any
226 alpha-amylase sequence in the genome, which could explain the fact that we showed that the
227 strain does not degrade starch. Moreover, the genome also encodes different types of
228 hydrogenases involved in energy conservation classically found in *Thermococcales*, such as
229 the membrane-bound hydrogenase Mbh (TEXT12C_0230-0246), the sulfidogenic hydrogenase
230 Mbs (TEXT12C_0689-0694) and the formate hydrogenlyase (TEXT12C_1011-1027).

231 In summary, strain EXT12c^T is a strict anaerobic archaeon growing
232 chemoorganoheterotrophically on complex proteinaceous substrates and which requires the

233 presence of elemental sulfur or L-cystine to produce a high biomass in culture, like the vast
234 majority of *Thermococcales*.

235 Antibiotic resistance of the strain was tested, in duplicates, in the presence of a variety of
236 antibiotics (chloramphenicol, kanamycin, rifampicin, and tetracycline), provided at four
237 concentrations (10, 25, 50 and 100 µg/mL). When the antibiotic was diluted in ethanol
238 (chloramphenicol) or DMSO (rifampicin), the same volume of solvent was added to control
239 cultures. Strain EXT12c^T was sensitive to rifampicin at 50 µg/mL and resistant to the other 3
240 antibiotics, at the concentrations tested.

241

242 The phylogenetic, phenotypic and physiological data shown in this article support the view that
243 strain EXT12c^T should be assigned to the genus *Thermococcus*. However, there are few
244 phenotypic differences, detailed in Table 1, between the novel isolate and its closely related
245 species. Strain EXT12c^T is distinguishable from *T. nautili* by a lower optimal growth
246 temperature and an ability to grow weakly by fermentation, in the absence of sulfur. It is able
247 to grow weakly on pyruvate, while *T. gammatolerans* and *T. eurythermalis* are not. Therefore,
248 from the genotypic and physiological evidence, we proposed to assign strain EXT12c^T to the
249 type strain of a novel species, for which the name *Thermococcus henrietii* sp. nov. is proposed.

250

251 **Description of *Thermococcus henrietii* sp. nov.**

252 *Thermococcus henrietii* (hen.ri.e'ti.i N.L. gen. n. *henrietii* of Henriet, in honour of Professor
253 Jean-Pierre Henriet, in recognition of his important contribution to the exploration of the sea-
254 bed).

255 Cells are irregular motile cocci (Diameter: $1.60 \pm 0.32 \mu\text{m}$). Obligately anaerobic. Growth is
256 observed at temperatures between 60 and 95 °C (optimum 80-85 °C), at NaCl concentration
257 from 1 to 5% (optimum 2 %) and at pH from 6 to 7.5 (optimum 6.5-7.0). Piezophilic growing
258 optimally under 30 MPa. S^o or L-cystine are not essential for growth but definitely stimulates
259 growth. Does not use sulfate, thiosulfate, sulfite, polysulfides, nitrate, nitrite or oxygen (0.5%,
260 5%, 20% v/v) as electron acceptors. Chemoorganoheterotrophic growth occurs on complex
261 proteinaceous substrates (yeast extract, peptone, tryptone, beef extract). Poor growth on
262 casamino acids, casein and pyruvate.

263 The type strain EXT12c^T (=UBOCC-M-2417^T =DSM 111004^T) was isolated from a deep-sea
264 sulfide sample collected at a depth of 2,496 m from a hydrothermal chimney at the East Pacific
265 Rise 9°N. The genomic DNA G+C content of the type strain is 54.58 mol%.

266 The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and draft genome
267 sequences are MT731286 and LT900021, respectively.

268

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275

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280

281 **Conflict of interest**

282 We declare no conflict of interest and no source of indirect financial support.

283

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370

371 **Figure and Tables legends**

372

373 **Fig. 1.** PhyML phylogenomic tree showing the phylogenetic positions of strain EXT12c^T and
374 representatives of some other related taxa, based on 40 concatenated ribosomal protein
375 sequences. *Pyrococcus* species were used as an outgroup. Branch supports, computed with the
376 aLTR SH-like method, are shown at branch nodes. Bar, 0.01 nucleotide substitution rate (K_{nuc})
377 units.

378

379 **Table 1.** Characteristics differentiating strain EXT12c^T from closest species of the genus
380 *Thermococcus*.

381 Strains: 1, EXT12c^T (data from this study); 2, *T. nautili* 30-1^T (Gorlas *et al.*, 2014); 3, *T.*
382 *gammatolerans* EJ3^T (Jolivet *et al.*, 2003); 4, *T. guaymasensis* DSM 1113^T (Canganella *et al.*,
383 1998); 5, *T. eurythermalis* A501^T (Zhao *et al.*, 2015). Characteristics are scored as: +, positive;
384 –, negative; v, variable; S, Stimulatory; R, required. §, data from the literature; *, data obtained
385 for the five strains under same experimental laboratory conditions.