Phylogenetic analysis of *Helicobacter* species based on partial *gyrB* gene sequences

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Analysis of 16S rRNA gene sequences is one of the most common methods for investigating the phylogeny and taxonomy of bacteria. However, several studies have indicated that the 16S rRNA gene does not distinguish between certain *Helicobacter* species. We therefore selected for phylogenetic analysis an alternative marker, *gyrB*, encoding gyrase subunit B. The aim of this investigation was to examine the applicability of *gyrB* gene fragments (~1100 bp) for the phylogenetic study of 16 *Helicobacter* species and a total of 33 *Helicobacter* strains included in this study. Based on the sequenced fragments, a phylogenetic tree was obtained that contained two distinct clusters, with gastric species forming one cluster and enterohepatic species the other. The only exception was the gastric species *Helicobacter mustelae*, which clustered with the enterohepatic species. The calculated similarity matrix revealed the highest interspecies similarity between *Helicobacter salomonis* and *Helicobacter felis* (89%) and the lowest similarity between *Helicobacter pullorum* and *H. felis* (60%). The DNA G+C content of the sequenced fragments was ≤40 mol% in enterohepatic species and >46 mol% in gastric species, excluding *Helicobacter pylori* and *H. mustelae*, with G+C contents of 34 and 42 mol%, respectively. In summary, the *gyrB* gene fragments provided superior resolution and reliability to the 16S rRNA gene for differentiating between closely related *Helicobacter* species. A further outcome of this study was achieved by designing *gyrB* gene-based species-specific PCR primers for the identification of *Helicobacter bizzozeronii*.

INTRODUCTION

The genus *Helicobacter* belongs to the *Epsilonproteobacteria* and at the time of writing comprised 23 recognized species (Euzéby, 1997). *Helicobacter* species infect and colonize the gastrointestinal tracts of a large variety of animal hosts. The most significant *Helicobacter* species, *Helicobacter pylori*, has been implicated as an important aetiological factor in a variety of human gastric disorders, such as duodenal and gastric ulcers and gastric cancer (Sanders & Peura, 2002).

Analysis of 16S rRNA gene sequences is the most common method used for the molecular study of the phylogeny and the taxonomy of bacteria, including *Helicobacter* species (Dewhirst *et al.*, 2000). However, several examples have indicated that the 16S rRNA gene does not differentiate between certain *Helicobacter* species, given that intraspecies diversity of 16S rRNA genes within some species may be higher than interspecies diversity. *Helicobacter bizzozeronii* and *Helicobacter salomonis*, for instance, share a 16S rRNA gene sequence similarity of more than 99% (Jalava *et al.*, 1997); by contrast, intraspecies diversity within *Helicobacter cinaedi* and *Helicobacter troglontum* can reach 4% (Hänninen *et al.*, 2003; Vandamme *et al.*, 2000). For this reason, other genes such as *hsp60* (Mikkonen *et al.*, 2004), the 23S rRNA gene (Dewhirst *et al.*, 2005) and *ureB* (Hänninen *et al.*, 2005)

have been used in phylogenetic studies on *Helicobacter*, each with particular strengths and weaknesses relating to the amount of sequence information obtained, applicability, labour-intensiveness and distinguishing power. Therefore, it seems evident that no single phylogenetic marker gene alone can clarify the phylogenetic relationships among *Helicobacter* species but that analyses of several well-established marker genes are needed. This idea was recently supported by Gevers *et al.* (2005), who discussed the value of multilocus sequence analysis in the phylogenetic analysis of bacteria.

The *gyrB* gene, which encodes the B-protein subunit of DNA gyrase, has also been utilized in phylogenetic studies. A- and B-proteins are the constituents of the gyrase enzyme, which functions in relieving supercoiling in DNA by creating transient breaks in the double helix (topoisomerase type II). The *gyrB* gene may be particularly suitable for bacterial identification because it is an evolving, single-copy house-keeping gene that has already proved to be a useful tool in phylogenetic studies of several complex taxa as well as in microarray-based applications (Kasai *et al.*, 2000; Yamamoto & Harayama, 1995; Soler *et al.*, 2004; Kakinuma *et al.*, 2003).

In the present study the applicability of the *gyrB* gene for the phylogenetic analysis of *Helicobacter* species was evaluated,

and the sequence data obtained from these experiments were employed to develop a PCR-based diagnostic test for the species-specific detection and identification of *H. bizzozeronii*.

METHODS

Bacterial strains. The partial *gyrB* gene was sequenced from 31 strains representing 15 *Helicobacter* species and one canine isolate of *Helicobacter* sp. 'flexispira' (Table 1). In addition, the corresponding sequences of *H. pylori* 26695 (=ATCC 700392) and J99 (=ATCC 700824) as well as *Campylobacter jejuni* NCTC 11168 were obtained from the NCBI nucleotide database.

DNA isolation and PCR amplification. Bacteria were cultured on Brucella blood agar plates (Oxoid) containing selective antimicrobial agents (Hänninen *et al.*, 1996) microaerobically at 37 °C for 2–3 days. Bacterial mass was collected from two or three plates and genomic DNA was isolated by the method of Pitcher *et al.* (1989). The preliminary *gyrB* gene sequence (1100 bp) of all the studied

strains was amplified with the degenerate primers UP1 and UP2r (Yamamoto & Harayama, 1995). Because the sequencing capacity of automated cycle sequencers is usually less than 700 bp, internal primers (HeliF1, HeliF1b, HeliR2, HeliR2b, HeliR2e and HeliR2g), producing DNA fragments of about 600–680 bp, were designed based on these preliminary sequence data, by using a primer design utility (Cybergene AB) (Table 2). The internal primers were then used together with specific primers UP1S and UP2Sr (Yamamoto & Harayama, 1995) so that, for each strain, two DNA fragments with slightly overlapping ends were amplified by PCR (Fig. 1). The number of primers was kept to the minimum by designing primers with broad species specificity (Table 2).

PCR was performed in a total volume of 50 µl with 2 U Dynazyme polymerase and 5 µl Dynazyme buffer (Finnzymes), 5 nmol each dNTP, 200 ng purified chromosomal DNA and 20 pmol each primer. The PCR thermal cycling conditions were as follows: denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C and extension for 1 min at 72 °C, for 30 cycles.

The PCR products were analysed by using 1.5% agarose gel electrophoresis. PCR products of the expected size were purified

Table 1. *Helicobacter* strains used in the study

Taxon	Strain (reference)	Source	EMBL accession number for partial <i>gyrB</i> gene sequence
<i>H. aurati</i>	CCUG 47791 ^T	Gastrointestinal tissue of Syrian hamster	AJ969179
<i>H. bilis</i>	ATCC 51630 ^T	Mouse faeces	AJ969180
	KO214 (Mikkonen <i>et al.</i> , 2004)	Canine faeces	AJ969181
	KO220 (Mikkonen <i>et al.</i> , 2004)	Canine faeces	AJ969182
	KI311 (Hänninen <i>et al.</i> , 2005)	Feline faeces	AJ969183
	ATCC 49314 (taxon 2)	Sheep	AJ969184
	ATCC 49320 (taxon 3)	Pig stomach; aborted fetus	AJ969185
	CCUG 23435 (taxon 8)	Human faeces	AJ969186
<i>H. bizzozeronii</i>	CCUG 35545 ^T	Canine gastric mucosa	AJ969187
	10 (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa	AJ969188
	Yrjälä (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa	AJ969189
	R-53 (Jalava <i>et al.</i> , 2001)	Human gastric mucosa	AJ969190
<i>H. canadensis</i>	CCUG 47163 ^T	Human faeces	AJ969191
<i>H. canis</i>	NCTC 12739 ^T	Canine faeces	AJ969178
<i>H. cholecystus</i>	CCUG 38167	Syrian hamster gall bladder	AJ969192
<i>H. cinaedi</i>	CCUG 33885	Canine or feline faeces	AJ969193
	CCUG 46353 'westmeadii'	Human blood	AJ969207
<i>H. felis</i>	ATCC 49179 ^T	Feline gastric mucosa	AJ969194
	DS3 (Paster <i>et al.</i> , 1991)	Canine gastric mucosa	AJ969195
	Lopki13 (Jalava <i>et al.</i> , 1998)	Feline gastric mucosa	AJ969196
<i>H. hepaticus</i>	CCUG 33637 ^T	Mouse liver	AJ969197
<i>H. muridarum</i>	CCUG 29262 ^T	Rat ileum	AJ969198
<i>H. mustelae</i>	CCUG 25715 ^T	Ferret gastric mucosa	AJ969199
<i>H. pametensis</i>	CCUG 29253	Tern faeces	AJ969200
<i>H. pullorum</i>	CCUG 33842	Human faeces	AJ969201
<i>H. salomonis</i>	CCUG 37845 ^T	Canine gastric mucosa	AJ969202
	CCUG 37848	Canine gastric mucosa	AJ969203
	HS100 (Mikkonen <i>et al.</i> , 2004)	Canine gastric mucosa	AJ969204
	Ko. K. III (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa	AJ969205
<i>H. trogonum</i>	ATCC 700114 ^T	Rat gut	AJ969206
<i>Helicobacter</i> sp., canine 'flexispira'	FL56 (Hänninen <i>et al.</i> , 2005)	Canine gastric mucosa	AJ969208

Table 2. Primers used for detection and sequencing of *gyrB* sequences of *Helicobacter* species

Primer	Direction	Species	Sequence (5'–3')
UP1	Forward	All	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA
UP2r	Reverse	All	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNCGTCAT
UP1S	Forward	All	GAAGTCATCATGACCGTTCTGCA
UP2Sr	Reverse	All	AGCAGGGTACGGATGTGCGAGCC
HeliF1	Forward	<i>H. bilis</i> , FL56, <i>H. trogonum</i> , <i>H. aurati</i> , <i>H. muridarum</i> , <i>H. cholecystus</i> , <i>H. pametensis</i> , <i>H. mustelae</i> , <i>H. salomonis</i> , <i>H. felis</i> , <i>H. bizzozeronii</i> , <i>H. acinonychis</i>	GATGGTGGCACGCATGAG
HeliF1b	Forward	<i>H. pullorum</i> , <i>H. canadensis</i> , <i>H. canis</i> , <i>H. cinaedi</i> , <i>H. hepaticus</i>	CGCACGAAGCAGGATTTAG
HeliR2	Reverse	<i>H. canadensis</i> , <i>H. pullorum</i> , <i>H. bilis</i> , FL56	CCTAGTTTACCCTTTGTTTG
HeliR2b	Reverse	<i>H. trogonum</i> , <i>H. aurati</i> , <i>H. muridarum</i> , <i>H. cholecystus</i> , <i>H. mustelae</i> , <i>H. cinaedi</i> , <i>H. hepaticus</i>	ATAATAGCAACAAGCCCCCTC
HeliR2e	Reverse	<i>H. pametensis</i> , <i>H. canis</i>	TACAAGTCCCTCACGCACATC
HeliR2g	Reverse	<i>H. bizzozeronii</i> , <i>H. felis</i> , <i>H. salomonis</i>	CTCCCTAATGTCCTCTTG

from 2 % NuSieve GTG low-melting-point agarose gel (BMA) with the Qiaquick gel extraction kit (Qiagen). Nucleotide sequencing was performed by automated cycle sequencing with Big Dye terminators (ABI 377XL; PE Applied Biosystems). Fragments were sequenced at least three times and in both directions to ensure accuracy. With the help of the overlapping ends, the sequence pairs were joined together to form the 1100-bp *gyrB* gene fragments used in the analysis.

Sequence analysis. The 1100-bp partial *gyrB* gene sequences obtained in this study and those available in public databases (*H. pylori* 26695, *H. pylori* J99 and *C. jejuni* NCTC 11168) were aligned using the CLUSTAL W computer program (Chenna *et al.*, 2003). The ExPASy Translate tool allowed us to translate the nucleotide sequences to the corresponding amino acid sequences. The BioNumerics software program, version 3.5 (Applied Maths), was used in subsequent phylogenetic tree constructions and analysis. The nucleotide and amino acid trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987). The topology of the trees obtained was evaluated by bootstrap analysis of 1000 replications and by introducing a range of different gap costs. In addition, a maximum-parsimony tree was constructed for comparison.

Species-specific PCR for *H. bizzozeronii*. A specific set of primers for the amplification of the partial *gyrB* gene sequence of *H. bizzozeronii* was designed on the basis of the acquired *gyrB* gene sequences using a primer design utility (Cybergene AB). The PCR

thermal cycling conditions were as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 1 min at 72 °C, for 30 cycles.

The *H. bizzozeronii*-specific primers were GyrbizzoF (5'-GTTGA-AATCGCCTTAGCC-3') and GyrbizzoR (5'-GTTCTAAAATCCGTG-CTGAG-3'). The specificity of these primers was tested with the following gastric strains: *H. bizzozeronii* CCUG 35545^T, 10, R-53 and Yrjälä, *Helicobacter felis* ATCC 49179^T, DS3 and Lopki13, *H. salomonis* CCUG 37848 and Ko. K. III, *H. pylori* 26695 and *Helicobacter acinonychis* ATCC 51104, with a representative of each of the enterohepatic species listed in Table 1 and with *C. jejuni* NCTC 11168.

RESULTS AND DISCUSSION

General topology of the phylogenetic tree

A neighbour-joining tree based on partial *gyrB* gene sequences (1100 bp), representing 47 % of the whole *gyrB* gene sequence, was constructed (Fig. 2). The topology of the resulting tree was supported by the following observations. (i) The overall topology of the tree resembled that described for other sequenced and phylogenetically analysed genes of *Helicobacter* species, such as the 16S rRNA gene (Gueneau & Loiseaux-De Goër, 2002), *hsp60* (Mikkonen *et al.*, 2004) and the 23S rRNA gene (Dewhirst *et al.*, 2005). The tree contained three distinguishable clusters, wherein the first contained most of the gastric *Helicobacter* species (gastric cluster), the second was formed mainly by enterohepatic species (enterohepatic cluster) and the third included the species *Helicobacter canadensis* and *Helicobacter pullorum*. (ii) The maximum-parsimony tree based on the same sequences had a topology nearly identical to that of the neighbour-joining tree (results not shown). The only significant difference was seen in the positioning of *Helicobacter mustelae*, which had moved closer to the other gastric species, forming an 'intermediate species' between these two clusters. (iii) The mean bootstrap value

Fig. 1. Sequencing strategy for the *gyrB* gene fragment of *Helicobacter* species.

Fig. 2. Neighbour-joining tree based on partial *gyrB* gene sequences showing the phylogenetic relationships among members of the genus *Helicobacter*. The sequences were rooted with *Campylobacter jejuni* NCTC 11168. Numbers at nodes are percentage occurrence in 1000 bootstrapped trees. Bar, 5% sequence divergence.

for all the nodes in the *gyrB* gene sequence neighbour-joining tree was 84 %, with only four bootstrap values under 60 %. The mean bootstrap value for the maximum-parsimony tree was 74 %. Compared with the respective mean bootstrap values for the 16S rRNA gene sequence tree, 67 and 52 % (Dewhirst *et al.*, 2005), the phylogenetic trees based on the *gyrB* gene sequences appear to be far more robust. (iv) Introduction of different gap costs did not influence the resulting neighbour-joining tree.

The derived amino acid tree was similar to the DNA tree, but the branch lengths were considerably shorter as a consequence of most of the nucleic acid variation being caused by silent mutations in the third bases of the codons (results not shown).

Interspecies DNA similarity values of partial *gyrB* gene sequences ranged from 60 % (between *H. pullorum* and *H. felis*) to 89 % (between *H. salomonis* and *H. felis*). The lowest

intraspecies similarity value, 94.6 %, was found among the two *H. pylori* strains tested.

The DNA G + C content of the fragments was ≤ 40 mol% (32.6–40.0 mol%) in the enterohepatic species and > 46 mol% (46.5–50.6 mol%) in most of the gastric species, excluding *H. pylori* and *H. mustelae*, with G + C contents of 34 and 42 mol%, respectively. The maximum difference between the lowest G + C content (*Helicobacter cholecystus*) and the highest G + C content (*H. felis*) was nearly 18 mol%, indicating high variability.

Gastric species

In the neighbour-joining tree, with the exception of *H. mustelae*, all the gastric *Helicobacter* species included in the study were grouped in the gastric cluster. Clustering of the gastric species was supported with high bootstrap values (100 %), indicating stable positions for these species (Fig. 2). The four *H. bizzozeronii*, three *H. felis* and four *H. salomonis* strains included in this cluster had intraspecies nucleotide similarity values of 96.2–96.9, 95.6–98.0 and 98.1–99.2 %, respectively, and interspecies similarity values of 74 % between *H. bizzozeronii* and *H. salomonis*, 76 % between *H. bizzozeronii* and *H. felis* and 89 % between *H. salomonis* and *H. felis*. These results indicate high intraspecies similarity and a high interspecies difference, which is congruent with the speciation among this group. This distinction cannot be obtained based on 16S rRNA gene sequences because they generate similarity values between these three species that are as high as 99 % (Jalava *et al.*, 1997).

Although *H. pylori* clustered with the other gastric species, its sequence similarity to other *Helicobacter* species was low. The mean *gyrB* gene sequence similarity to the other gastric species was only 64 % and to the enterohepatic species was 63 %. The DNA G + C content of the *gyrB* gene fragment in *H. pylori* was 8–17 mol% lower than in other gastric species and about 5 mol% lower than the whole-genome G + C content of *H. pylori* (Alm *et al.*, 1999). These results suggest that *H. pylori* differentiated a long time ago from animal gastric *Helicobacter* species and became adapted for the colonization of the human gastric area.

According to the partial *gyrB* gene sequences, *H. mustelae* clustered with the enterohepatic *Helicobacter* species and its *gyrB* DNA G + C content was 42 mol%, which is closer to the enterohepatic values (≤ 40 mol%) than to the gastric values (> 46 mol%). These results support the findings of Gueneau & Loiseaux-De Goër (2002) and Mikkonen *et al.* (2004) and suggest that *H. mustelae* is an enterohepatic species by origin, but has relatively recently been able to acquire characteristics (e.g. the *ureI* gene) enabling it to alter its colonization niche from the intestinal to the gastric environment.

Enterohepatic species

The second cluster included most of the enterohepatic species. A tight subcluster was formed by the seven

Helicobacter bilis strains, with 96.1–99.8% intraspecies similarity at the nucleotide level. This further confirms the results of our previous study, in which phylogenetic analysis of fragments of *ureB* and *hsp60* gene sequences led us to conclude that the species *H. bilis* can be extended to include the reference strains of *Helicobacter* species 'flexispira' taxa 2, 3 and 8 (Hänninen *et al.*, 2005). In the *gyrB* gene sequence tree all the *H. bilis* strains clustered tightly together and the other 'flexispira-like' *Helicobacter* species, *H. trogonum*, *Helicobacter muridarum* and *Helicobacter aurati*, were positioned in the vicinity of the *H. bilis* strains (Fig. 2). However, in the 16S rRNA gene sequence tree, the *H. bilis* strains were dispersed with weak bootstrap values within a cluster that also included the type strains of *H. cinaedi*, *Helicobacter hepaticus* and *Helicobacter canis* (Hänninen *et al.*, 2005).

The canine strain FL57 is highly similar to *H. bilis* in terms of cell morphology and biochemical characteristics, but possesses 16S rRNA, *hsp60*, *ureB* (Hänninen *et al.*, 2005) and, as shown in the present study, *gyrB* gene sequences that are distinct from the corresponding *H. bilis* sequences. Further studies are needed to establish whether this bacterium represents a novel *Helicobacter* taxon.

Another subcluster within the enterohepatic cluster was formed by *H. mustelae*, *Helicobacter pametensis* and *H. cholecystus*. There are few apparent characteristics that unify this subcluster, although similar clustering has been observed in 16S rRNA and 23S rRNA gene sequence trees (Dewhirst *et al.*, 2005).

In the trees based on the ribosomal sequences, *H. cinaedi* strains are anomalously positioned among the taxa with flexispira morphology (Dewhirst *et al.*, 2005). In the *gyrB* gene sequence tree, the *H. cinaedi* strains formed a separate subcluster clearly distinct from the species with 'flexispira' morphology.

The third cluster included the two helicobacters with unsheathed flagella, *H. canadensis* and *H. pullorum*. These species colonize the lower part of the gastrointestinal tract of birds. *H. canadensis* has also been isolated from pigs (Inglis *et al.*, 2006). Their position in the tree was supported by a strong bootstrap value (100%). The interspecies *gyrB* gene sequence similarity value between these two species was 85.1%. The formation of a separate cluster for *H. canadensis* and *H. pullorum* can be explained by their sequence similarity to the other enterohepatic species, which was on average about 4% lower than within the enterohepatic species in general. The separate clustering of these species has also been observed in the 23S rRNA gene sequence tree (Dewhirst *et al.*, 2005).

Development of species-specific PCR

H. bizzozeronii is difficult to distinguish from *H. felis* and *H. salomonis* owing to high similarity in phenotypic and biochemical characteristics. Development of diagnostic

methods for the detection of *H. bizzozeronii*, *H. salomonis* and *H. felis* in human gastric samples is now justified even more strongly following the publication of an investigation in which these species were identified, respectively, from 2.4, 11.4 and 8.9% of 123 human gastric biopsy specimens (Van den Bulck *et al.*, 2005). These results indicate that these species, previously detected mainly from dogs and cats, also seem capable of infecting humans. The *gyrB* gene fragment analysis revealed differences in the nucleotide sequence of *H. bizzozeronii* relative to *H. felis* and *H. salomonis* that allowed us to develop a species-specific PCR method for the rapid diagnosis of *H. bizzozeronii*. As a result of PCR amplification using *H. bizzozeronii*-specific primers for several *Helicobacter* species and strains, only the four *H. bizzozeronii* strains produced a single band of 219 bp in the electrophoresis gel.

In conclusion, the partial *gyrB* gene sequence analysis proved to be a particularly well-suited tool for phylogenetic studies. The overall topology of the phylogenetic tree based on the *gyrB* gene resembled that described for other sequenced and phylogenetically analysed genes of *Helicobacter* species, such as the 16S rRNA gene (Gueneau & Loiseaux-De Goër, 2002), *hsp60* (Mikkonen *et al.*, 2004) and the 23S rRNA gene (Dewhirst *et al.*, 2005). However, the *gyrB* gene seems to evolve at a much faster rate than, for example, the 16S rRNA and 23S rRNA genes, allowing the formation of clear interspecies differences at the sequence level (60–89%) compared with the intraspecies similarity values (94.6–99.8%). For example, the ribosomal sequences are unable to distinguish between certain *Helicobacter* species, such as *H. felis* and *H. bizzozeronii*, due to overlapping interspecies and intraspecies similarity values (Dewhirst *et al.*, 2005). In addition, *gyrB* gene sequencing is straightforward as the sequences contain no intervening sequences and only a moderate number of primers are required, although consequently the amount of sequence information obtained (~1100 bp) is less than can be obtained with the more labour-intensive 23S rRNA gene sequencing (~2700 bp). It is therefore our recommendation, congruently with the multilocus sequence analysis approach suggested by Gevers *et al.* (2005), that studies on *Helicobacter* phylogeny should include the analysis of more than one established phylogenetic marker gene. Therefore, a reliable foundation for phylogenetic analysis of *Helicobacter* species could be based on joint data obtained from sequences such as the universally applied 16S rRNA gene and a suitable protein encoding gene, such as the *gyrB* gene.

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