

Description of 'Candidatus Helicobacter heilmannii' based on DNA sequence analysis of 16S rRNA and urease genes

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While *Helicobacter pylori* is accepted as the major bacterial agent of gastric disease in humans, some patients and many animals are infected with a larger, tightly helical-shaped bacterium previously referred to as '*Helicobacter heilmannii*' or '*Gastrospirillum hominis*'. Taxonomic classification of these bacteria has been hampered by the inability to cultivate them *in vitro* and by the inadequate discriminatory power of 16S rRNA gene sequence analysis. This study describes the detection and phylogenetic analysis of 26 different gastrospirillum isolates from humans and animals, which incorporates sequence data based on the 16S rRNA and urease genes. Fifteen gastrospirilla detected in humans, primates and pigs clustered with '*Candidatus Helicobacter suis*', thus expanding the host range for this organism. By comparison, based on 16S rRNA data, the remaining 11 gastrospirilla could not be differentiated from *Helicobacter felis*, *Helicobacter bizzozeronii* and *Helicobacter salomonis*. However, urease gene sequence analysis allowed for the discrimination of this latter group into four discrete clusters, three of which contained the above recognized species. The fourth cluster contained isolates from human and feline hosts, and should provisionally be considered a unique bacterial species, for which the name '*Candidatus Helicobacter heilmannii*' is proposed.

INTRODUCTION

The discovery of *Helicobacter pylori* in 1983 and the illustration of its role in the aetiology of gastric disease refocused attention on the stomach as a site capable of harbouring autochthonous bacteria (Marshall, 1983; Warren, 1983). Studies of the gastric mucosa of animals, however, revealed they were colonized with organisms that are quite distinct from *H. pylori* (Solnick *et al.*, 2003). The bacterium most commonly seen is a large helical-shaped organism with three to eight turns (Baskerville & Newell, 1988; Curry *et al.*, 1987; Henry *et al.*, 1987; Lee *et al.*, 1988). Dent *et al.* (1987) reported the presence of these larger organisms

in a small number of patients presenting with upper-gastrointestinal symptoms. These bacteria were referred to as '*Gastrospirillum hominis*' (McNulty *et al.*, 1989), adding to the plethora of historic names that have been used to describe them (Bergey, 1948). A phylogenetic study of two such gastrospirillum isolates from humans found that they were most closely related to *Helicobacter felis* (Solnick *et al.*, 1993). The name '*Helicobacter heilmannii*' was subsequently proposed in honour of the German pathologist Konrad Heilmann, who described a large study of these bacteria in humans (Heilmann & Borchard, 1991). However, analysis of the 16S rRNA gene sequences derived from clones from each patient showed they were not identical (96.6% similarity) and they subsequently became known as '*H. heilmannii*' type one (clone G1A1 from patient one) and type two (clone G2A9 from patient two).

Neither '*G. hominis*' nor '*H. heilmannii*' currently has any official standing in bacterial nomenclature. Morphologically similar organisms have, however, been validly described in cats and dogs (*H. felis* and *Helicobacter bizzozeronii*; Hanninen *et al.*, 1996; Paster *et al.*, 1991) and pigs ('*Candidatus Helicobacter suis*'; De Groote *et al.*, 1999). These gastric helicobacters, together with *Helicobacter salomonis*

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Abbreviations: UFP, universal eubacterial forward primer; URP, universal eubacterial reverse primer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *ureAB* gene sequences reported in this study can be found in the figure legends.

Transmission electron micrographs of gastric specimens and a table listing the primers used for sequencing and PCR are available as supplementary material in IJSEM Online.

(Jalava *et al.*, 1997), form a distinct subgroup based on their 16S rRNA gene sequence data (De Groot *et al.*, 1999). Although 16S rRNA gene sequence analysis is an extraordinarily useful phylogenetic tool, in this instance it is not sufficiently sensitive to discriminate among known unique species. For example, *H. felis*, *H. bizzozeronii*, *H. salomonis* and the '*H. heilmannii*' type two isolate share > 98 % 16S rRNA gene sequence similarity (Hanninen *et al.*, 1996; Jalava *et al.*, 1997; Paster *et al.*, 1991).

In order to further discriminate among the gastrospirilla, alternative taxonomic methodologies were sought. One possible method is the examination of sequence similarity clustering in protein-encoding genes (Palys *et al.*, 1997). The rationale behind this methodology is that highly conserved genes, such as the 16S rRNA gene, may not reflect recent adaptations of specific groups of bacteria to their distinct ecological niches, an evolutionary factor that could be detected by analysis of certain protein-encoding genes. This method has been successfully used to describe other species, especially those sharing very high or almost identical levels of 16S rRNA gene sequence similarity (Ambler, 1996; Palys *et al.*, 2000, 1997; Soini *et al.*, 1994). The urease gene was chosen for further phylogenetic study of the gastrospirilla as all gastric helicobacters possess this enzyme; it is critical for their survival in the acidic environment of the stomach, and is also implicated in their pathogenesis. The goal of this study was to examine the 16S rRNA and *ureAB* gene sequences from a large number of gastrospirilla derived from diverse hosts, in order to clarify the phylogeny of these bacteria.

METHODS

Human isolates. Human isolates were obtained from patients presenting at endoscopy clinics with upper-gastrointestinal symptoms in which bacteria with a '*H. heilmannii*'-like morphology, rather than *H. pylori*, were found. Patient details for three of the isolates (HU1–3) have been published previously (Lee *et al.*, 1989; Solnick *et al.*, 1993). Isolates four (HU4) and five (HU5) were obtained from 31- and 71-year-old females, respectively, who were diagnosed with mild antral gastritis (biopsies courtesy of Dr G. Daskalopoulos, Sydney and Dr Roberto Fiocca, Pavia, Italy, respectively). Isolates one to four were obtained from patients in Sydney and Canberra, Australia, and isolate five from a patient in Pavia, Italy.

Animal isolates. The gastric mucosa from 29 animals that had died from natural causes at Taronga Zoo, Sydney, was screened for the presence of large helical bacteria by direct phase microscopy and urease assay. Additional isolates were obtained from gastric biopsies obtained from three cheetahs undergoing health checks at Taronga Zoo and from a Rhesus macaque housed at the California National Primate Research Centre (University of California, Davis, CA). Ten pigs, obtained from a local slaughterhouse (Burns Pet Foods, Sydney, Australia), were also screened for the presence of gastrospirilla.

Reference strains. For comparative purposes, DNA was extracted (Puregene DNA Purification Kit; Gentra Systems) from the following organisms: *H. pylori* strains 26695, the Sydney strain (SS1) and four fresh clinical strains (OB, PA, DA and MB) (Lee *et al.*, 1997; Tomb *et al.*, 1997); *H. felis* strains CS1^T (ATCC 49179^T), CS2, DS2, DS5 and Into (Jalava *et al.*, 1999a; Paster *et al.*, 1991); *H. bizzozeronii*

CCUG 35545^T and CCUG 35546 (Hanninen *et al.*, 1996); *H. salomonis* CCUG 37845^T and CCUG 37848 (Jalava *et al.*, 1997); a clinical strain of *Campylobacter jejuni* (clinical strain); and a clinical strain of *Campylobacter coli* (clinical strain). In addition, sequences for the 16S rRNA gene from seven additional *Helicobacter* strains were obtained from GenBank. To avoid confusion, throughout this manuscript we refer to bacteria that can be cultivated *in vitro* as 'strains', while uncultivated bacteria are referred to as 'isolates'.

In vivo culture. Mucus scrapings from animals and human patients showing the presence of gastrospirilla were homogenized and inoculated into specific-pathogen-free mice, as described elsewhere (Dick *et al.*, 1989; Lee *et al.*, 1989).

In vitro culture. Attempts were made to culture the gastrospirilla using methods described elsewhere (Hanninen *et al.*, 1996; Jalava *et al.*, 1997; Paster *et al.*, 1991). Briefly, gastric mucus scrapings from the host animal, patient or infected mouse were inoculated onto a 0.65 µm filter placed on non-selective blood agar plates (Blood agar base no. 2; Oxoid) or directly onto selective agar plates [Blood agar supplemented with Skirrow's selective supplement (Oxoid) and Fungizone (Squibb & Sons)]. A number of other agar bases, including brain heart infusion and Brucella medium, were also tested. All plates were incubated for up to 14 days at 37 °C under both microaerobic and anaerobic conditions. For primary gastric samples, culture was undertaken as soon as feasible, which varied from hours to 1–2 days after the collection of the biopsy specimens or the death of the animal. Numerous culture attempts were also made using freshly obtained gastric samples from the infected mice.

Histology and electron microscopy. Fresh stomach biopsies from the original host or infected mice were fixed in 10 % formalin for routine histology and Karnovsky's fixative for electron microscopy (Karnovsky, 1965). Formalin-fixed samples were stained with May–Grunwald–Giemsa or a modified Steiner silver stain to assess colonization. Samples for electron microscopy were embedded in Spurr's Epoxy Resin (Polysciences), stained with uranyl acetate and lead citrate, and viewed with an Hitachi 7000 transmission electron microscope. Any bacterial cultures successfully cultivated were negatively stained with 1 % phosphotungstic acid and viewed as above.

DNA extraction. DNA was extracted directly from gastric biopsies of infected patients or animals or from the gastric mucosa of infected mice using the Puregene DNA Purification Kit. The DNA from the isolates originating in the USA and Italy (HU5 and CM2) was extracted from frozen gastric biopsies using the QIAamp Tissue Kit (Qiagen) (Solnick *et al.*, 1993). The DNA was stored at –20 °C until required.

PCR amplification and sequencing. PCR amplification reactions involved 1 × reaction buffer [67 mM Tris/HCl, 16 mM (NH₄)₂SO₄, 0.45 % Triton X-100, 0.2 % gelatin], one unit of *Taq* DNA polymerase (Biotech International), 200 µM of each deoxynucleotide triphosphate, 2 mM MgCl₂, 10 pmol of each oligonucleotide primer and 1 µl diluted DNA (usually a 1:10 dilution of original sample containing approximately 20–100 ng µl⁻¹) in a final volume of 50 µl. The amplification parameters for each specific reaction are listed below. All reactions were carried out using a Perkin Elmer PE2400 thermocycler. PCR products were separated on agarose mini-gels in TAE buffer (40 mM Tris/acetate, 1 mM EDTA) and photographed under UV transillumination after staining with ethidium bromide.

PCR products were purified prior to sequencing with Wizard PCR Preps DNA Purification System (Promega) or Centricon-100 filters (Amicon). The amplified DNA was then directly sequenced using the ABI PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems) and the GeneAmp[®] PCR System 2400

(Perkin Elmer) according to the manufacturers' protocols. Sequencing products were separated on model 377 DNA Sequencer machines and analysed using programs contained in the INHERIT package (PE Applied Biosystems). In all cases both strands of DNA were sequenced with contiguous overlaps.

Amplification and sequencing of the 16S rRNA gene. Two PCRs were used to amplify *Helicobacter*-genus-specific DNA directly from the gastric samples. These reactions each incorporated one universal eubacterial primer for the 16S rRNA gene and one *Helicobacter*-specific primer, namely 27F (universal eubacterial forward primer, UFP) with H676R (*Helicobacter*-16S rRNA reaction one) and 1494R (universal eubacterial reverse primer, URP) with H276R (*Helicobacter*-16S rRNA reaction two) (Neilan *et al.*, 1997; Riley *et al.*, 1996; Weisburg *et al.*, 1991). The cycling conditions for the *Helicobacter*-16S rRNA reaction one were initial denaturation at 94 °C for 4 min, then 35 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 1.5 min, followed by a final extension step of 72 °C for 5 min. The conditions for the *Helicobacter*-16S rRNA reaction two were identical except the annealing was performed at 45 °C for 40 s. This allowed for the sequencing of the near complete 16S rRNA gene using primers listed in Table S1 (available as supplementary material in IJSEM Online) as appropriate.

Amplification and sequencing of the urease gene. Amplification of either a 1224 bp or a 1752 bp section of the *ureA* and *ureB* genes (including the intergenic spacer region) from the gastrospirilla and a number of reference strains was performed by the use of primers designed in this study (Table S1, IJSEM Online). Amplification of the urease genes was achieved by the use of either primers U430F and U1735R (urease one, 1224 bp) or primers U430F and U2235R (urease two, 1752 bp). The cycling conditions for the urease one reaction were initial denaturation at 94 °C for 3 min, then 35 cycles of 94 °C for 10 s, 52 °C for 30 s and 72 °C for 1.5 min, followed by a final extension step of 72 °C for 5 min. For the urease two reaction the conditions were as above with the following modifications: annealing at 42 °C for 30 s and extension at 72 °C for 2 min. These products were then sequenced with primers listed in Table S1 (in IJSEM Online).

Urease-based PCR for the putative gastrospirillum species. Analysis of the urease sequences of the isolates and strains in this study permitted identification of regions of the *ureA* gene that differed between the gastrospirillum isolates, *H. felis*, *H. bizzozeronii* and *H. salomonis*. Using this information, PCRs specific for the type one and type two isolates were designed, employing oligonucleotides T1ureF and T1ureR, and T2ureF and T2ureR, respectively (Table S1, supplementary material). The cycling conditions for the type one PCR were an initial denaturation at 94 °C for 5 min, 35 cycles of amplification (94 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min), followed by 72 °C for 4 min. A similar protocol was used for the type two reaction with the exception that the annealing temperature was increased to 58 °C.

Phylogenetic analysis. Sequences were aligned using the GCG program PILEUP, version 8 (Genetics Computer Group, Program Manual for the Wisconsin Package, 1994) and the multiple-sequence alignment and profile alignment tools in the CLUSTAL W package (Thompson *et al.*, 1994). Aligned sequences were corrected manually and nucleotide positions that contained ambiguities were removed from further analyses. Genetic distances, corrected for multiple base changes by the method of Jukes & Cantor (1969), were calculated using the DNADIST program in the PHYLIP package, version 3.57c (Felsenstein, 1989). The phylogenetic tree was reconstructed by the neighbour-joining method of Saitou & Nei (1987), and the tree was plotted using NJplot from within the CLUSTAL W package. Bootstrap values were obtained from analysis of 1000 resamplings of the corrected alignment using the programs SEQBOOT and CONSENSE

(PHYLIP package) (Felsenstein, 1985). Trees were also constructed using parsimony (DNAPARS) and maximum-likelihood (DNAML) algorithms (PHYLIP package).

RESULTS AND DISCUSSION

Morphology, ultrastructure and bacterial culture

Examination revealed the presence of helical-shaped bacteria in the gastric mucus from 22 of the 33 animals from the zoo and primate centre, five of the ten pigs from the slaughterhouse and the five patients. The majority of the bacteria observed had a similar ultrastructural appearance. They were large (5–6 µm × 0.5–0.6 µm) with a distinctive helical shape consisting of five to seven turns and bipolar tufts of sheathed flagella. Examples of the ultrastructural appearance of some of these isolates are available in IJSEM Online as supplementary material (Fig. S1).

Using the *in vivo* culture technique, isolates of gastrospirilla were obtained from two mandrill monkeys (*Papio sphinx*) (isolates MM1 and MM2), two crab-eating macaques (*Macaca fascicularis*) (isolates CM1 and CM2), a Rhesus macaque (*Macaca mulatta*) (isolate RM1), a bobcat (*Felis rufus*) (isolate BC1), a New Guinea wild dog (*Canis familiaris*) (isolate WD1), five pigs (*Sus scrofa*) (isolates P1 to P5) and four human patients (isolates HU1 to HU4). In addition, DNA was obtained directly from gastric biopsies from six additional animals and one human patient (HU5). The animals included a white tiger (*Panthera tigris*) (isolate T1), an Asian dhole (*Cuon alpinus*) (isolate AD1), six individual biopsies from three different cheetahs (*Acinonyx jubatus*) (isolates C2S, C2E, C3S, C3E, C4S, C4E) and an additional Rhesus macaque (isolate RM2). All isolates were obtained from Australia except for isolates HU5 (Italy) and RM2 (USA).

Attempts to culture the bacteria *in vitro* were all unsuccessful, except in the case of mice infected with mucus from the New Guinea wild dog. From this sample a thin water-like film was detected on blood agar plates after 3–5 days incubation under microaerobic conditions. Electron microscopic analysis of the cultured bacterium and of gastric tissue obtained from the wild dog both showed a bacterium that morphologically resembled *H. felis*, i.e. a large helical organism with bipolar flagella and periplasmic fibres in pairs.

Analysis of the 16S rRNA gene

Using the two *Helicobacter*-genus-specific PCRs we amplified a 627 bp and a 1213 bp region of the 16S rRNA gene, including a 376 bp overlapping area. No amplicons were obtained from the closely related *Campylobacter* species (data not shown). This species specificity allowed direct sequencing of the PCR products without the need to clone them. In addition, sequencing of multiple isolates from infected mice showed that the length of colonization in the mouse and passing isolates in mice did not affect the 16S

rRNA gene sequence to a significant level (0–8 bp differences over 1464 bp, 0–0.5%). The 16S rRNA gene sequences from the 26 isolates in this study have been deposited in GenBank/EMBL/DDBJ with the accession numbers shown in the legend to Fig. 1.

The phylogenetic relationship among the 26 gastrospirilla and other *Helicobacter* species was determined by pairwise distance matrix, parsimony and maximum-likelihood methods. All three algorithms gave similar results in topology and geometry (data not shown). A phylogenetic tree was reconstructed from the calculated genetic distances (Fig. 1). It divides the 26 isolates into two discrete clusters. Cluster 1 contained four of the isolates obtained from humans, and all those obtained from the mandrill monkeys, the crab-eating macaques, the Rhesus macaques, the pigs and 'Candidatus *H. suis*'. These isolates all showed a very high degree of sequence homology, $\geq 99.3\%$. This high degree of sequence homology was also seen when our data were compared to other studies in pigs (Cantet *et al.*, 1999; Choi *et al.*, 2001; De Groote *et al.*, 2000; Roosendaal *et al.*, 2000). Cluster 2 included human isolate two (HU2), along with isolates from the cheetahs, the bobcat, the Asian dhole, the tiger, the wild dog, an isolate from a cat (Norris *et al.*, 1999), the putative '*H. heilmannii*' isolate cultured from a human (subsequently shown to be *H. bizzozeronii*) (Andersen *et al.*, 1999; Jalava *et al.*, 2001) and the recognized species *H. salomonis*, *H. felis* and *H. bizzozeronii*. All these bacteria showed levels of sequence homology between 95 and 98%. Clusters 1 and 2 were distinct from other gastric helicobacters including *H. pylori*, *Helicobacter acinonychis*, *Helicobacter mustelae* and 'Candidatus *Helicobacter bovis*'.

Analysis of the urease gene

The 16S rRNA gene sequence analysis showed that cluster 2 was more heterogeneous than cluster 1, and contained more than one recognized species. Previous investigations have demonstrated that light microscopy studies and analysis of the 16S and 23S rRNA genes do not allow for the discrimination of the recognized species within cluster 2 (Jalava *et al.*, 1999b). We therefore sought to use an alternative method that might provide increased resolution. We chose to examine a protein-encoding gene (Palys *et al.*, 1997), since previous work has shown that this strategy can group bacterial strains into discrete similarity clusters that correspond to ecologically distinct populations. The basic premise is that the mean sequence divergence between strains of the same cluster is less than that seen between strains of different clusters. Few organisms fall in between these clusters so that the sequence clusters correspond to recognized species or subspecies (Ambler, 1996; Cohan, 1994). This is due to 'periodic selection' whereby natural selection favours adaptive alleles thus purging diversity at specific loci. We selected the urease gene for further study because this enzyme is central to the gastric helicobacters for metabolism and virulence.

Preliminary attempts to amplify a section of the urease gene complex from the gastrospirilla in this study, using previously described methods, were unsuccessful (Neiger *et al.*, 1998; Solnick *et al.*, 1994). We therefore designed new primers (U430F, U1735R and U2235R), based on consensus regions in the urease gene sequences for *H. pylori*, *H. felis* and '*H. heilmannii*' (GenBank/EMBL/DDBJ accession nos M60398, X69080 and L25079, respectively), that allowed the amplification of an approximately 1500–1600 bp region of the urease gene complex. Further oligonucleotides for sequencing of this region were designed by primer walking (Table S1 in IJSEM Online).

The section of the urease gene complex that was amplified included ~75% of the *ureA* gene (3' end), ~62% of the *ureB* gene (5' end) and the intergenic spacer region. This region was successfully amplified and sequenced for 18 of the isolates in this study. The same segment of the urease gene complex was amplified and sequenced from five *H. felis* strains, two *H. bizzozeronii* strains, two *H. salomonis* strains and six *H. pylori* strains, to test the integrity of this method as a phylogenetic tool. A corresponding region of the *ureA* and *ureB* DNA sequence for *H. mustelae* and *Helicobacter hepaticus* was obtained from GenBank. The phylogenetic relationships determined by the genetic distance method (as shown in Fig. 2) were representative of results obtained by maximum-parsimony and maximum-likelihood methods (data not shown). In contrast to the data obtained from sequence analysis of the 16S rRNA gene, five clusters were apparent from analysis of the urease sequences: '*H. heilmannii*' type one isolates (cluster A), '*H. heilmannii*' type two isolates (cluster B), *H. bizzozeronii* (cluster C), *H. felis* (cluster D) and *H. salomonis* (cluster E). These five clusters were also distinct from other known *Helicobacter* species. Therefore, urease sequence analysis permitted discrimination of the 16S rRNA cluster 2 into four discrete clusters, three of which (C, D and E) contained a single recognized species and thus preserved the integrity of known phylogenetic relationships.

Examination of multiple strains within the individual species studied (*H. pylori*, *H. felis*, *H. bizzozeronii* and *H. salomonis*) and the '*H. heilmannii*' type one and two isolates showed that the level of urease gene sequence similarity within a species or group of isolates was very high (>97%). However, the value was much lower between the different recognized and putative species. For example '*H. heilmannii*'-like isolates (type one or two) showed sequence similarities of ~73–76%, 77–80%, 78–80% and 81–83% with *H. pylori*, *H. felis*, *H. salomonis* and *H. bizzozeronii*, respectively. In agreement with previous studies, the intergenic spacer regions for *H. pylori* and *H. felis* were 3 bp and 9 bp, respectively (Ferrero & Labigne, 1993; Labigne *et al.*, 1991). Five of the cheetah isolates and the isolate from the wild dog also had an intergenic spacer region of 9 bp. The remaining gastrospirilla, proposed to be representative of either type one or type two isolates, had an intergenic spacer

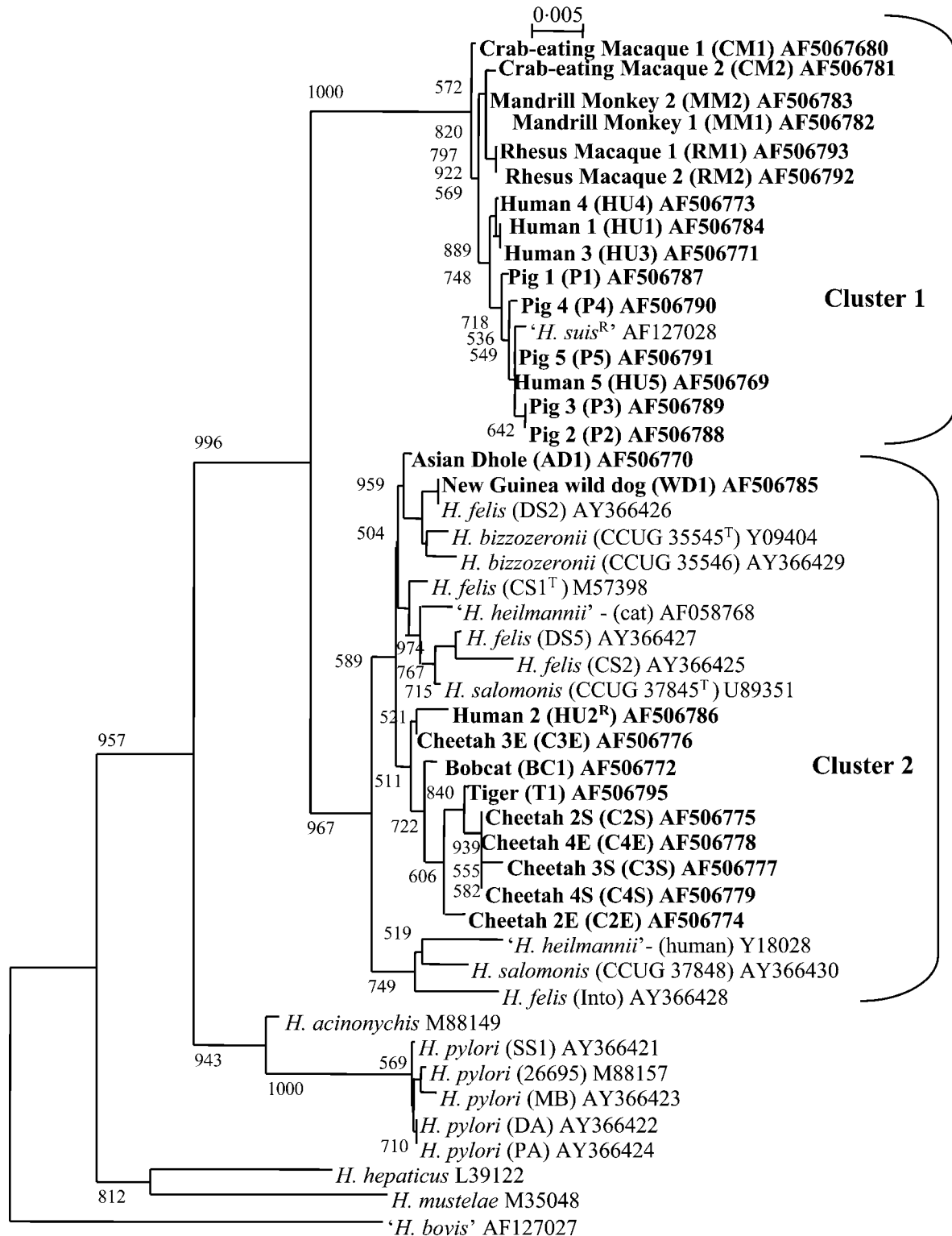


Fig. 1. A phylogenetic tree, reconstructed from genetic distances, based on the near-complete 16S rRNA gene sequences for the gastrospirilla (shown in bold) and other closely related bacteria. Bootstrap values (for branches present in more than 50% of 1000 resamplings of the data) are indicated at the nodes. Two major clusters of gastrospirilla, the '*H. heilmannii*'-like bacteria and the *H. felis*-like bacteria, can be seen.

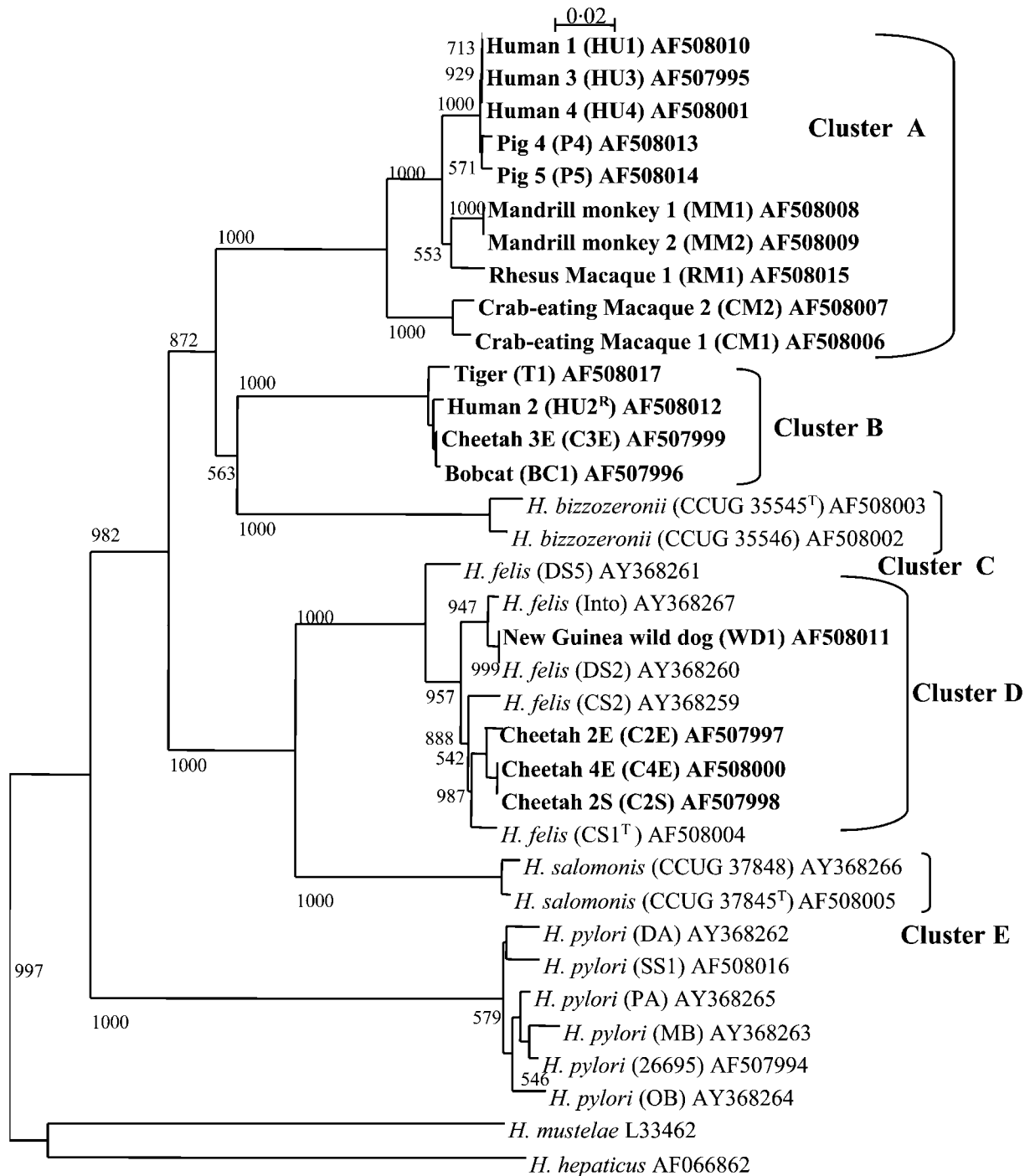


Fig. 2. A phylogenetic tree, reconstructed from genetic distances, based on the partial *ureA* and *ureB* gene sequences for the gastrospirilla (shown in bold) and other closely related bacteria. Bootstrap values (for branches present in more than 50% of 1000 resamplings of the data) are indicated at the nodes. The gastrospirilla could be differentiated into five clusters, the '*H. heilmannii*'-like type one/'*Candidatus Helicobacter suis*' strains, '*H. heilmannii*'-like type two strains, *H. felis*, *H. bizzozeronii* and *H. salomonis*.

region of 14 bp, compared to 15 bp for *H. bizzozeronii* and 10 bp for *H. salomonis*.

Urease-based PCRs for the putative '*H. heilmannii*' species

Two urease-based species-specific PCRs were designed in this study. The type one reaction (primers T1ureF and T1ureR) successfully amplified a 323 bp product from the '*H. heilmannii*' type one isolates originating from primates, pigs and humans (Fig. 2, cluster A). By comparison, the type two reaction (primers T2ureF and T2ureR) amplified a 376 bp product from the four '*H. heilmannii*' type two isolates only (Fig. 2, cluster B). The strain from the wild dog (WD1), *H. pylori* (26695) and the type strains of *H. felis*, *H. bizzozeronii* and *H. salomonis* were negative in both reactions. Therefore, the urease sequence analysis permitted the development of PCR probes that enable the detection of the uncultivated species directly from gastric material.

Proposal regarding the taxonomy of type one and type two gastrospirilla

Data from analysis of the urease gene (phylogenetic trees and species-specific PCRs) provide further evidence for the existence of several species within this collection of gastrospirillum isolates. The isolates from the primates, pigs and humans (Fig. 2, cluster A, which corresponds with cluster one in Fig. 1) could be easily differentiated from other isolates. Together with the 16S rRNA results, these data suggest that this group of bacteria, the '*H. heilmannii*' type one gastrospirilla, constitutes a single species, and, in accordance with current taxonomic regulations, should be described as '*Candidatus H. suis*'. This study thus greatly expands the host range for these isolates compared to their original description in pigs (De Groote *et al.*, 1999).

On the other hand, the strains and isolates belonging to 16S rRNA cluster two (Fig. 1) could be further differentiated into four clusters (clusters B–E, Fig. 2) by urease DNA sequence. Three of these clusters (C, D and E) correspond to known species and the fourth cluster (B) constitutes a putative new species. While the isolates in cluster B (type two isolates) are closest, both morphologically and genetically, to *H. bizzozeronii* (cluster C), several aspects of the data suggest that they are distinct: (1) a significant difference in their urease sequence homology (82–84%); (2) a size difference in their intergenic spacer regions; (3) *H. bizzozeronii* was negative in the type-two-specific urease PCR; (4) type two isolates are as yet uncultivable; and (5) type two isolates are seen commonly in feline rather than canine hosts. The difference in host range between clusters B and C is also supported by the extensive electron microscopic studies by Weber & Schmittiel (1962), in which the size and number of coils of spiral bacteria from cats and dogs were measured. They described two dominant forms, a thicker, shorter form in dogs (*H. bizzozeronii*

cluster C in our study) and a longer, thinner form in cats (cluster B in our study).

Clusters D and E corresponded with *H. felis* and *H. salomonis*, respectively. *H. salomonis* can be differentiated from the other gastrospirilla due to its slightly different morphology. Based on urease gene sequence similarities and intergenic spacer regions, five of the isolates from the cheetahs and the strain from the wild dog could be considered as *H. felis*. For the latter strain electron microscopic examination of the original host tissue and the cultured bacterium, which showed helical organisms entwined with periplasmic fibres in pairs, supported this. Unfortunately due to the nature of the specimens obtained from the cheetahs (biopsies from live animals collected by endoscopy) similar analysis was not possible. The urease gene sequence data are thus in agreement with DNA–DNA hybridization and protein profiles of the cultivable bacteria, which allowed for their discrimination into three species, a differentiation not possible using the 16S rRNA gene (Hanninen *et al.*, 1996; Jalava *et al.*, 1997). As expected, the urease sequences of the six *H. pylori* strains formed a discrete cluster separate from the other *Helicobacter* species.

This study expands our current knowledge of the gastrospirilla and describes PCR probes that permit discrimination of the uncultivable isolates. In addition, an alternative tool to study the phylogeny of these closely related organisms is described. Analysis of the genes encoding the urease protein was shown to be more discriminatory for species differentiation within the gastrospirilla than more commonly used methods, such as morphology and rRNA gene sequence analysis. This latter methodology allowed for a more sensitive discrimination of what previously were referred to as '*H. heilmannii*' type one and type two isolates. The former can be classified as '*Candidatus H. suis*' confirming the speculation of De Groote *et al.* (1999) that taxa found in pigs, humans and primates all constitute one species. We propose that the latter isolates, found in humans and domestic and exotic feline species, be classified as '*Candidatus Helicobacter heilmannii*'.

Taxonomic description of '*Candidatus Helicobacter heilmannii*'

Heilmannii after Konrad Heilmann who described the first large case study of gastrospirilla infections in humans (Heilmann & Borchard, 1991), *ε-Proteobacteria*, genus *Helicobacter*, NC. Large helical-shaped cells with five to seven turns, 0.5–0.6 µm × 5–10 µm in size. Gram-negative cell wall structure. Motile by means of bipolar tufts of 10–20 sheathed flagella. Strong urease activity. Can be propagated in mice but not *in vitro*. Currently isolated from humans and feline species. NAS of 16S rRNA gene and partial urease gene for the reference strain, isolate HU2^R, obtained from a human patient, GenBank/EMBL/DDBJ accession nos AF506786 and AF508012, respectively. Oligonucleotide sequence complementary to unique region of *ureA* gene 5'-AAG TGG GGA TTG AAG CGG GC-3'.

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