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Usefulness of Housekeeping Genes for the Diagnosis of *Helicobacter pylori* Infection, Strain Discrimination and Detection of Multiple Infection

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Helicobacter pylori, housekeeping genes, detection, genotyping, multiple infection.

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Abstract

Background: *Helicobacter pylori* infects human stomachs of over half the world's population, evades the immune response and establishes a chronic infection. Although most people remains asymptomatic, duodenal and gastric ulcers, MALT lymphoma and progression to gastric cancer could be developed. Several virulence factors such as flagella, lipopolysaccharide, adhesins and especially the vacuolating cytotoxin VacA and the oncoprotein CagA have been described for *H. pylori*. Despite the extensive published data on *H. pylori*, more research is needed to determine new virulence markers, the exact mode of transmission or the role of multiple infection.

Materials and Methods: Amplification and sequencing of six housekeeping genes (*amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS*) related to *H. pylori* pathogenesis have been performed in order to evaluate their usefulness for the specific detection of *H. pylori*, the genetic discrimination at strain level and the detection of multiple infection. A total of 52 *H. pylori* clones, isolated from 14 gastric biopsies from 11 patients, were analyzed for this purpose.

Results: All genes were specifically amplified for *H. pylori* and all clones isolated from different patients were discriminated, with gene distances ranged from 0.9 to 7.8%. Although most clones isolated from the same patient showed identical gene sequences, an event of multiple infection was detected in all the genes and microevolution events were showed for *amiA* and *cpn60* genes.

Conclusions: These results suggested that housekeeping genes could be useful for *H. pylori* detection and to elucidate the mode of transmission and the relevance of the multiple infection.

Helicobacter pylori chronically infects more than half of the world's population because the host immune response fails to eliminate the infection. In most infected people, the bacterium acts as a commensal organism inducing chronic asymptomatic gastritis that can last for life. In other cases, however, it is responsible for a heavy toll of morbidity and mortality as a consequence of peptic ulcers and gastric cancer. Chronic gastritis may progress to intestinal metaplasia, dysplasia and eventually gastric cancer. This multi-step process is known as the Correa pathway [1]. The clinical outcome of *H. pylori* infection depends on strain virulence, host response, and environmental factors.

Several virulence factors have been identified in *H. pylori*: gastric colonization mediated by flagella, urease, outer membrane proteins (OMPs), phospholipids, glycolipids, and other adhesins (*babA*, *hpA*, *napA*, *sabA*, etc.), toxins such as lipopolysaccharide (LPS) and the vacuolating cytotoxin VacA, a type IV secretion system encoded by the cag pathogenicity island (*cag*PAI) containing the effector protein CagA, a γ-Glutamyl transpeptidase (GGT) associated with colonization and

cell apoptosis, the duodenal ulcer-promoting gene A (*dupA*), and others [2–4]. Among virulence factors identified in *H. pylori*, *cagA,* and *vacA* genes are the main virulence markers as shown in an study conducted by [5], in which patients infected with high virulence strains (*cagA*+, *vacA* s1i1–m1) had a higher risk of progression to preneoplastic lesions and gastric cancer in comparison with patients infected with low virulence strains.

Strains of *H. pylori* exhibit considerable genetic diversity following a panmictic (non-clonal) population structure due to horizontal gene transfer and frequent recombination. In contrast, since *H. pylori* does not spread epidemically, phylogenetic studies based on multilocus sequence analysis (MLSA) of housekeeping genes are contributing to trace human migrations [6,7]. On the other hand, intrapatient diversity of *H. pylori* needs to be reevaluated because results about the prevalence of multiple infection are controversial, and it could have implications related to the mode of transmission, antibiotic resistance and virulence of *H. pylori* [2,8,9]. Moreover, there is a lack of studies regarding *H. pylori* detection from specimens other than gastric biopsies [10].

Other genes have likewise been related to *H. pylori* pathogenesis: *amiA* (N-acetylmuramoyl-L-alanine amidase) and *luxS* (autoinducer-2 synthase) genes are involved in biofilm formation although the former is also related to bacterial adhesion, cell morphology and immune escape [11]. Others such the cholesterol-α-glucosyltransferase gene (*cgt* or *capJ*) glycosilates host cholesterol contributing to pathogenicity and antimicrobial resistance while *cpn40* (*dnaJ*), *cpn60* (*groEL*), and *cpn70* (*dnaK*) genes have been suggested as a risk factor for oncogenesis and are recommended as serological markers of *H. pylori* infection [3,12,13].

In this study, *H. pylori* specific PCR amplification and sequencing of *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ,* and *luxS* genes have been developed in order to evaluate their usefulness in the detection of *H. pylori* infection, to genetic discrimination at strain level, to detection of multiple infection and to MLSA.

Materials and Methods

Helicobacter pylori Clones and DNA Extraction

Ten *H. pylori* strains (APP134, B247, B271, B319, B355, B491, B508S, B508T, CRL122, and SVC135) from the *H. pylori* collection of the Digestive Diseases Department of the Hospital Taulí (Sabadell, Barcelona, Spain) were included in this study. Outpatients sent to the Endoscopy Unit of the Hospital Taulí for evaluation of dyspeptic symptoms from February 2006 to November 2015 were recruited in this collection. Patients were contacted prior to the endoscopy and were asked to participate. Before the endoscopy, the patients signed informed consent. During endoscopy, antral and corpus biopsies were obtained for histology, rapid urease test (RUT) and molecular studies. Isolation, culture and identification of *H. pylori* were performed after a positive RUT test. The RUT biopsy was plated on Pylori Agar (bioMérieux) in microaerophilic conditions in microaerophilic jars (Jar Gassing System; Don Whitley Scientific Limited, UK). After a maximum of a week, *H. pylori* isolates were subcultured on Columbia plates (bioMérieux) and identified by colony morphology, Gram-negative staining and a positive result for urease, catalase, and oxidase tests. The strains were frozen in Brucella Broth with 10% glycerol and stored at −80 °C until analysis [14].

The strains were recovered on Columbia agar with 5% sheep blood (bioMérieux) and incubated at 37 °C under microaerophilic conditions. These strains were previously isolated from antral biopsies of different patients, except for B508S and B508T, which were isolated from the same patient, B508S from normal tissue and B508T from gastric adenocarcinoma (Table 1). For this study, these *H. pylori* strains were not considered as pure cultures because they were originally obtained by picking up most of the growth on Pylori agar (bioMérieux) after seeding of gastric biopsies, and not

Table 1 Samples included in this study

Gastric biopsies (n = 14)	Endoscopic/Histopathological diagnosis	Clones analyzed (n = 52)
APP134	Duodenal ulcer/moderate gastritis	APP134-1, -2, -3
B247[a]	Neoplasia/adenocarcinoma	B247-1, -2, -3
B271	Duodenal ulcer/moderate gastritis	B271-1, -2, -3
B319	Normal/mild gastritis	B319-1, -2, -3
B355	Duodenal ulcer/mild gastritis	B355-1, -2, -3
B491[a]	Neoplasia/adenocarcinoma	B491-1, -2, -3
B508S[a]	Neoplasia/adenocarcinoma	B508S-1, -2, -3, -4, -5, -6
B508T	Neoplasia/adenocarcinoma	B508T-2A, -2B, -3, -4, -5, -6
B657A	Normal/mild gastritis	B657A-1, -2, -3, -4
B657C	Normal/mild gastritis	B657C-1, -2, -3, -4
B659A	Normal/moderate gastritis	B659A-1, -2, -3, -4
B659C	Normal/moderate gastritis	B659C-1, -2, -3, -4
CRL122	Normal/mild gastritis	CRL122-1, -2, -3
SVC135	Normal/moderate gastritis	SVC135-1, -2, -3

[a]Gastric biopsies obtained from normal tissue of patients with adenocarcinoma.

from individual colonies. In order to obtain pure cultures, between three and six isolated colonies were selected from each blood agar culture.

Helicobacter pylori colonies were also isolated from antral and corpus biopsies of two different patients (B657A/B657C and B659A/B659C) (Table 1) seeded onto Pylori agar.

Helicobacter pylori strains ATCC 49503 and ATCC 51932 were also included as controls in the present study.

Subcultures of individual colonies were performed on Columbia blood agar or Brucella agar (BD Diagnostics) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

DNA Extraction, PCR Amplification and Sequencing

DNA of each clone was extracted from 4 ml of cultures in Brucella broth supplemented with 10% FBS following the manufacturers' instructions (REAL pure genomic DNA extraction kit).

Primers for *H. pylori* specific PCR amplification and sequencing of the housekeeping genes *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ,* and *luxS* (Table 2) were designed from 43 *H. pylori* complete genome sequences (see Table S1). To evaluate the specificity of the PCR assays, the following four strains, belonging to different bacterial species, were included as negative controls: *Campylobacter jejuni* ATCC 33291, *Pseudomonas aeruginosa* 42A2, *Aeromonas molluscorum* 848T^{T}, and *Aeribacillus pallidus* DR03.

PCR amplifications were carried out in a total volume of 50 μL containing 50 mM KCl, 15 mM Tris/HCl, pH 8.0, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Amersham Biosciences), 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 40 pmol of each primer (Sigma), and 250 ng DNA. The reaction mixtures were subjected to the following thermal cycling program in a 2720 Thermal Cycler (Applied Biosystems): denaturation at 95 °C for 5 minutes, 35 cycles of 95 °C for 1 minutes, 54–64 °C (Table 2) for 1 minute and 72 °C for 1 minute and a final extension step at 72 °C for 5 minutes.

The amplified products were purified using the MSB® Spin PCRapace kit (Stratec) or the ExoSAP-IT® (Affymetrix) and sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with the following thermal cycling conditions: 96 °C for 1 minute and 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes. Nucleotide sequences were determined in an ABI PRISM 3730 DNA analyzer by the Genomics Unit of Scientific and Technological Centers from University of Barcelona (CCiTUB).

Data Analyses

Partial sequences of *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ,* and *luxS* genes were aligned independently and phylogenetic trees were constructed by neighbor-joining method and Jukes–Cantor distance estimation model using MEGA 6.0 [15]. Concatenated sequences of all genes obtained by the online sequence toolbox FaBox [16] were also analyzed (Table 3). The topological robustness of the phylogenetic trees was evaluated by a bootstrap analysis through 1000 replicates.

Results

A total of 52 *H. pylori* clones were isolated from 14 gastric biopsies from 11 patients (Table 1).

Table 2 Primers designed for specific PCR amplification and partial sequencing of *Helicobacter pylori* housekeeping genes

Gene	Length (pb)[a]	Primer	Sequence 5′→3′	Ta	Amplicon (pb)
amiA	1323	amiA-658	GTTTTRGACGCYGGGCATGG	64 °C	635
		amiA-1292	CCATCAGCAATGCCCTTAGC		
cgt	1170	cgt-252	GGCTTTTAAGGGAGCGGATA	60 °C	615
		cgt-866	ATCGCTTCGCTYTCCACATT		
cpn60	1641	Hp156	CGTGAGCGTGGCTAARGAG	54 °C	801
		Hp956	GCTTTGCCTAAAAACTCYACTT		
cpn70	1863	cpn70-982	ATTTCAGAAGTGGTGATGGT	54 °C	612
		cpn70-1593	GTTTCTCGCTTCAATCACTT		
dnaJ	1110	dnaJ-259	TTTGAAGATTTAGGCTCGTT	54 °C	588
		dnaJ-846	TAAAGAYGGCACTTTAATCG		
luxS	468	luxS-38	TGGATCACACYAAAGTCAAAG	54 °C	429
		luxS-466	AAACCCCCACTTCAGACCA		

Ta, annealing temperature.

[a]Data from the complete genome of *Helicobacter pylori* 26695 (NC_000915).

Table 3 Genetic distance values of individual and concatenated genes

Gene	Sequence (nt)	Distances values		
		Range	Minimum[a]	Mean ± SE
amiA	576	0–0.052	0.019	0.031 ± 0.004
cgt	558	0–0.044	0.009	0.025 ± 0.004
cpn60	555	0–0.039	0.009	0.023 ± 0.004
cpn70	588	0–0.040	0.015	0.027 ± 0.004
dnaJ	564	0–0.061	0.013	0.032 ± 0.004
luxS	405	0–0.078	0.028	0.046 ± 0.006
Concatenated	3246	0–0.036	0.024	0.030 ± 0.002

[a]Minimum distances values between sequences from clones isolated from biopsies of different patients.

All housekeeping genes (*amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS*) were specifically amplified for the bacterial species *H. pylori* with the primers designed in this study, none gene was amplified for the other bacterial species analyzed. Amplicons and partial gene sequences were 429–635 nt and 405–588 nt long, respectively (Table 2, Table 3).

Gene sequences from the 52 *H. pylori* clones and from *H. pylori* ATCC 49503 and ATCC 51932 were compared. All gene sequences from clones isolated from biopsies of different patients were different, whose gene distances ranged from 0.9 to 7.8% and the highest distance values were obtained from *luxS*. Distance rates calculated from concatenated sequences ranged from 2.4 to 3.6%, with a mean distance of 3% (Table 3).

All clones isolated from the same patient showed identical sequences except in the case of B508S/B508T and B657A/B657C. Two different *amiA* and *cpn60* sequences were detected from B508T clones: B508T-2A, B508T-2B, B508T-3, B508T-5, and B508T-6 showed identical sequences, which were different to those from clone B508T-4. A unique nucleotide difference (0.2% divergence) was detected in the gene *amiA*, meanwhile six nucleotide differences (1.1% divergence) were found in *cpn60*. B508S clones presented identical *amiA* and *cpn60* sequences to those from B508T-4. However, on the basis of *cgt*, *cpn70*, *dnaJ*, and *luxS* sequence comparison, all B508T and B508S clones analyzed were identical. Phylogenetics trees for *amiA*, *cpn60*, *luxS*, *cgt*, *cpn70*, and *dnaJ* are shown in Figure 1A, B, C and Fig. S1A, B, C, respectively.

On the other hand, all six genes allowed to differentiate two groups of clones from B657A and B657C biopsies. A group was formed by B657A-1, B657A-2, and B657A-3 and another group by B657A-4 and all four clones from B657C (Fig. 1B, C and Fig. S1A, B, C), with the following intergroup distance rates: 3.6, 2.2, 3.5, 3.1, 1.6, and 7.3% for the genes *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS*, respectively. Moreover, two nucleotide differences (0.3% divergence) were observed between B657A-4 and B657C clones for *amiA* (Fig. 1A).

The phylogenetic tree obtained from concatenated sequences (3246 nt) is shown in Figure 2.

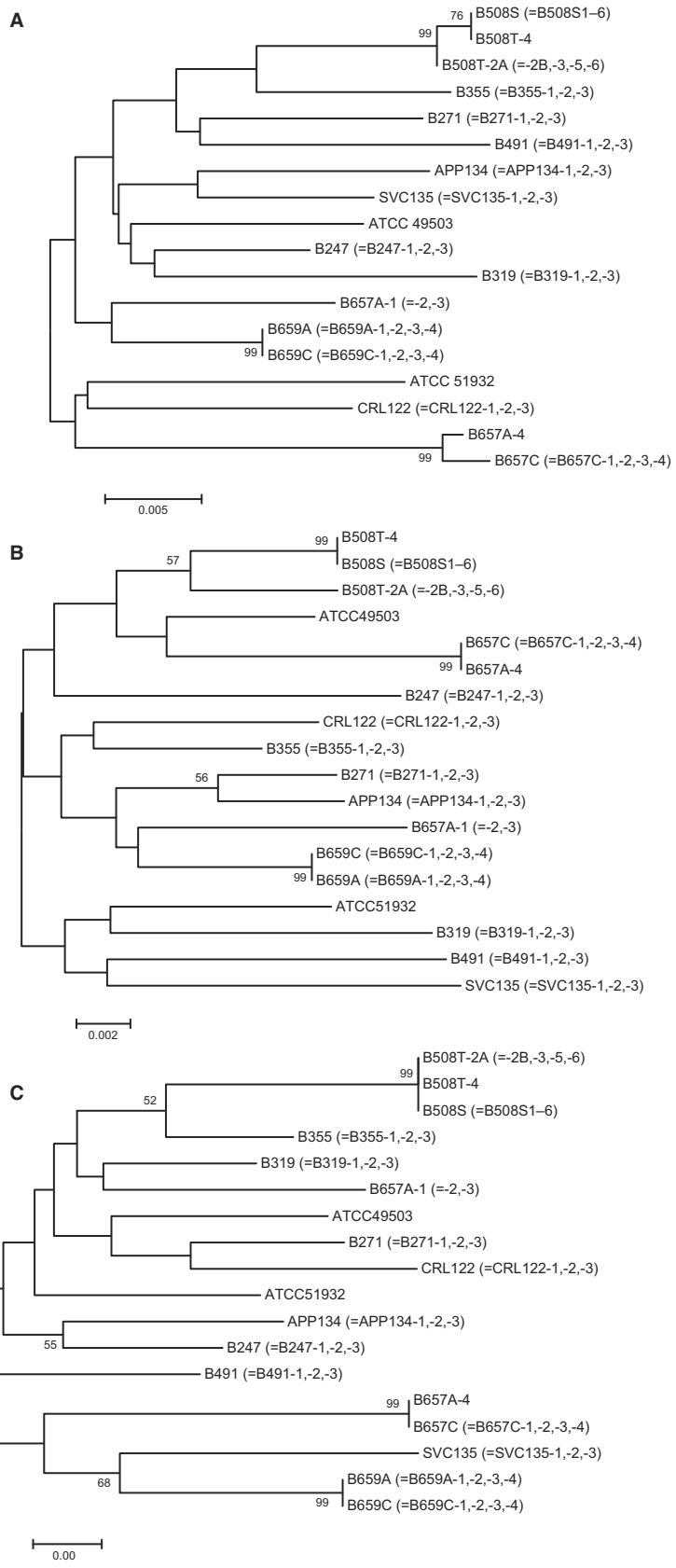
Discussion

In this study, specific PCR detection of *H. pylori* has been achieved for the housekeeping genes *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS*. These genes are potential candidates to detect *H. pylori* infection in gastric biopsies and other specimens (as gastric juice, stool, saliva, dental plaque, water, and food samples), together with others, mainly used for gastric biopsies, as *ureA*, *vacA*, 16S rRNA, and 23S rRNA genes, previously described [17,18].

Sequencing of any of these six genes has allowed a clear differentiation between all clones isolated from different patients (0.9–7.8% divergence). These results suggest that these housekeeping genes could be useful to elucidate the mode of transmission of *H. pylori*, an issue that is still controversial. While oral-oral, gastro-oral, and fecal-oral transmission are the most probable ways, *H. pylori* isolation from stools or the oral cavity is difficult and different environmental sources of *H. pylori* (food, animals, water) could play a role as reservoirs [10].

Two different strains isolated from the antral biopsy B657A were clearly detected by all genes with high distance values (1.6–7.3%). One strain was represented by the identical clones B657A-1, -2 and -3, and the other strain by the clone B657A-4, which was identical to clones isolated from B657C (corpus biopsy from the same patient), except in the case of *amiA* as mentioned below. This high divergence observed between both strains indicates an event of multiple infection in the antrum of the stomach. Horizontal gene transfer and genetic recombination by mixed infection is crucial for the acquisition of the high genetic diversity of *H. pylori* [19]. Although some authors have detected mixed infections, different studies have reported very variable prevalence rates and little is known about the role of multiple infections on disease outcome [8,20,21].

Figure 1 Consensus neighbor-joining phylogenetic trees obtained from 18 *Helicobacter pylori* sequences of genes *amiA* (A) *cpn60* (B) and *luxS* (C). Bar, distance values as calculated by MEGA 6.0. Bootstrap values (>50%) after 1000 replicates are shown as percentages.



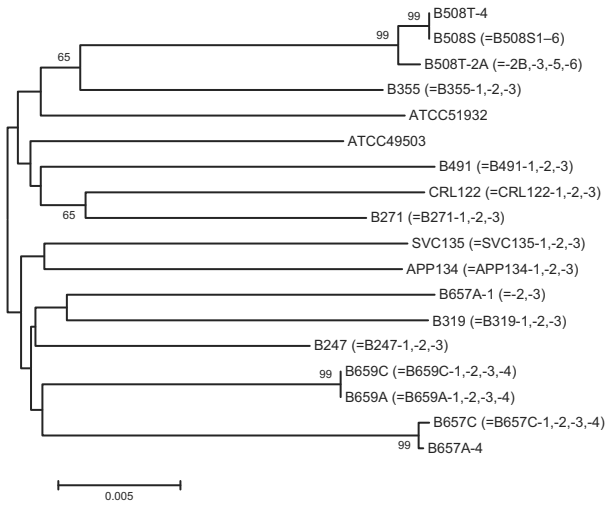


Figure 2 Consensus neighbor-joining phylogenetic tree obtained from *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, *and luxS* concatenated sequences. Bar, distance of 0.005 substitutions per nucleotide position as calculated by MEGA 6.0. Bootstrap values (>50%) after 1000 replicates are shown as percentages.

Although *luxS* gene showed the highest distance values, the genes *amiA* and *cpn60* were more useful to discriminate clones isolated from the same biopsy and patient. Two different clones isolated from the sample B508T, gastric biopsy collected from tissue with adenocarcinoma, were only detected by partial sequencing of *amiA* and *cpn60* (one and six nucleotide differences, respectively). One of both clones (B508T-4) was identical to clones isolated from B508S, gastric biopsy from the same patient but collected from normal tissue. On the other hand, two *amiA* nucleotide differences were observed between B657A-4 and B657C clones. These small differences between clones suggest events of microevolution rather than related to multiple infection. Evidences that *H. pylori* can establish a chronic infection after infection with a single strain have been described [22,23] and, recently, Linz et al. [24] have revealed that the mutation rate during the acute infection phase is over 10 times faster than during chronic infection.

Concatenation of *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS* partial sequences allowed us to conduct a multilocus sequence analyses (MLSA) approach (Fig. 2), which is useful to elucidate intra- and interspecies phylogenetic relationships [25,26] and, in the case of *H. pylori*, the phylogeographic differentiation of bacterial populations associated to the migration of human populations [6,27]. Most of these genes have never been used for these purposes except *dnaJ* and, especially, *cpn60*, that is useful for microbial phylogeny, detection and identification, ecology, and evolution through the analysis of the 555 bp region known as universal target (UT) analyzed in this study [28,29].

In conclusion, *H. pylori* specific detection has been developed, all *H. pylori* clones isolated from different patients have been discriminated, and microevolution and mixed infection have been detected by means of amplification and sequencing of housekeeping genes *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS* for the first time. Further studies based on these housekeeping genes are needed to explore their potential applications for *H. pylori* detection in different specimens, the mode of transmission of *H. pylori*, the role associated to virulence or the phylogeographic differentiation of *H. pylori* populations.

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Competing interests: The authors have declared that no competing interests exist.

Note

The GenBank/EMBL/DDBJ accession numbers for the *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ* and *luxS* gene sequences are KU053341–58, KU053359–76, KU053377–94, KU053395–412, KU053413–30 and KU053431–48, respectively.

References

1 Correa P, Houghton J. Carcinogenesis of *Helicobacter pylori*. *Gastroenterology* 2007;133:659–72.
2 Mobley HLT, Mendz GL, Hazell SL. *Helicobacter pylori:* Physiology and Genetics. Washington (DC): ASM Press, 2001.
3 Mascellino MT, Margani M, Oliva A. *Helicobacter pylori*: determinant and markers of virulence. *Dis Markers* 2009;27:137–56.
4 Backert S, Clyne M. Pathogenesis of *Helicobacter pylori* infection. *Helicobacter* 2011;16(Suppl. 1):19–25.
5 González CA, Figueiredo C, Lic CB, Ferreira RM, Pardo ML, Ruiz Liso JM, et al. *Helicobacter pylori cagA* and *vacA* genotypes as predictors of progression of gastric preneoplastic lesions: a long-term follow-up in a high-risk area in Spain. *Am J Gastroenterol* 2011;106:867–74.
6 Linz B, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, et al. An African origin for the intimate association between humans and *Helicobacter pylori*. *Nature* 2007;445:915–8.
7 Moodley Y, Linz B, Yamaoka Y, Windsor HM, Breurec S, Wu JY, et al. The peopling of the Pacific from a bacterial perspective. *Science* 2009;323:527–30.
8 Sheu SM, Sheu BS, Lu CC, Yang HB, Wu JJ. Mixed infections of *Helicobacter pylori*: tissue tropism and histological significance. *Clin Microbiol Infect* 2009;15:253–9.

9 Talarico S, Gold BD, Fero J, Thompson DT, Guarner J, Czinn S, et al. Pediatric *Helicobacter pylori* isolates display distinct gene coding capacities and virulence gene marker profiles. *J Clin Microbiol* 2009;47:1680–8.

10 Khalifa MM, Sharaf RR, Aziz RK. *Helicobacter pylori*: a poor man's gut pathogen? *Gut Pathog* 2010;2:2.

11 Chaput C, Ecobichon C, Cayet N, Girardin SE, Werts C, Guadagnini S, et al. Role of AmiA in the morphological transition of *H. pylori* and in immune escape. *PLoS Pathog* 2006;2:e97.

12 Di Felice V, David S, Cappello F, Farina F, Zummo G. Is chlamydial heat shock protein 60 a risk factor for oncogenesis? *Cell Mol Life Sci* 2005;62:4–9.

13 McGee DJ, George AE, Trainor EA, Horton KE, Hildebrandt E, Testerman TL. Cholesterol enhances *H. pylori* resistance to antibiotics and LL-37. *Antimicrob Agents Chemother* 2011;55:2897–904.

14 Lario S, Ramírez-Lázaro MJ, Aransay AM, Lozano JJ, Montserrat A, Casalots Á, et al. microRNA profiling in duodenal ulcer disease caused by *Helicobacter pylori* infection in a Western population. *Clin Microbiol Infect* 2012;18:E273–82.

15 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;30:2725–9.

16 Villesen P. FaBox: an online toolbox for FASTA sequences. *Mol Ecol Notes* 2007;7:965–8.

17 Ramírez-Lázaro MJ, Lario S, Casalots A, Sanfeliu E, Boix L, García-Iglesias P, et al. Real-time PCR improves *Helicobacter pylori* detection in patients with peptic ulcer bleeding. *PLoS One* 2011;6:e20009.

18 Wang YK, Kuo FC, Liu CJ, Wu MC, Shih HY, Wang SS, et al. Diagnosis of *Helicobacter pylori* infection: current options and developments. *World J Gastroenterol* 2015;21:11221–35.

19 Falush D, Kraft C, Taylor NS, Correa P, Fox JG, Achtman M, et al. Recombination and mutation during long-term gastric colonization by *Helicobacter pylori*: estimates of clock rates, recombination size, and minimal age. *Proc Natl Acad Sci USA* 2001;98:15056–61.

20 Raymond J, Thiberg JM, Chevalier C, Kalach N, Bergeret M, Labigne A, et al. Genetic and transmission analysis of *Helicobacter pylori* strains within a family. *Emerg Infect Dis* 2004;10:1816–21.

21 Ghose C, Pérez-Pérez GI, van Doorn LJ, Domínguez-Bello MG, Blaser MJ. High frequency of gastric colonization with multiple *Helicobacter pylori* strains in Venezuelan subjects. *J Clin Microbiol* 2005;43:2635–41.

22 Kennemann L, Didelot X, Aebischer T, Kuhn S, Drescher B, Droege M, et al. *Helicobacter pylori* genome evolution during human infection. *Proc Natl Acad Sci USA* 2011;108:5033–8.

23 Toita N, Yokota S, Fujii N, Konno M. Clonality analysis of *Helicobacter pylori* in patients isolated from several biopsy specimens and gastric juice in a Japanese urban population by random amplified polymorphic DNA fingerprinting. *Gastroenterol Res Pract* 2013;2013:721306.

24 Linz B, Windsor HM, McGraw JJ, Hansen LM, Gajewski JP, Tomsho LP, et al. A mutation burst during the acute phase of *Helicobacter pylori* infection in humans and rhesus macaques. *Nat Commun* 2014;5:4165.

25 Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, et al. Opinion: re-evaluating prokaryotic species. *Nat Rev Microbiol* 2005;3:733–9.

26 Tindall BJ, Rosselló-Móra R, Busse HJ, Ludwig W, Kämpfer P. Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol* 2010;60:249–66.

27 Suzuki R, Shiota S, Yamaoka Y. Molecular epidemiology, population genetics, and pathogenic role of *Helicobacter pylori*. *Infect Genet Evol* 2012;12:203–13.

28 Hill JE, Paccagnella A, Law K, Melito PL, Woodward DL, Price L, et al. Identification of *Campylobacter* spp. and discrimination from *Helicobacter* and *Arcobacter* spp. by direct sequencing of PCR-amplified *cpn60* sequences and comparison to cpnDB, a chaperonin reference sequence database. *J Med Microbiol* 2006;55:393–9.

29 Miñana-Galbis D, Farfán M, Lorén JG, Fusté MC. The reference strain *Aeromonas hydrophila* CIP 57.50 should be reclassified as *Aeromonas salmonicida* CIP 57.50. *Int J Syst Evol Microbiol* 2010;60:715–7.

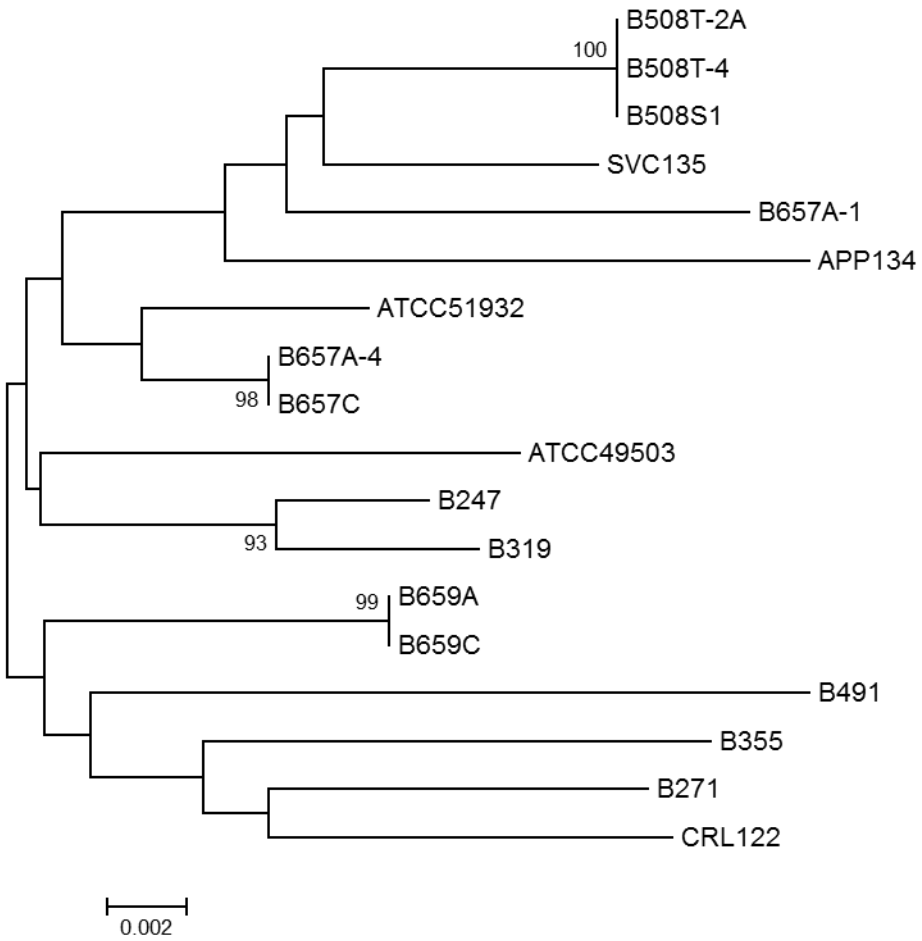
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Consensus neighbor-joining phylogenetic trees obtained from 18 *H. pylori* sequences of genes *cgt* (A), *cpn70* (B), and *dnaJ* (C). Bar distance values as calculated by MEGA 6.0. Bootstrap values (>50%) after 1000 replicates are shown as percentages.

Table S1 *Helicobacter pylori* complete genome sequences used to primer design.

Figure S1 (A)


B508T-2A
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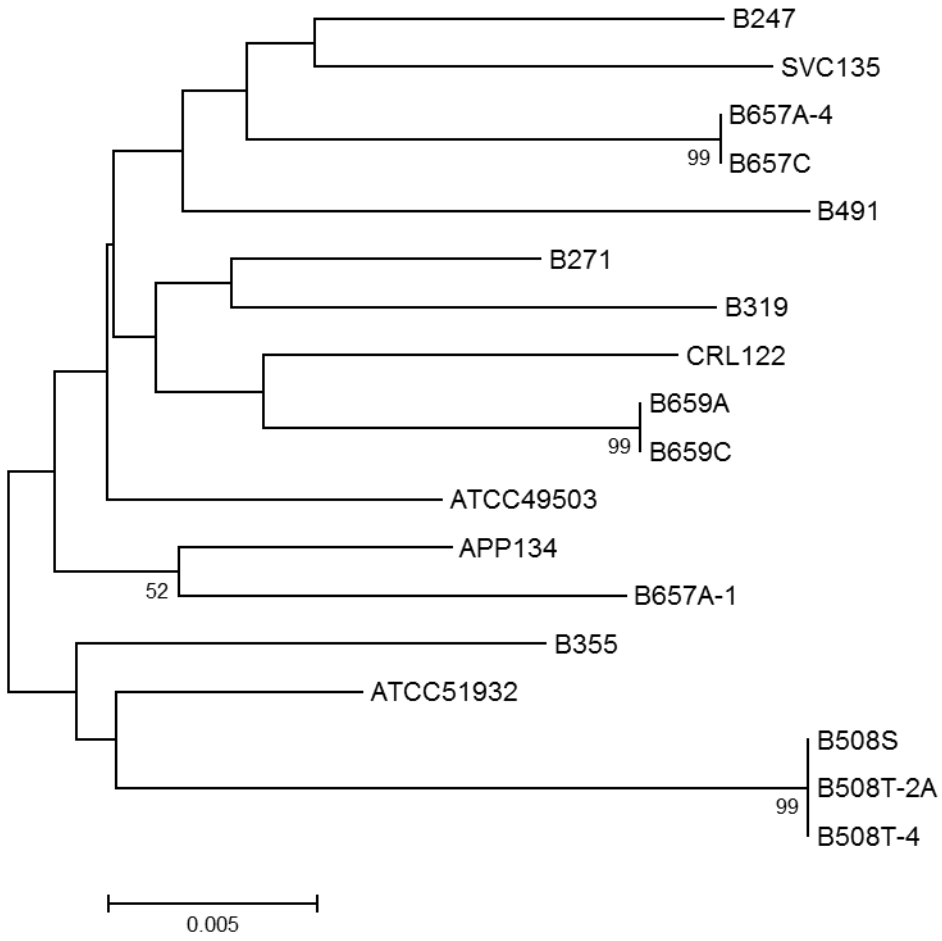
B508T-4
B508S1
SVC135
B657A-1
APP134
ATCC51932
B657A-4
98
B657C
ATCC49503
B247
93
B319
99
B659A
B659C
B491
B355
B271
CRL122
0.002

Figure S1 (B)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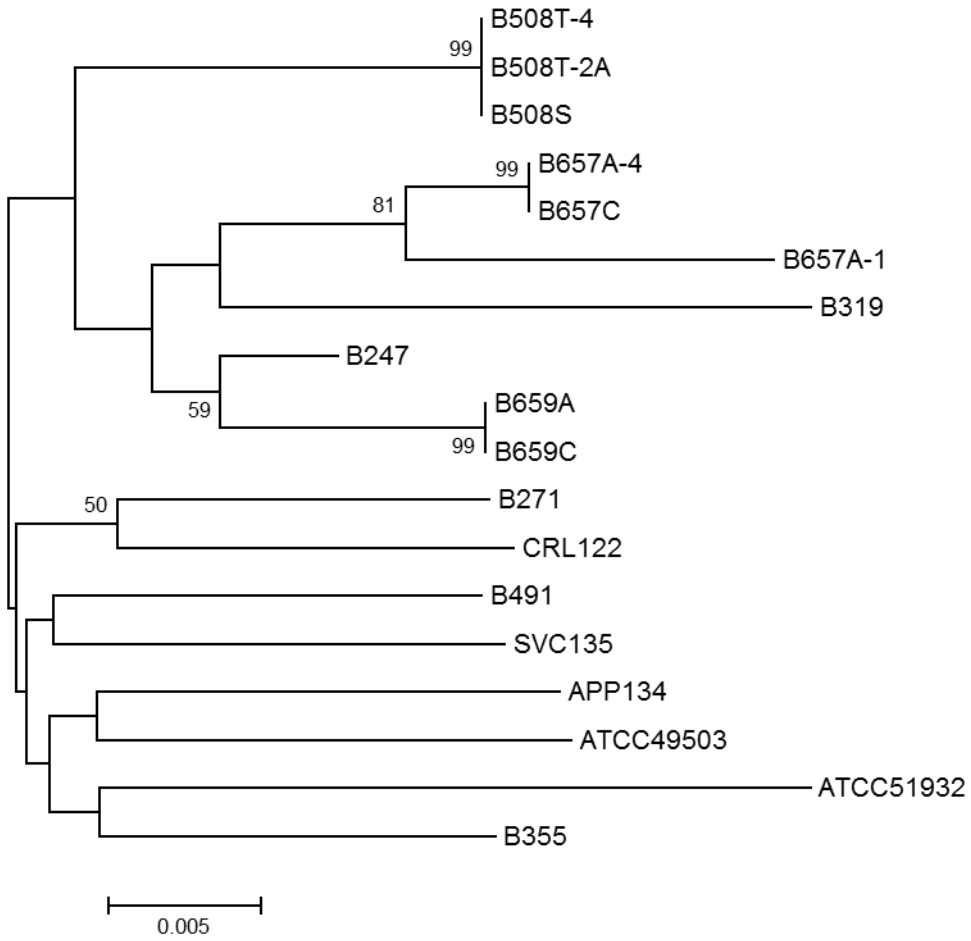
B657A-4
99
B657C
B491
B271
B319
CRL122
B659A
99
B659C
ATCC49503
APP134
52
B657A-1
B355
ATCC51932
B508S
B508T-2A
99
B508T-4
0.005

Figure S1 (C)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B508T-2A
B508S
99
B657A-4
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B657C
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B319
B247
59
B659A
99
B659C
50
B271
CRL122
B491
SVC135
APP134
ATCC49503
ATCC51932
B355
0.005

Table S1 *H. pylori* complete genome sequences used to primer design

***H. pylori* strain**	**Reference sequence number**
51	CP000012
52	CP001680
83	CP002605
908	CP002184
2017	CP002571
2018	CP002572
26695	CP003904
26695	NC_000915
35A	CP002096
Aklavik117	CP003483
Aklavik86	CP003476
B38	NC_012973
B8	NC_014256
Cuz20	CP002076
ELS37	CP002953
F16	AP011940
F30	AP011941
F32	AP011943
F57	AP011945
G27	NC_011333
Gambia94/24	CP002332
HPAG1	NC_008086
HUP-B14	NC_017733
India7	CP002331
J99	NC_000921
Lithuania75	CP002334
P12	NC_011498
PeCan18	NC_017742
PeCan4	NC_014555
Puno120	CP002980
Puno135	CP002982
Rif1	CP003905
Rif2	CP003906
Sat464	CP002071
Shi112	NC_017741
Shi169	NC_017740
Shi417	NC_017739
Shi470	NC_010698
SJM180	NC_014560
SNT49	CP002983
SouthAfrica7	CP002336
v225d	CP001582
XZ274	CP003419