Proposal of *Helicobacter higonensis* sp. nov. isolated from a human clinical specimen, and emended description of *Helicobacter valdiviensis* collado, 2014.

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ABSTRACT

We have previously isolated a gram-negative microaerophilic strain, PAGU 2000^{T} from a patient presenting with a fever in Kumamoto Prefecture, Japan. The present study aimed to comprehensively analyze the taxonomy of the isolated strain using a polyphasic approach. The 16S rRNA gene sequence analysis indicated that the strain was a member of enterohepatic *Helicobacter*. The strain PAGU2000^{T} shared a 97.5% 16S rRNA gene nucleotide identity with *Helicobacter valdiviensis*, and this taxonomic position was confirmed by phylogenetic analysis of the GyrA amino acid sequences. The proposed strain PAGU2000^{T} has a 1.482 Mbp chromosome with a DNA G+C content of 31.3 mol% and encodes 1520 predicted coding sequences. The average nucleotide identity between the strain PAGU 2000^{T} and type strain of *H. valdiviensis* was 70.3%, which was lower than the recommended threshold of 95% for species delineation. The strain PAGU2000^{T} was a motile,

non-spore-forming, and spiral-shaped bacterium, exhibiting catalase and oxidase activities but not urease and nitrate reduction. This study demonstrates that the isolate represents a novel species within enterohepatic *Helicobacter*, for which the name *Helicobacter higonensis* is proposed (type strain: PAGU 2000^T =GTC 16811^T=LMG 33095^T). In this study, we describe the phenotypic and morphological features of this strain and propose an emended description of some biochemical traits of *H. valdiviensis.*

Key words: genus *Helicobacter*, *Helicobacter higonensis*, *Helicobacter valdiviensis,* taxonomy, phylogenetic analysis, genomic sequence

INTRODUCTION

The genus *Helicobacter* is classified in the family *Helicobacteriaceae* of the order *Campylobacterales*, with *Helicobacter pylori* as the type species (1). Members of the genus *Helicobacter* are typically curved, helical, spiral, or fusiform rod-shaped bacteria, with or without periplasmic fibers (2). *Helicobacter* species are gram-negative, cytochrome oxidase-producing, and non-spore-forming bacteria (1, 2). Currently, 52 *Helicobacter* species have valid names (3).

Over the past 25 years, isolated cases of enterohepatic *Helicobacter* infections in adults and children have been reported (2). The isolates are primarily from blood and, to a lesser

58 extent, from fecal samples (4, 5). Among the enterohepatic *Helicobacter* species that have
59 been isolated from humans, *Helicobacter cinaedi* infection exhibits various clinical
60 manifestations, including fever, gastroenteritis, cellulitis, diarrhea, arthritis, and bacteremia
61 (6). *Helicobacter fennelliae* is associated with human gastroenteritis and bacteremia (7).
62 *Helicobacter canis* is associated with bacteremia and inflammatory bowel disease (8).
63 *Helicobacter pullorum* is associated with several cases of human gastroenteritis (9). Other
64 enterohepatic helicobacters are less frequently isolated and include *Helicobacter canadensis*
65 and *Helicobacter hepaticas* (4, 10).
66 We have successfully isolated a strain of the genus *Helicobacter* from the blood of a patient
67 with a fever, and this isolate was positioned within enterohepatic helicobacters by
68 phylogenetic analysis. Therefore, the present study aimed to determine their taxonomic
69 positions more accurately using a polyphasic approach. We propose that the strain PAGU
70 2000^T constitutes a novel species within the genus *Helicobacter*.
71
72 **MATERIALS AND METHODS**
73 **Bacterial strains and culture conditions**
74 The strain PAGU 2000^T was isolated from a blood culture of a febrile patient (Kumamoto,
75 Japan, 2017). *Helicobacter valdiviensis* LMG 27920^T (=PAGU 2070^T) was directly purchased
76 from the BCCM/LMG Bacteria Collection (Gent, Belgium). These strains were inoculated on

77 trypticase soy agar (TSA; Difco Laboratories, Franklin Lakes, NJ, USA) supplemented with
78 5% defibrinated horse blood at 37 °C for 3 days under microaerobic conditions (6% O_2, 7%
79 CO_2, 7% H_2, and 80% N_2) generated using an ANOXOMAT apparatus (MART II; MART
80 Microbiology, Drachten, Netherlands). Strains were stored at -80 °C in tryptic soy broth
81 supplemented with 15% (v/v) glycerol.
82
83 **Gene sequence determination and phylogenetic analysis**
84 The 16S rRNA genes of the strains PAGU 2000^T and PAGU 2070^T were sequenced and
85 used to determine their positions within the genus *Helicobacter*. Polymerase chain reaction
86 (PCR) amplification targeting the 16S rRNA gene was carried out (11) and the gene sequence
87 of each amplicon determined using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied
88 Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and an automatic DNA sequencer
89 (3130 Genetic Analyzer, Applied Biosystems). To ascertain the phylogenetic positions of the
90 isolates, the 16S rRNA gene sequences of the strains PAGU 2000^T and PAGU 2070^T were
91 compared with sequences of related taxa obtained from the DDBJ (DNA Data Bank of Japan;
92 http:// http://www.ddbj.nig.ac.jp)(12). Sequence alignments were performed using Clustal X
93 software (13), and the phylogenetic tree was constructed using the NJ plot software (14).
94 Phylogenetic analyses of the 23S rRNA (15) and GyrA sequences (16) were also
95 performed using the methods described above. *GyrA* gene sequences were translated into

amino acids to determine their phylogenetic relationships with other strains of *Helicobacter* species.

The genomic sequences of strain PAGU 2000^T was determined using a previously described procedure (17). DNA libraries were prepared using the Nextera XT Library Kit (Illumina Inc., San Diego, CA, USA). We generated 150 bp paired-end reads with greater than 190-fold genomic coverage using the HiSeq system (Illumina). After trimming the sequences based on base quality (quality score limit, 0.05), all reads generated were assembled *de novo* using CLC Genomics Workbench version 11.01 (CLC Bio, Aarhus, Denmark). To determine the relationships between PAGU 2000^T and the type strain of *H. valdiviensis* PAGU 2070^T, we defined their degrees of identity at the whole-genome level using the average nucleotide identity (ANI) analysis method (18). We used the Rapid Annotation Using Subsystem Technology (RAST) server (19) to compare the presence or absence of genes in strains PAGU 2000^T and PAGU 2070^T.

Biochemical characteristics

Gram staining was performed following the method described by Bartholomew and Mittwer (20). The morphological features of the cells were observed using scanning electron microscopy (SEM). The slides were fixed using 5% (v/v) glutaraldehyde in phosphate buffer overnight. The slides were then serially dehydrated with increasing concentration of ethanol

and kept in a desiccator overnight. The slide was spur-coated with platinum before observed by SEM (JEOL, Tokyo, Japan).

Catalase activity was determined by bubble production using 3%(v/v) H_2O_2. Oxidase activity was determined using oxidase test strips (Eiken Chemical, Tokyo, Japan). Growth of the strain PAGU2000^T was evaluated on TSA supplemented with 5% horse blood at 25, 37, and 42 °C under microaerobic conditions, and 37 °C under aerobic or anaerobic conditions. Growth on media containing glycine (1%), NaCl (1.5 %), and susceptibility to nalidixic acid (30 μg/mL) and cephalothin (30 μg/mL) was carried out using previously described methods (21, 22). All media were cultured for up to seven days. The indoxyl acetate hydrolysis test was performed according to the method described by Popovic-Uroic *et al.* (23). Other biochemical characteristics were determined using the API-Campy system, according to the manufacturer's instructions (bioMérieux, Marcy-l'Étoile, France). Using the API-Campy identification system, the following biochemical analyses were performed: urease activity, reduction of nitrates, esterase activity, hydrolysis of hippurate, γ-glutamyltransferase activity, reduction of triphenyl-tetrazolium chloride (TTC), and alkaline phosphatase, pyrrolidonyl, l-arginine, and l-aspartate arylamidase activities. The G+C mol% DNA content was calculated from whole-genome sequence data.

RESULTS

Genetic analysis

The 16S rRNA gene sequencing revealed that the clinical isolate clustered within the genus *Helicobacter* (Figure 1). The sequence was compared with those of species of the genus *Helicobacter* available in GenBank. The 16S rRNA gene sequence of PAGU 2000^T shared the highest identity (97.5%) with that of *Helicobacter valdiviensis* PAGU 2070^T, which was isolated from wild bird fecal samples (24), followed by *Helicobacter pametensis* CCUG 29255^T (96.9%), *Helicobacter brantae* MIT04-9366^T (96.5%), and *Helicobacter pullorum* CIP104787^T (96.5%).

Although 16S rRNA gene sequence analysis has become a standard method for determining prokaryotic phylogeny, studies have shown that it is not suitable for species identification in the genus *Helicobacter* because some species can have as high as 99% similarity (24). Dewhirst *et al.* have suggested that 16S rRNA gene sequence data do not always faithfully reflect phylogenetic relationships and that 23S rRNA gene sequence data are more reliable for the identification and classification of helicobacters because of the 3-fold higher number of informative bases (15, 25, 26). Therefore, we performed 23S rRNA gene sequence analysis, which showed that PAGU 2000^T was most closely related to *H. valdiviensis* (98.2% sequence similarity) (Figure 2). Moreover, the isolated strain exhibited 96.7% and 96.4% sequence similarity with *Helicobacter canadensis* MIT98-5491^T and *H.*

153 *pullorum* CIP104787^T, respectively.

154 In addition, Ménard *et al.* have demonstrated that the *gyrA* gene-encoded protein is a more
155 reliable and stable marker for studying relationships among *Helicobacter* species (16). In the
156 present study, the amino acid sequences of GyrA were compared to those with sequences of
157 related taxa obtained from the DDBJ (Figure 3). Fragments of approximately 700 residues of
158 the GyrA were obtained, which showed 92.2, 91.3, and 90.3% amino acid sequence similarity
159 with *H. valdiviensis*, *H. pullorum*, and *H. canadensis*, respectively. The phylogenetic tree
160 based on the 16S rRNA, 23S rRNA, and GyrA sequences revealed that the isolate PAGU
161 2000^T formed an independent species (Figure 1-3).

162 The whole-genome sequence of strain PAGU 2000^T has been deposited at
163 GenBank/EMBL/DDBJ with accession number DRR511427. The strain PAGU2000^T has a
164 1.482 Mbp chromosome with a DNA G+C content of 31.3 mol% and encodes 1520 predicted
165 coding sequences. We also used the whole-genome in-silico DNA similarity analysis method
166 to determine the exact relationships between PAGU 2000^T and *H. valdiviensis* PAGU2070^T
167 (DRR511428). This analysis, based on full genome sequences, indicated that PAGU 2000^T
168 shared 70.3% ANIb with *H. valdiviensis*. The ANI values between strain PAGU 2000^T and
169 other closely related species, *H. pametensis* ATCC51478^T (GCA_000518225.1), *H. brantae*
170 MIT04-9366^T (GCA_003364205.1) or *H. pullorum* CCUG33837^T (GCA_017979475.1) were
171 66.1%, 65.8% or 69.9%, respectively. RAST analysis comparing the PAGU 2000^T and PAGU

172 2070^T genomes revealed functioning parts present only in PAGU 2000^T (Fig S1 and Table
173 S1).
174
175 **Phenotypic characteristics**
176 We investigated the morphology of the strain PAGU2000^T using SEM. The cells presented
177 slightly curved rods and were 1.4-5.3 μm in length and 0.3–0.5 μm in width. The organism
178 has a single, unsheathed, bipolar flagellum. The SEM image of the PAGU2000^T is shown in
179 Figure 4. No periplasmic fibrils were observed. Moreover, although *H. valdiviensis* reportedly
180 has a single, monopolar flagellum (24), we observed a single, bipolar flagellum (Figure 4).
181 A detailed list of phenotypic characteristics of the species is described below (after the
182 Discussion section), and a comparison of the most important phenotypic characteristics of
183 strain PAGU 2000^T with those of other species of the genus *Helicobacter* including *H.*
184 *valdiviensis* is shown in Tables 1 and S2.
185 Cell growth of the strain PAGU 2000^T occurred at 37 °C and 42 °C under microaerobic
186 conditions but not at 25 °C. The isolate tested positive for catalase, oxidase, esterase, and
187 alkaline phosphatase, but did not hydrolyze indoxyl acetate, urease, γ-glutamyltransferase,
188 pyrrolidonyl arylamidase, L-arginine arylamidase, L-aspartate arylamidase, TTC reduction,
189 nitrate reduction, hippurate hydrolysis, and H_2S production. No bacterial growth was
190 observed in 1% glycine or 1.5% NaCl. Moreover, the strain PAGU 2000^T was resistant to

cephalothin (30 μg/mL) but sensitive to nalidixic acid (30 μg/mL).

The *H. valdiviensis* PAGU 2070^{T} strain was positive for nitrate reduction, TTC reduction, alkaline phosphatase activity, and H_2S production.

DISCUSSION

In the present study, we used a polyphasic approach to comprehensively evaluate the taxonomy of a strain of the genus *Helicobacter* which we have isolated from the blood of a patient with a fever. We determined the similarity values between the 16S rRNA gene sequence of strain PAGU 2000^{T} and the type strains of all members of the genus *Helicobacter* using the Clustal X, obtaining a similarity range of 91.2–97.5%. The phylogenetic tree based on 16S rRNA gene sequences clearly indicated that the isolated strain PAGU 2000^{T} represents a single species within the genus *Helicobacter*, most closely related to *H. valdiviensis* (97.5% similarity). We confirmed the taxonomic status of the novel strains within the genus *Helicobacter* and their representation of a novel lineage using 23S rRNA phylogenetic trees. Additionally, the results of phylogenetic analysis, using approximately 700 residues of the GyrA sequence obtained from isolate PAGU2000^{T}, were consistent with the results of the 16S and 23S rRNA gene analyses. The isolate shared only 51.9-92.2% similarity with these species, representing sufficient difference to assign the isolate PAGU

2000^T to a new taxon.

The boundary of the definition of prokaryotic species based on whole-genome sequence similarities has been proposed to be approximately 94% (26), 95% (28), or approximately 95–96% ANI (29). These values correspond to the traditional 70% DNA-DNA hybridization (DDH) values of the current species definition. In the present study, the in-silico DNA similarities indicated that the strain PAGU 2000^T shared less than 70.3% ANIb with *H. valdiviensis* type species, which is significantly lower than the boundary, confirming that it is an independent species. *H. valdiviensis* strains, the most closely related species of the PAGU 2000^T strain, were isolated from wild bird fecal samples (24) whereas the PAGU 2000^T strain was isolated from a human blood sample.

In the present study, the *H. valdiviensis* PAGU 2070^T strain was positive for nitrate reduction, TTC reduction, alkaline phosphatase activity, and H_2S production, which is inconsistent with the results described by Collado *et al*. (24). To confirm the difference in the biochemical test results of *H. valdiviensis* between our study and that by Collado *et al.*, we performed tests in two independent laboratories, *i.e.* at Aichi Gakuin University, Japan, and at Ghent University, Belgium, and both analyses confirmed that the two strains are positive for these biochemical parameters. The PAGU 2000^T and *H. valdiviensis* LMG 27920^T strains can easily be distinguished based on various biochemical properties; the strain PAGU 2000^T can be distinguished from *H. valdiviensis* PAGU 2070^T by hydrolysis of indoxyl acetate

hydrolysis, H_2S production, and nitrate and TTC reduction.

In conclusion, in the present study, the combined evidence derived from phylogenetic analysis, whole-genome sequencing, and biochemical characteristics demonstrates that the strain PAGU 2000^T represents a novel species within the genus *Helicobacter*.

Description of *Helicobacter higonensis* sp. nov.

Helicobacter higonensis (hi.go.nen'sis. N.L. fem. adj. higonensis pertaining to Higo, a traditional geographical name for Kumamoto Prefecture in Japan, the source of the type strain).

Cells are gram-negative, non-spore-forming curved rods (0.3–0.5 μm wide and 1.4-5.3 μm long). Cells are motile using a single unsheathed bipolar flagellum. The bacterium grows under microaerobic conditions at 37 °C or 42 °C, but not at 25 °C. No growth was observed under aerobic conditions at 37 °C. The bacterium is positive for oxidase, catalase, alkaline phosphatase, and esterase. Other tests from the API-Campy systems yielded negative results. The organism does not hydrolyze the indoxyl acetate and does not grow either in 1% glycine or 1.5% NaCl. The bacterial cells are resistant to cephalothin (30 μg/mL) but sensitive to nalidixic acid (30 μg/mL). The type strain, PAGU2000^T, isolated from the blood of a patient with fever in Kumamoto, Japan, 2017 was deposited in Gifu Type Culture Collection as GTC

16811^T and BCCM/LMG Bacteria Collection as LMG 33095^T. It has a DNA G+C content of 31.3 mol% and a genome size of 1.482 Mbp. The 16S rRNA sequence of the type strain has been deposited in GenBank under the accession number LC671231.

Emended description of the species *Helicobacter valdiviensis* Collado *et al.* 2014

The species was described by Collado *et al.* (2014). We propose the following corrections. Cells are motile through a single unsheathed bipolar flagellum and grow on horse blood agar at 37 °C under anaerobic conditions. This bacterium exhibits alkaline phosphatase activity, H_2S production, and nitrate and TTC reduction.

DISCLOSURE

The authors have no conflict of interest to declare.

Board members are co-authors: Kawamura, Yoshiaki, Sawa, Tomohiro, and Miyoshi-Akiyama, Tohru are an Editorial Board members of Microbiology and Immunology and co-authors of this article. To minimize bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication.

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Figure legends

Fig. 1 Phylogenetic tree based on the partial nucleotide sequences of 16S rRNA gene of type strains and '*Helicobacter higonensis*' PAGU2000^{T}. The scale bar represents 1 inferred nucleotide substitution per 100 nucleotides. The numbers at the nodes are bootstrap values. The phylogenetic tree was constructed using NJplot software (14).

Fig. 2 Phylogenetic tree based on the partial nucleotide sequences of 23S rRNA gene of type strains and '*Helicobacter higonensis*' PAGU2000^{T}. The scale bar represents 1 inferred nucleotide substitution per 100 nucleotides.

Fig. 3 Phylogenetic tree based on the GyrA gene-encoded protein showing the position of '*Helicobacter higonensis*' within the genus *Helicobacter*. The scale bar represents 5 inferred amino acid substitutions per 100 amino acids.

Fig. 4 Scanning electron micrograph of '*H. higonensis*' PAGU 2000^{T} (A) and *H. valdiviensis* PAGU 2070^{T} (B) (Scale bar = 1 um).

List of abbreviations

LMG: Belgian Coordinated Collections of Microorganisms/ LMG Bacteria Collection

PAGU: Pharmaceutical Sciences Aichi Gakuin University

rRNA: ribosomal RNA

DDH: DNA-DNA hybridization

Table 1. Phenotypic characteristics that differentiate '*Helicobacter higonensis*' sp. nov. from other related species.

	H. higonensis	*H. valdiviensis*	*H. pametensis*	*H. brantae*	*H. ganmani*	*H. pullorum*
Microaerobic growth at 42°C	+	+	+	+	-	+
Aerobic growth at 37°C	-	-	-	-	-	-
Anaerobic growth at 37°C	+	+	w+	-	+	-
Oxidase production	+	+	+	+	+	+
Catalase production	+	+	+	+	-*	+
Indoxyl acetate hydrolysis	-	+	-	+	-	-
Urease activity	-	±	-	-	-	-
Nitrate reduction	-	+	+	-	+	+
Triphenyl-tetrazolium chloride (TTC) reduction	-	+	nd	nd	+	nd
Alkaline phosphatase activity	+	w+	+	-	-	-
H_2S production	-	+	-	nd	nd	nd
Growth on 1% glycine	-	+	+	+	-	-
Number of flagella	2	2	2	2	2	1
Distribution of flagella	B	B	B	ST	B	M

Symbols: +, 100% of strains positive; −, 100% of strains negative;±, 42–66% of strains positive; w, weak; nd, not determined; M, monopolar; B, bipolar; ST, subterminal.

*Weak reaction detected (in two out of seven strains).

Data taken from this study and Refs.(30, 31, 32).

Figure 1

16S rRNA gene

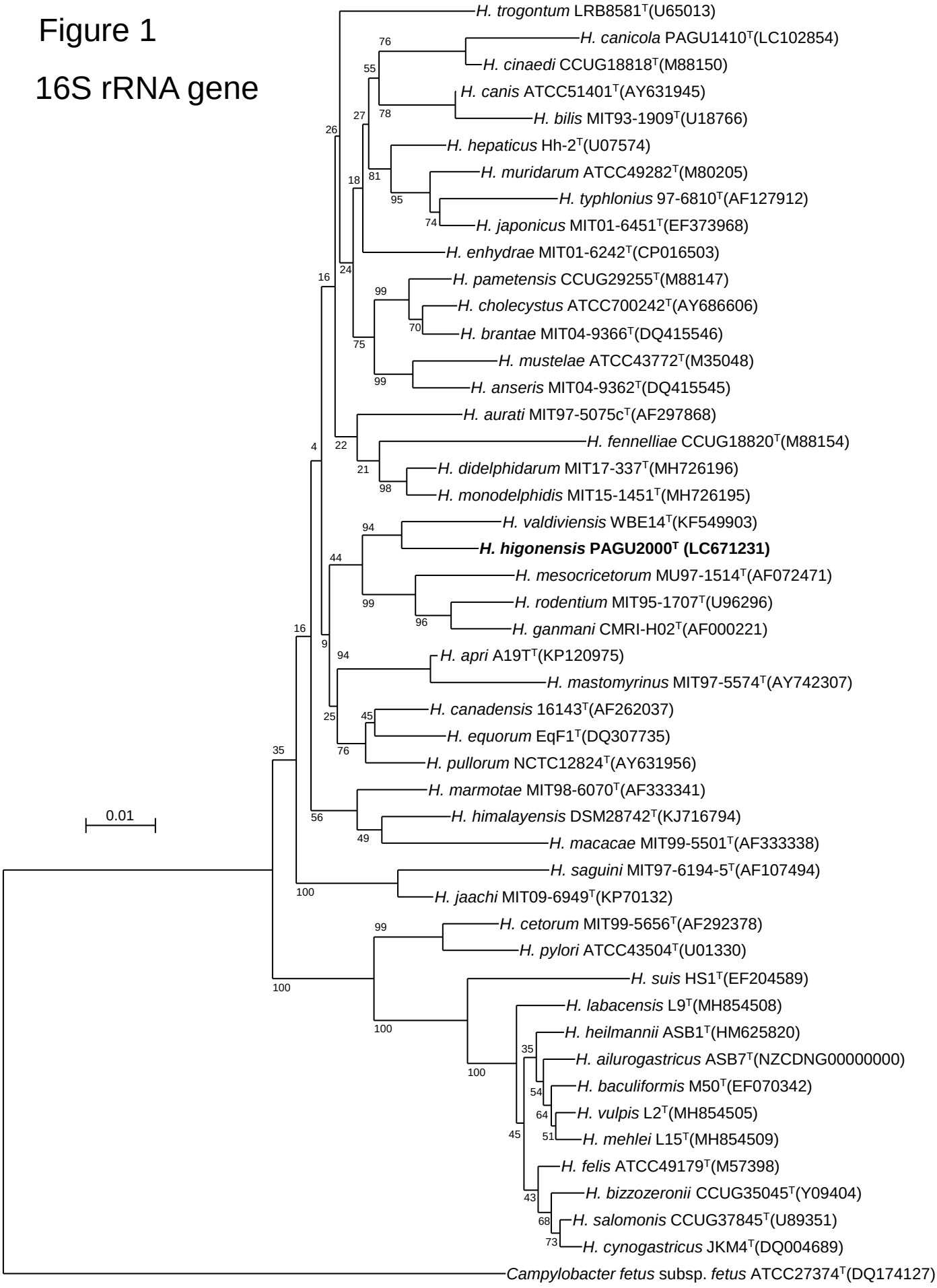

Figure 2

23S rRNA

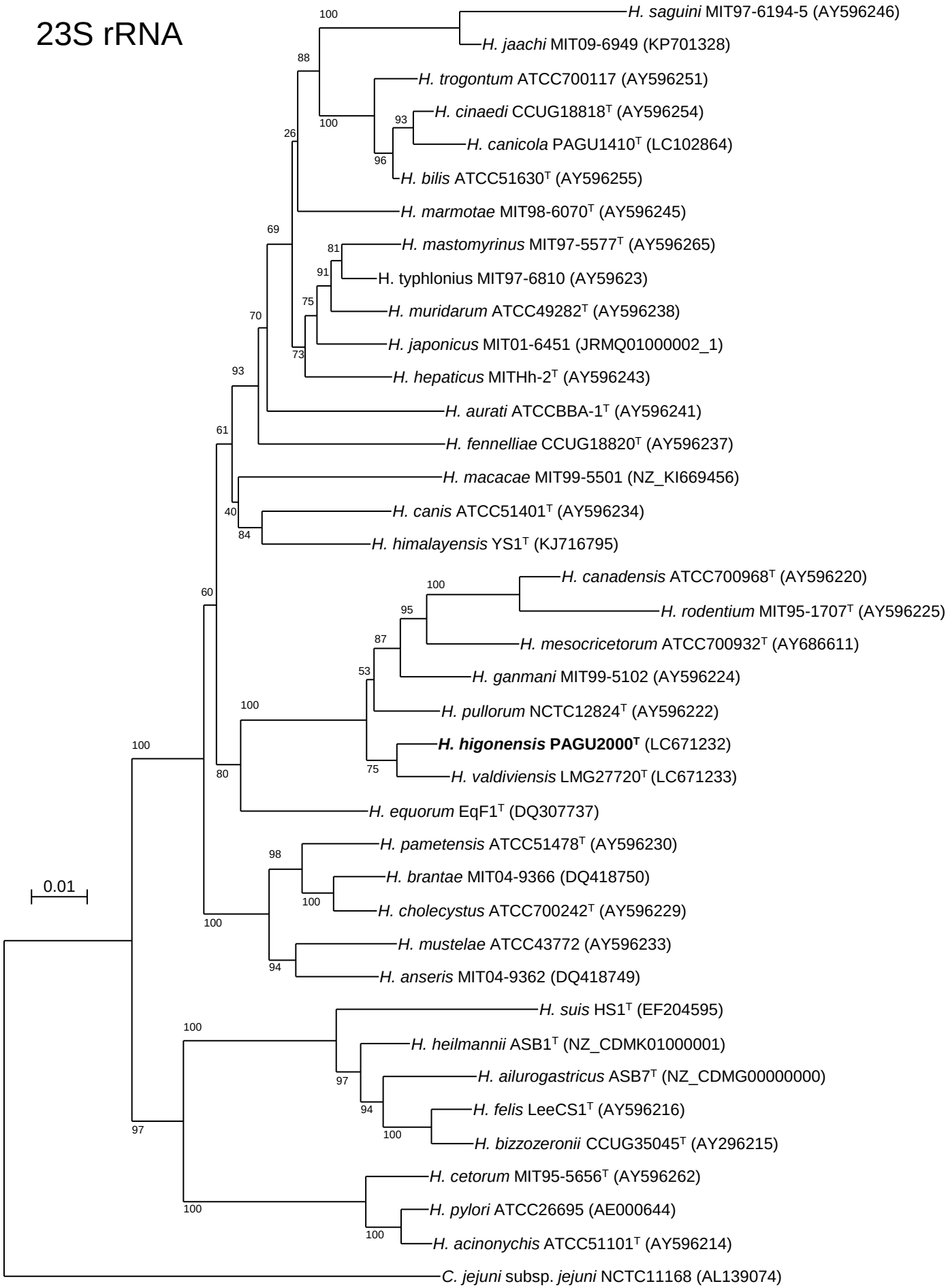

Figure 3

GyrA amino acid

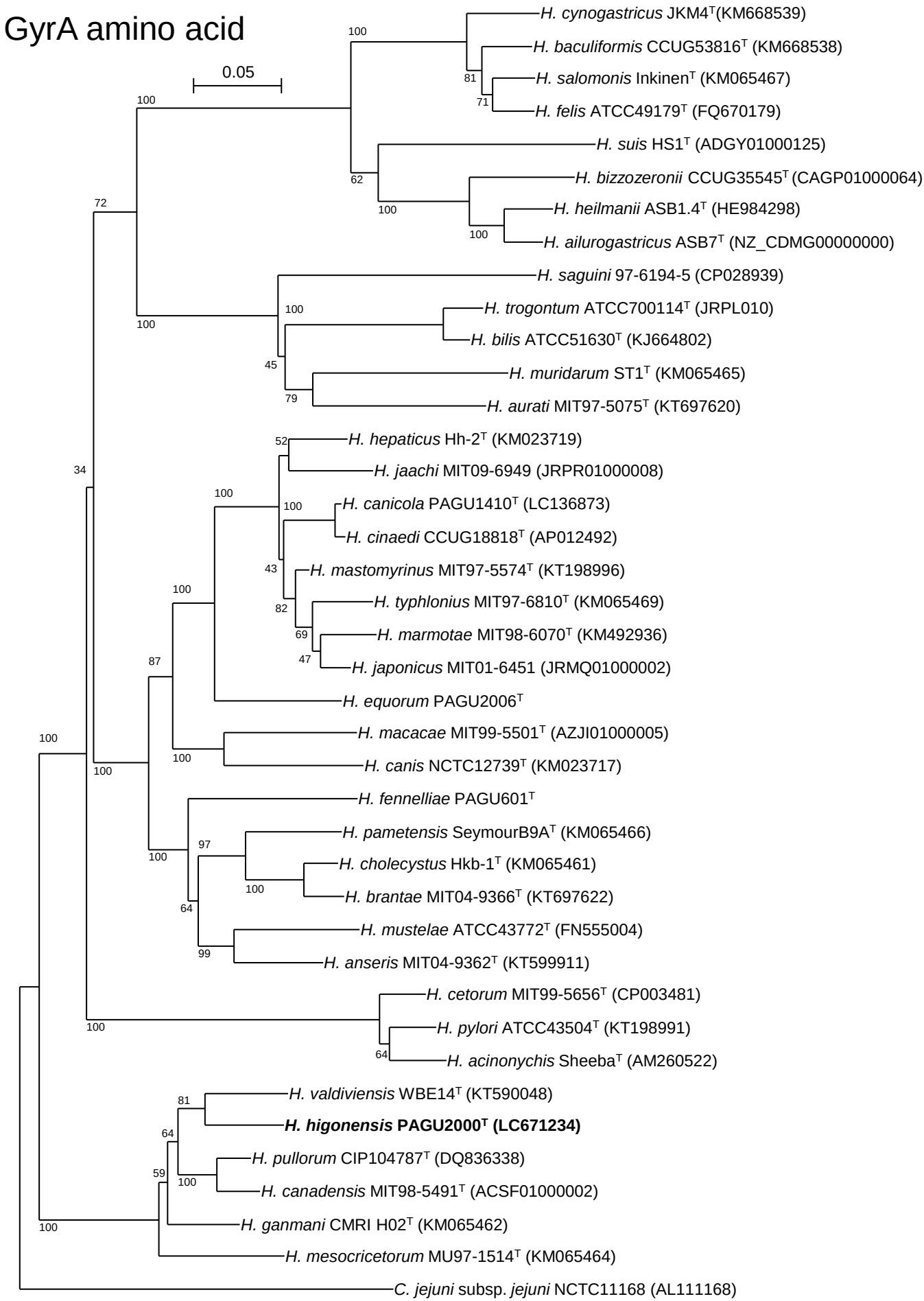

Figure 4

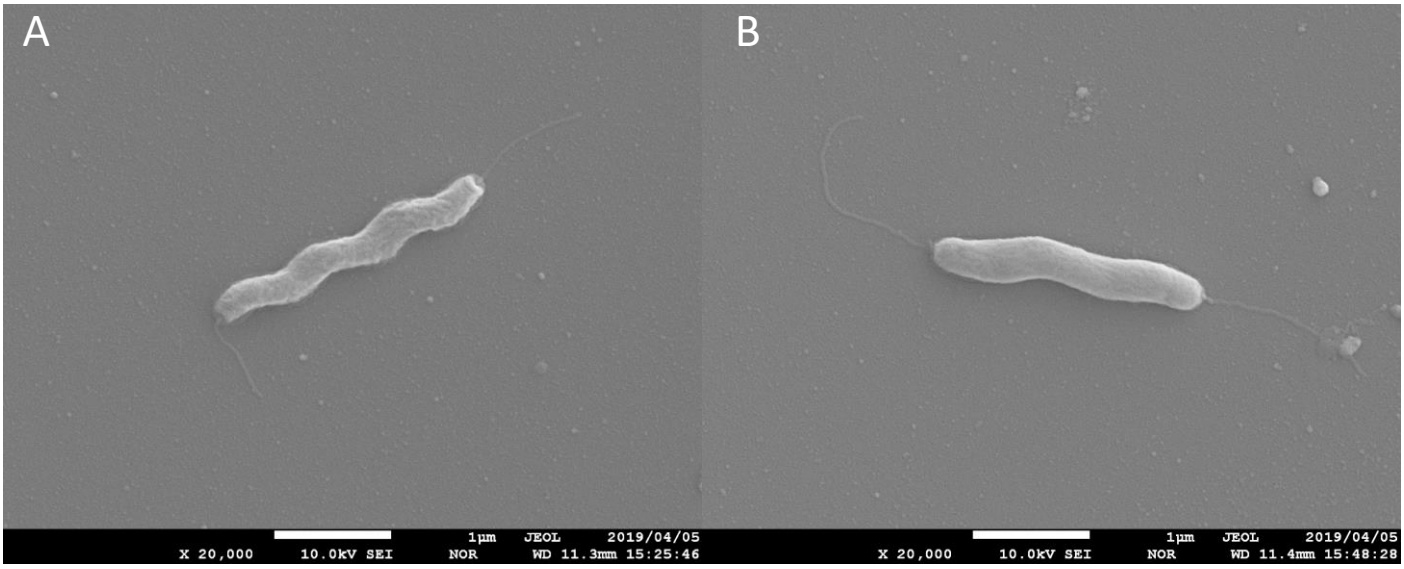

Fig. 4. Scanning electron micrograph of '*H. higonensis*' PAGU 2000^{T} (A) and *H. valdiviensis* PAGU 2070^{T} (B) (bar = 1 um).

Table S1. List of the functioning parts* possessed by PAGU 2000^{T}.

Subsystem	Role
Glycine and Serine Utilization	L-serine dehydratase, alpha subunit (EC 4.3.1.17)
	L-serine dehydratase, beta subunit (EC 4.3.1.17)
Chorismate: Intermediate for synthesis of Tryptophan, PAPA antibiotics, PABA, 3-hydroxyanthranilate and more.	Isochorismatase (EC 3.3.2.1)
Rhamnose containing glycans	Glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24)
CBSS-272943.3.peg.1367	Rossmann fold nucleotide-binding protein Smf possibly involved in DNA uptake
CBSS-331978.3.peg.2915	Preprotein translocase subunit SecG (TC 3.A.5.1.1)
DNA repair, bacterial	DNA recombination protein RmuC
	DNA-cytosine methyltransferase (EC 2.1.1.37)
	Methyl-directed repair DNA adenine methylase (EC 2.1.1.72)
DNA replication strays	DNA polymerase III polC-type (EC 2.7.7.7)
Restriction-Modification System	Putative DNA-binding protein in cluster with Type I restriction-modification system
	Putative predicted metal-dependent hydrolase
	Type III restriction-modification system DNA endonuclease res (EC 3.1.21.5)
	Type III restriction-modification system methylation subunit (EC 2.1.1.72)
Hemin transport system	Ferric siderophore transport system, periplasmic binding protein TonB
Ton and Tol transport systems	putative TolA function
Potassium homeostasis	Potassium efflux system KefA protein
Aminopeptidases (EC 3.4.11.-)	Xaa-Pro aminopeptidase (EC 3.4.11.9)
Oxidative stress	Superoxide dismutase [Cu-Zn] precursor (EC 1.15.1.1)
Copper homeostasis	Multidrug resistance transporter, Bcr/CflA family
Multidrug Resistance Efflux Pumps	Probable outer membrane component of multidrug efflux pump

*The notion of functioning is defined by having genes for all the functional roles that compose a variant of a subsystem (RAST server).

Table S2. Phenotypic characteristics that differentiate Helicobacter higonensis sp. nov. from other speciesof the genus *Helicobacter* : 1, *H. higonensis* ; 2, *H. acinonychis* ; 3, *H. ailurogastricus* ; 4, *H. anseris* ; 5, *H. apri* ; 6, *H. aurati* ; 7, *H. baculiformis* ; 8, *H. bilis* ; 9, *H. bizzozeronii* ; 10, *H. brantae* ; 11, *H. canadensis* ; 12, *H.canicola* ; 13, *H. canis* ; 14, *H. cetorum* ; 15, *H. cholecystus* ; 16, *H. cinaedi* ; 17, H. colisuis ; 18, *H. cynogastricus* ; 19, *H. didelphidarum* ; 20, *H. delphinicola* ; 21, *H. enhydrae* ; 22, *H. equorum* ; 23, *H. felis* ; 24, *H. fennelliae* ; 25, *H. ganmani* ; 26, *H. heilmannii* ; 27, *H. hepaticus* ; 28, *H. himalayensis* ; 29, *H. jaachi* ; 30, *H. japonicus* ;31, *H. kayseriensis* ; 32, *H. kumamotonensis* ; 33, *H. labacensis* ; 34, *H. macacae* ; 35, *H. marmotae* ; 36, *H. mastomyrinus* ; 37, *H. mehlei* ; 38, *H. mesocricetorum* ; 39, *H. monodelphidis* ; 40, *H. muridarum* ; 41, *H. mustelae* ; 42, *H. pametensis* ; 43, *H. pullorum* ; 44, *H. pylori* ; 45, *H. rodentium* ; 46, *H. saguini* ; 47, *H. salomonis* ; 48, *H. suis* ; 49, *H. trogontum* ; 50, *H. turcicus* ; 51, *H. typhlonius* ; 52, *H. valdiviensis* ; 53, *H. vulpis* .

+, All strains examined give a positive result; -, all strains examined give a negative result; (+), 80–94 % strains positive; ±, 33–66 % strains positive; (-), 7–33 % strains positive; V, 26-74% strains positive; NA, nalidixic acid; CE, cephalotin; I, intermediate resistance; B, bipolar; M, monopolar; St, subterminal; Pt, peritrichous; U, undetermined. Data are from references (32-34).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
Growth at 37°C (microaerobic)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 42°C (microaerobic)	+	−	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−	(+)	−	−	−	+	+	+	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	+	−	−	+	+	+	−
Growth at 25°C (microaerobic)	−	−	−	U	U	U	−	U	−	U	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth at 37°C (anaerobic)	+	−	U	−	U	−	+	U	U	−	−	−	−	−	+	−	+	+	U	−	U	−	−	−	+	+	+	+	−	−	+	−	U	−	−	−	U	−	−	−	+	+	−	−	+	−	+	+	U	+	−	−	U
Growth at 37°C (aerobic)	−	−	U	−	U	U	−	U	U	−	−	−	−	−	−	−	−	−	U	−	U	−	−	−	−	−	−	−	−	−	−	−	U	−	−	−	U	−	−	−	−	+	−	−	−	−	U	−	U	−	−	−	U
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	U	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	(+)	+	+	+	+	+	+	+	(+)	(−)	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	(+)	+	+	+	+	+	−	+	+	+
Nitrate reduction	−	−	+	−	+	−	+	+	+	−	±	−	−	−	−	+	+	+	−	−	−	+	+	−	+	+	+	+	−	−	−	−	+	−	−	−	−	+	(−)	−	+	+	+	−	+	−	−	+	−	+	−	+	+
Indoxyl acetate hydrolysis	−	−	−	+	−	+	−	−	+	+	+	−	+	−	−	(−)	−	−	−	−	−	−	−	+	+	−	−	+	−	+	−	−	−	+	−	−	−	U	−	−	+	+	−	−	(−)	−	−	(−)	−	U	+	−	+	−
Urease	−	+	+	+	−	+	+	+	+	−	−	−	−	+	−	−	−	+	+	−	−	−	+	−	−	+	+	−	+	−	−	−	+	−	−	−	−	+	+	+	+	−	−	(+)	−	−	+	+	+	+	−	−	(±)	+
Alkaline phosphatase	+	+	+	−	+	−	+	U	+	−	−	+	+	−	+	(−)	+	+	±	−	−	+	+	±	−	−	U	+	−	−	+	+	+	−	+	−	+	+	−	+	+	+	−	+	−	+	±	+	(−)	+	−	−	+
Hippurate hydrolysis	−	−	+	U	U	U	−	−	−	U	U	−	−	U	U	−	−	−	−	U	U	−	−	−	−	+	−	−	U	U	−	−	−	U	U	U	−	−	U	−	−	−	−	−	−	U	−	−	−	−	−	−	−
γ-Glutamyl transpeptidase	−	U	U	−	−	+	+	U	+	−	−	−	U	+	−	U	v	−	+	+	−	−	U	U	U	+	U	+	−	−	−	(−)	+	−	−	−	+	−	+	U	U	U	U	+	−	+	+	+	+	−	−	+
Growth on 1 % glycine	−	−	U	+	−	−	−	+	−	+	−	−	−	U	+	−	−	+	−	−	+	−	−	−	−	−	+	−	+	−	+	−	−	+	+	+	−	−	−	−	−	+	−	−	+	+	−	−	−	+	+	−
Reduction of Triphenyltetrazolium chlorides (TTC)	−	−	+	U	U	U	U	+	−	U	−	+	V	U	U	+	v	U	−	U	U	U	−	+	+	U	+	U	U	U	−	−	+	U	U	V	V	U	U	−	U	U	−	U	U	+
Resistance to: NA (30 µg)	−	+	U	−	−	−	I	+	+	−	±	−	−	±	I	−	+	I	−	−	−	+	−	−	−	U	+	−	−	+	−	+	−	+	+	+	−	−	+	−	−	+	(+)	+	+	−	U	+	+	−	(±)	−
Resistance to CE (30 µg)	+	−	U	+	+	+	+	+	−	+	+	+	(−)	+	+	+	+	+	+	−	−	+	−	−	+	U	+	+	+	+	+	+	−	+	+	+	−	+	+	−	(−)	+	+	±	U	+	+	+	+	−
Periplasmic fibres	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Distribution of flagella	B	M	B	St	B	B	B	B	B	St	B	B	B	B	M	B	B	B	B	B	M	M	B	B	B	B	B	B	B	M	St	B	B	B	B	B	B	B	B	B	Pt	B	M	M	B	B	B	B	B	B	B	M	B
Number of flagella	2	2–5	6–8	2	2	7–10	11–22	3–4	10–20	2	2	2	2	2	2	1–2	2	6–12	6–12	2–6	1	1	14–20	2	2	4–10	2	1–2	7–14	1	2	2	8–10	2	2	2	5–10	2	7–14	10–14	4–8	2	1	4–8	2	6–12	10–23	4–10	5–7	2	1–2	1	5–10
DNA G+C content (mol%)	31	30	37	30	40	36	45	35	46	39	34	39	48	36	35	37–38	34	44	32	U	41	38	45	35	37	47	36	40	41	38	38	38	48	41	40	U	46	34	35	34	43	38	34–35	35–37	37	35	46	40	33	35	39	32	47

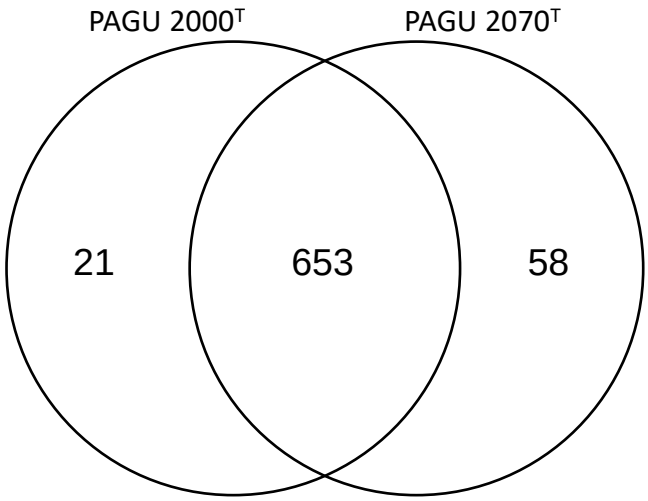

Fig. S1 Venn diagram representing not-shared and common subsystems among *H. higonensis* PAGU 2000^T and *H. valdiviensis* PAGU 2070^T (based on RAST server).