Extension of the species *Helicobacter bilis* to include the reference strains of *Helicobacter* sp. flexispira taxa 2, 3 and 8 and Finnish canine and feline flexispira strains

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The evolution and taxonomy of enterohepatic *Helicobacter* species with flexispira morphology were studied by a polyphasic approach including phenotypic characterization, analysis of 16S rRNA and *ureB* gene sequences and dot-blot DNA–DNA hybridization of whole genomic DNA. In addition, available phylogenetic data on the HSP60 gene were used in the analysis. The study included 14 Finnish canine and feline flexispira strains, the reference strains of *Helicobacter* sp. flexispira taxa 2, 3 and 8 and *Helicobacter bilis* ATCC 51630^T. Phenotypically, all canine and feline strains were similar to *H. bilis*. Analysis of 16S rRNA gene sequences of these strains revealed a similarity of 97–99.5%. Similarity of *ureB* nucleotide and amino acid sequences within the studied flexispira group was 97–100% and 99–100%, respectively, revealing close relatedness. *ureB* sequences of *Helicobacter hepaticus* had only 64–66% similarity to the flexispira group. The similarity to *Helicobacter troglodytes* was 81.5–82.1%. High levels of DNA–DNA hybridization between the strains were found in dot-blot tests. Polyphasic analysis of the phenotypic and genotypic characteristics of the Finnish flexispira strains and the reference strains of taxa 2, 3 and 8 showed that they differed from other *Helicobacter* species and are members of the previously described species *H. bilis*. In addition, canine strain F56 differed in all phylogenetic analyses from the *H. bilis* group and probably represents a novel *Helicobacter* species.

INTRODUCTION

The taxonomy and diversity of the genus *Helicobacter* has been extended during the last 10 years to contain 23 species with validly published names (<http://www.bacterio.cict.fr/h/helicobacter.html>; last updated January 2004). *Helicobacter aurati*, *Helicobacter bilis*, *Helicobacter canis*, *Helicobacter cinaedi*, *Helicobacter cholecystus*, *Helicobacter fennelliae*, *Helicobacter hepaticus*, *Helicobacter marmotae*, *Helicobacter muridarum*, *Helicobacter pametensis*, *Helicobacter pullorum* and *Helicobacter troglodytes* colonize the lower intestinal tract of various animal species (Solnick & Schauer, 2001; Fox *et al.*, 2002). Some of these species seem to be strictly host-adapted, such as *H. canis* in dogs and *H. pametensis* in birds (Solnick & Schauer, 2001). Certain enterohepatic *Helicobacter* species, e.g. '*Flexispira rappini*' and *H. pullorum*,

have shown potential for zoonotic transmission (Archer *et al.*, 1988; Romero *et al.*, 1988; Stanley *et al.*, 1994). Moreover, DNA of *H. bilis*, *H. hepaticus*, '*F. rappini*', *H. pullorum* and *Helicobacter pylori* has been detected by PCR from the bile and gall bladder of human patients with various hepatobiliary diseases (Fox *et al.*, 1998; Ljungh & Wadström, 2002; Silva *et al.*, 2003). An increased seropositivity for non-gastric *Helicobacter* species has also been detected in serum of patients with autoimmune liver diseases (Nilsson *et al.*, 2003). The host spectrum of several intestinal *Helicobacter* species is not fully known, due to limited studies on a large variety of animals. Cultivation of intestinal *Helicobacter* species requires fresh samples and adequate culture techniques, limiting the knowledge gained on their taxonomy, ecology and epidemiology.

'*F. rappini*' is a provisional name given by Bryner and colleagues (Bryner, 1987; Bryner *et al.*, 1988) to a group of organisms originally isolated from aborted sheep fetuses (Kirkbride *et al.*, 1986). Flexispiras, which have spindle-shaped cells surrounded by periplasmic fibrils and bipolar tufts of sheathed flagella, were later shown to be members

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of the genus *Helicobacter* (Paster *et al.*, 1991; Dewhirst *et al.*, 2000a). The flexispira group includes the four named species *H. bilis* (Fox *et al.*, 1995), *H. trogontum* (Mendes *et al.*, 1996), *H. muridarum* (Lee *et al.*, 1992) and *H. aurati* (Patterson *et al.*, 2000), originally isolated from mice, rats, mice and hamsters, respectively, and several unnamed taxa based on 16S rRNA gene sequence analysis (Dewhirst *et al.*, 2000a). Recently, the species *H. trogontum* was extended when a polyphasic approach in studies of their taxonomy was used to include some porcine *Helicobacter* strains as well as the reference strains of provisional taxa 1, 4 and 5 (Hänninen *et al.*, 2003). *H. bilis* and taxa 2, 3 and 8 have been shown to produce cytolethal distending toxin but no toxin production was found in *H. trogontum* (Kostia *et al.*, 2003). Dewhirst *et al.* (2000a) proposed the provisional name *Helicobacter* sp. flexispira taxa 1 to 8 and 10 for unnamed *Helicobacter* species with flexispira morphology based on 16S rRNA gene sequences. A group of *Helicobacter* sp. flexispira without validly published taxonomy has been isolated from pigs (taxon 2), sheep (taxon 3), dogs (taxa 7 and 8) and humans (taxon 8) (Dewhirst *et al.*, 2000a). Bacteria with flexispira morphology have also been isolated from blood of patients with bacteraemia (Tee *et al.*, 1998; Sorlin *et al.*, 1999; Weir *et al.*, 1999) and in faecal samples of patients with diarrhoea, and their DNA has been detected in bile and liver samples in association with various hepatobiliary diseases (Fox *et al.*, 1998; Matsukura *et al.*, 2002).

Analysis of 16S rRNA gene sequences has been one of the most common methods used for studies of phylogeny and taxonomy of bacteria, including *Helicobacter* species (Vandamme *et al.*, 1996; Dewhirst *et al.*, 2000b; Stackebrandt *et al.*, 2002). However, several examples among *Helicobacter* indicate that 16S rRNA gene sequences are not good

markers for species identification of certain *Helicobacter* species. *Helicobacter bizzozeronii* and *Helicobacter salomonis*, for instance, have sequences that can be more than 99% similar (Jalava *et al.*, 1997). By contrast, the intraspecies diversity within *H. cinaedi* and *H. trogontum* can be 4%, which is higher than the interspecies diversity between some enterohepatic *Helicobacter* species (Vandamme *et al.*, 2000; Hänninen *et al.*, 2003). The name flexispira is sometimes used for bacteria that do not have spiral morphology or fibrils outside the cell and that have only one flagellum at each end of the cell, based on the high similarity of their 16S rRNA gene sequence to a strain of *Helicobacter* sp. flexispira taxon 8 (Iten *et al.*, 2001).

Here, we report, using a polyphasic approach, that *Helicobacter* sp. flexispira taxa 2, 3 and 8, some canine and feline flexispira strains and *H. bilis* constitute a species. In addition, we found that a flexispira strain isolated from a dog differs from *H. bilis* in several genetic characteristics.

METHODS

Bacterial strains. Bacteria with flexispira morphology were isolated from 14 dogs and cats using either a filtration technique on Brucella blood agar or culture of faecal material on freshly prepared Brucella blood agar medium containing (ml^{-1}) 2.5 U polymyxin B, 5 μg trimethoprim, 10 μg vancomycin and 2 μg amphotericin B (Hänninen *et al.*, 2003). The medium was incubated at 37 °C for up to 7 days in a microaerobic atmosphere containing 5% oxygen, 10% CO_2 and 3% H_2 and observed for spreading growth. Bacteria with flexispira morphology were identified using light microscopy and certain cultures (F56, F57, KI900, KO220, KI520, KI311 and KO214) were later confirmed by transmission electron microscopy to have typical flexispira morphology (Hänninen *et al.*, 2003). During earlier studies, the strains were tested several times for catalase, oxidase and urease production (Hänninen *et al.*, 1996, 2003). In addition, the strains were characterized for the following features:

Fig. 1. Phylogenetic tree of representative enterohepatic *Helicobacter* species based on 16S rRNA gene sequences (>1400 nucleotides). The tree was constructed using a neighbour-joining method rooted with *Campylobacter jejuni*. GenBank accession numbers are given in parentheses. Numbers at each node represent percentages of bootstrap support calculated from 1000 probable trees. Bar, 2% sequence divergence.

nitrate reduction, urease, γ -glutamyl transferase and esterase using API CAMPY (bioMérieux), growth at 25 and 42 °C and on 1% glycine and sensitivity to nalidixic acid and cephalothin (Hänninen *et al.*, 1996, 2003).

Sequencing. For sequencing studies, DNA was isolated as described elsewhere (Hänninen *et al.*, 1996, 2003; Mikkonen *et al.*, 2004). The nearly complete (at least 1400 bases sequenced) 16S rRNA genes of eight Finnish canine and feline flexispira strains were amplified by PCR with universal primer pair F19-38 (5'-CTGG-CTCAGGAYGAACGCTG-3') and R1541-1522 (5'-AAGGAGGTG-ATCCAGCCGCA-3'). Sequencing of the purified (QIAquick PCR purification kit; Qiagen) PCR products was performed by Sanger's dideoxynucleotide chain-termination method using primers F19-38, R1541-1522, F908-926 (5'-AACTCAAAGGAATTGACGG-3') and R536-519 (5'-GTATTACCGCGGCTGCTG-3'). Samples were run in a Global IR² sequencing device with e-Seq 1.1 software (LiCor) according to the manufacturer's instructions. Overlapping complementary sequences were joined using the Align IR 1.2 program (LiCor). The consensus sequences of strains belonging to *Helicobacter* species and *Campylobacter jejuni* (outgroup) [retrieved from/ deposited in GenBank (<http://www.ncbi.nlm.nih.gov>) using BLASTN 2.2.6 (Altschul *et al.*, 2001)] were aligned and a phylogenetic tree was constructed from the global alignment by the neighbour-joining algorithm using the BioNumerics 3.0 software package (Applied Maths). Bootstrap probability values were calculated from 1000 resampled trees. Fig. 1 shows the distance matrix tree based on 16S rRNA gene sequences and the accession numbers of the 16S rRNA gene sequences used/deposited. The specificity of the primers designed for identification of *H. bilis* based on 16S rRNA gene amplification was tested by the method of Fox *et al.* (1995).

Partial sequences of the *ureB* gene of 12 *Helicobacter* sp. flexispira strains (Table 1) were produced by automated cycle sequencing with Big Dye terminators (ABI 377XL; PE Applied Biosystems). The sequenced area (519 bp) corresponded to positions 139–658 of the partial 732 bp *ureB* gene sequence of *Helicobacter* sp. flexispira ATCC 49315. The primers (ureBFF, 5'-GAGATGGTATGGCGCAATCT-3'; ureBFR1, 5'-CCCAATCTTCATGCACCTTT-3') amplified a fragment of the expected size from all *Helicobacter* sp. flexispira strains, except *H. trogonum*. Using another reverse primer (5'-TCAATGCCAC-AGCAGTAAC-3'), a 247 bp fragment was also amplified from *H. trogonum*. Additional sequences were available from public databases (Fig. 2). After pairwise and multiple alignments, a phylogenetic tree was constructed by the neighbour-joining method using the Jukes-Cantor coefficient (Fig. 2). The topology of the tree was evaluated by 1000 trials of bootstrap analysis. The *ureB* sequences were translated to the corresponding protein sequences with the TRANSEQ program (EMBOSS). The resulting protein sequences were aligned and the neighbour-joining tree was calculated with CLUSTAL W. The phylogenetic tree based on the protein sequences was drawn by the TREEVIEW program 1.6.6 (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

DNA–DNA hybridization. Dot-blot hybridization of whole genomic DNA was performed for the reference strains of taxa 2, 3 and 8, *H. bilis* ATCC 50160^T, *H. trogonum* ATCC 700114^T, *H. canis* NCTC 12739^T and *H. cinaedi* CCUG 18818^T as well as for 14 canine and feline flexispira strains (Table 1) by the technique employed in our previous studies (Hänninen *et al.*, 1996; Jalava *et al.*, 1997, 2001). The concentrations of DNA used were 50, 5 and 0.5 ng. The strains used as probes were reference strains of taxa 2 (ATCC 49314), 3 (ATCC 49320) and 8 (CCUG 23435) (Table 1), *H. bilis* ATCC 50160^T, *H. trogonum* ATCC 700114^T and strains FL56 and FL57. The probes were labelled with digoxigenin with a DNA labelling and detection kit (Roche). Hybridization and all washings were performed at 58 °C. The intensity of hybridization was assessed by

Table 1. *Helicobacter* strains used in the study

Strain	Source
<i>Helicobacter bilis</i> ATCC 51630 ^T	Mouse faeces
<i>Helicobacter</i> sp. flexispira	
FL56	Canine faeces
KO214	Canine faeces
KO220	Canine faeces
KO534B	Canine faeces
KO114	Canine faeces
KO115	Canine faeces
KO794	Canine faeces
FL57	Canine faeces
FL76	Canine faeces
KI900	Feline faeces
KI311	Feline faeces
KI520	Feline faeces
KI901	Feline faeces
KI902	Feline faeces
<i>Helicobacter canis</i> NCTC 12739 ^T	Canine faeces
<i>Helicobacter cholecystus</i> CCUG 38167 ^T	Syrian hamster gall bladder
<i>Helicobacter cinaedi</i> CCUG 18818 ^T	Human faeces
<i>Helicobacter hepaticus</i> CCUG 33637 ^T	Mouse liver
<i>Helicobacter muridarum</i> CCUG 29262 ^T	Rat ileum
<i>Helicobacter mustelae</i> CCUG 25715 ^T	Ferret gastric mucosa
<i>Helicobacter pametensis</i> CCUG 29253	Tern faeces
<i>Helicobacter pullorum</i> NCTC 12826 ^T	Human faeces
<i>Helicobacter trogonum</i> ATCC 700114 ^T	Rat
<i>Helicobacter</i> sp., taxon 2 86-2279 (= ATCC 49314)	Sheep
<i>Helicobacter</i> sp., taxon 3 88-2491 (= ATCC 49320)	Pig stomach; aborted fetus
<i>Helicobacter</i> sp., taxon 8 CCUG 38944 (= ATCC 43880)	Human stool
<i>Helicobacter</i> sp., taxon 8 CCUG 23435 (= ATCC 43879)	Human stool

comparing the intensities of the colour that developed as described previously (Hänninen *et al.*, 1996; Jalava *et al.*, 1997, 2001).

RESULTS AND DISCUSSION

Morphology

Light and electron microscopy characterization of the strains showed that they all had cigar-like morphology with external fibrils outside the cell and tufts of sheathed flagella at both ends of the cell, findings typical of organisms (Weir *et al.*, 1999; Sorlin *et al.*, 1999; Jalava *et al.*, 1998; Hänninen *et al.*, 2003) given the provisional name 'flexispira' by Bryner *et al.* (1987).

Phenotypic characteristics

The strains were all urease-positive, negative in nitrate reduction, had esterase and γ -glutamyl transferase activity

Fig. 2. Phylogenetic tree of representative enterohepatic and gastric *Helicobacter* species based on partial *ureB* gene sequences (518 nucleotides). The tree was constructed using a neighbour-joining method rooted with *Klebsiella aerogenes*. GenBank accession numbers are given in parentheses. Numbers at each node represent percentages of bootstrap support calculated from 1000 probable trees. Bar, 5% sequence divergence.

and grew at 42 °C but not at 25 °C and did not grow in 1% glycine (Table 2). In catalase tests, repeated several times, 10/14 isolates were catalase-negative. In the taxonomic description of the species *H. bilis*, the type strain ATCC 51630^T and all other isolates were described as reducing nitrate to nitrite (Fox *et al.*, 1995). In our studies, the type strain ATCC 51630^T did not reduce nitrate, even after several separate experiments. Whether the type strain had lost its nitrate reduction capacity during subculture is unknown. Earlier studies have shown that canine flexispiras

can be either catalase-negative or catalase-positive (Dewhirst *et al.*, 2000a). These results were confirmed here.

Phylogeny based on the 16S rRNA gene

Sequences of *H. bilis* ATCC 51630^T, *Helicobacter* sp. flexispira taxa 2, 3 and 8 reference strains and Finnish canine and feline flexispira strains and sequences of flexispira strains deposited in GenBank under the names *H. bilis* (HR17 and MIT 93-4659) and '*F. rappini*'

Table 2. Characteristics of selected non-gastric *Helicobacter* species

Taxa: 1, *H. bilis*, 14 Finnish strains; 2, *H. bilis* ATCC 53630^T; 3, *Helicobacter* sp. flexispira, taxon 8; 4, *Helicobacter* sp. flexispira, taxon 2; 5, *Helicobacter* sp. flexispira, taxon 3; 6, *Helicobacter* sp. flexispira, taxon 7; 7, *H. aurati*; 8, *H. trogonum*; 9, *H. pullorum*; 10, *H. muridarum*; 11, *H. hepaticus*; 12, *H. cinaedi*; 13, *H. canis*; 14, *Helicobacter* sp. flexispira, taxon 10; 15, *H. rodentium*. Data were obtained from Dewhirst *et al.* (2000a), Fox *et al.* (1995), Hänninen *et al.* (2003), Mendes *et al.* (1996), Patterson *et al.* (2000), Saunders *et al.* (1999), Shen *et al.* (1997) and Robertson *et al.* (2001). ND, Not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Catalase production	– (10/14)	+	+/-	+	+	–	+	+	+	+	+	+	+	+	+
Nitrate reduction	– (14/14)	–	–	–	–	–	–	–	+	–	+	+	–	–	+
Urease	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–
γ-Glutamyl transferase	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–
Tolerance of glycine, 1%	– (14/14)	+	–	–	–	–	–	ND	–	–	+	+	–	+	+
Growth at 42 °C	+	+	+	+	+	+/-	+	+	+	–	–	–	+	+	+
Susceptibility to:*															
Nalidixic acid (30 mg l ⁻¹)	R (14/14)	R	R	R	R	R	S	R	R	R	R	S	S	R	R
Cephalothin (32 mg l ⁻¹)	R (14/14)	R	R	R	R	R	R	R	S	R	R	I	I	R	R
Periplasmic fibrils	+	+	+	+	+	+	–	+	–	+	–	–	–	+	–
Number of flagella	10–14	3–14	3–14	ND	ND	ND	7–10	6–14	1	10–14	2	1–2	2	6–12	2
Distribution of flagella†	BP	BP	BP	BP	BP	BP	BP	BP	MP	BP	BP	BP	BP	BP	BP

*R, Resistant; S, susceptible; I, intermediate.

†BP, Bipolar; MP, monopolar.

(AF034135, AF118017, AF225550, AF336948) were widely dispersed into a cluster, which also included some other enterohepatic *Helicobacter* species (*H. canis* NCTC 12739^T, *H. cinaedi* CCUG 18818^T and ATCC 30365 and *H. hepaticus* ATCC 51448^T). *H. troglodytes* was in the same cluster as *H. muridarum*, *H. aurati* and *H. fennelliae*. The bootstrap values supporting the topology of the branching of enterohepatic species were usually low. These results show trends similar to those of several previous studies (Sorlin *et al.*, 1999; Dewhirst *et al.*, 2000a; Vandamme *et al.*, 2000; Hänninen *et al.*, 2003), indicating extensive diversity within enterohepatic *Helicobacter* species and revealing that the phylogeny of the 16S rRNA gene is inconsistent with the cellular phylogeny and taxonomy of these enterohepatic *Helicobacter* species. Finnish canine and feline flexispira strains had similarity of 94.8–96.8% to *H. troglodytes*, 98–100% to *H. bilis* ATCC 51630^T, 97% to the reference strains of taxa 2 and 3 and 97–99.5% to taxon 8. Taxa 2 and 3 were in their own branch, although the bootstrap value supporting their position was low (Fig. 1). The sequence of strain FL56 had only 91% similarity to taxon 8, *H. bilis* ATCC 51630^T and Finnish canine and feline strains. It had the highest similarity to the taxon 7 reference strain (98.5%). *H. aurati* had 95–96% similarity to the other flexispira strains. The incoherence of the evolution of the 16S rRNA gene with that of enterohepatic *Helicobacter* species studied by a polyphasic taxonomic approach could be explained by frequent genetic exchanges between enterohepatic species in this genomic region. Several enterohepatic *Helicobacter* species may simultaneously colonize the intestines of animal hosts, e.g. cats, dogs, mice and rats (Solnick & Schauer, 2001), enabling genetic recombination between different *Helicobacter* species. We also tested the specificity of the *H. bilis* 16S rRNA gene primers (Fox *et al.*, 1995) and found that they produced fragments from *H. troglodytes* ATCC 700114^T, *H. bilis* ATCC 51630^T, *H. cinaedi* CCUG 18818^T, the taxon 2 reference strain and five of the Finnish canine flexispira strains examined. These studies revealed that the primers were not specific to *H. bilis* but amplified fragments from other related enterohepatic *Helicobacter* species as well. These results are concordant with the observation of rather high interspecies 16S rRNA gene similarity. High intraspecies diversity of the 16S rRNA gene sequence (Vandamme *et al.*, 2000; Hänninen *et al.*, 2003) also suggests that design of a species-specific PCR test for identification of related enterohepatic *Helicobacter* species will be a difficult task.

Insertion sequences in the 16S rRNA gene

Insertion sequences have been demonstrated to be rather common in both the 16S and 23S rRNA genes in *Helicobacter* species (Hurtado *et al.*, 1997). They seem to be identical or highly similar within a *Helicobacter* species and interspecies similarities also exist (Hurtado *et al.*, 1997). The insert usually starts in the same region. In our studies, an identical or highly similar (98–100% similarity) 187 bp insertion sequence, starting at position 220, was detected in

11 of the flexispira sequences (Fig. 1). Insertion sequences were lacking in six sequences, including the reference strain of taxon 8, ATCC 43880 (Table 1). Insertion sequences of strain FL56 and the taxon 7 reference strain were highly similar and differed from other studied intervening sequences. Previous studies have shown that bacteria with flexispira morphology possess intervening sequences (Dewhirst *et al.*, 2000a). The high similarity of the intervening sequences of *H. bilis*, taxa 2, 3 and 8 and some Finnish canine and feline flexispira strains suggests that these bacteria are also taxonomically highly related, as has been shown to be the case for other *Helicobacter* species (Linton *et al.*, 1994; Fox *et al.*, 1995). A PCR amplification test specific for intervening sequences can be used as a diagnostic tool to identify those *H. bilis* strains that have an intervening sequence in their 16S rRNA gene, as earlier proposed by Fox *et al.* (1995).

Phylogeny based on *ureB* sequences

ureB has been shown in some previous studies to be suitable for studying the phylogeny and evolution of gastric *Helicobacter* species (Gueneau & Loiseaux-De Goër, 2002). Evaluation of the suitability of the *ureB* gene for the taxonomy of enterohepatic *Helicobacter* species has been limited by the small number of sequences available. *ureB* sequence analysis could not, in any case, be used for the whole genus because several urease-negative enterohepatic species exist (Table 2; Solnick & Schauer, 2001). As expected, the fragment was amplified from the reference strains of taxa 2, 3 and 8 (CCUG 223435). In addition, the same product was amplified from *H. bilis* ATCC 53160^T and from all eight Finnish canine and feline strains studied.

The 479 bp *ureB* sequences were used for phylogenetic analysis and a tree was constructed (Fig. 2). The sequences of *H. bilis*, taxa 2, 3 and 8 reference strains, Finnish canine and feline flexispira strains and the four flexispira sequences from GenBank [*F. rappini*? ATCC 43879 (AF116549), '*F. rappini*? ATCC 49315 (AF1165509), '*F. rappini*? NIH (AF116551) and '*Flexispira*' sp. (AJ130884)] clustered tightly together, having 97–100% similarity. The sequence of *H. bilis* ATCC 51630^T was 97–98.7% similar to the flexispira sequences listed above. The flexispira group mentioned above had similarity of 64.4–65.8% to *H. hepaticus* ATCC 51448^T. These results indicate a close genetic relationship between the studied bacteria with flexispira morphology as well as their clear separation from the studied urease-positive enterohepatic *Helicobacter* species. The only exception was the sequence of strain FL56, which had a similarity of only 91.4–93.5% to other flexispira strains. In the phylogenetic tree, strain FL56 was also distinguished from the other flexispira strains, having a high bootstrap value of 100 (Fig. 2). *H. hepaticus* was represented by six sequences (five from GenBank and a sequence from our study) and they formed their own cluster in the tree. The similarity of the six *H. hepaticus* sequences varied from 99.2 to 99.4%, indicating a higher intraspecies similarity than that found for the flexispira

group. *Helicobacter mustelae* clustered with the enterohepatic branch and not with the gastric *Helicobacter* species (Fig. 2), even though it is a gastric-area-colonizing species (Fox *et al.*, 1990). The type strains of the gastric species *H. pylori*, *Helicobacter felis*, *H. bizzozeronii* and *H. salomonis* formed a gastric branch. Other studies concentrating more on the analysis of *ureB* gene phylogeny of gastric *Helicobacter* species have also shown that gastric and enterohepatic species are located in separate clusters (Gueneau & Loiseaux-De Goër, 2002). Comparison of the respective amino acid sequences indicated 98–100% similarity for *H. bilis*, *Helicobacter* sp. flexispira taxa 2, 3 and 8 strains, seven Finnish canine and feline flexispira strains and the four '*F. rappini*' sequences from GenBank. FL56 had a similarity of 96–97% to other strains. Our results suggest that, based on the high similarity of their *ureB* sequences, *H. bilis*, taxa 2, 3 and 8 strains and canine and feline flexispira strains are members of the same species. The lower similarity of FL56 to other flexispira strains suggests that this strain might belong to another species.

Comparison of the sequences of *H. trogontum* with sequences of other flexispira strains was not possible because the primers used failed to amplify its *ureB* sequence. We therefore performed a limited study on seven strains by developing primers that amplified both canine and feline flexispiras (KO220, FL57, KI311), *H. bilis* ATCC 51630^T, taxa 2 and 8 reference strains and *H. trogontum*. Sequencing and analysing this 247 bp fragment revealed that *H. trogontum* ATCC 700411^T had only 81.5–82.1% similarity to *H. bilis* and other strains studied, suggesting that the phylogeny of this fragment of *ureB* was concordant with speciation. The sequence similarity within the other flexispira strains was high, from 96.5 to 99.5%. In conclusion, these studies showed that *ureB* sequence analysis can differentiate *H. trogontum* from other flexispiras, including *H. bilis*. PCR with primers ureBFF and ureBR1 can be used for specific amplification of *H. bilis* DNA.

Whole genome DNA–DNA hybridization

Dot-blot hybridization using three concentrations of genomic DNA revealed a high level of hybridization between *H. bilis* ATCC 51630^T, taxa 2, 3 and 8 reference strains and 14 canine and feline flexispira strains. This indicates high whole genomic DNA similarity (results not shown) and suggests that all of these strains are members of a single species, i.e. *H. bilis*. Because 16S rRNA gene sequences of *H. canis* and *H. cinaedi* can show high similarity to *H. bilis* and other flexispiras (Fig. 1), DNAs of *H. canis* and *H. cinaedi* were hybridized with probe DNAs as well. The probes hybridized only weakly with the DNAs of *H. canis* and *H. cinaedi*, indicating only low relatedness and revealing that the flexispira group is not part of either *H. canis* or *H. cinaedi*. Whole genomic DNA–DNA hybridization has been one of the key taxonomic markers in the delineation of a bacterial species (Stackebrandt *et al.*, 2002). DNA–DNA hybridization studies have not been performed extensively for *Helicobacter* species. In our previous studies,

we used the present dot-blot technique, which requires less than 100 ng DNA, to describe two novel *Helicobacter* species, *H. bizzozeronii* (Hänninen *et al.*, 1996; Jalava *et al.* 2001) and *H. salomonis* (Jalava *et al.*, 1997), as well as to describe the extension of *H. trogontum* to include reference strains ATCC 43968, ATCC 43966 and ATCC 49310 (Hänninen *et al.*, 2003).

Phylogeny of HSP60

Using approximately 600 bp sequences of the HSP60 gene as a phylogenetic marker, we have earlier shown that evolution of the HSP60 gene tends to correspond much better to the taxonomy of the genus *Helicobacter* than does the 16S rRNA gene (Mikkonen *et al.*, 2004). The study also included *H. bilis* ATCC 51630^T, taxa 2, 3 and 8 reference strains, some of the canine and feline flexispira strains included in the present study (KO214, KO534B, KO220 and FL56), *H. cinaedi* and *H. canis*, as well as several *H. trogontum* strains. Analysis of HSP60 sequences showed that *H. bilis*, the taxon 8 reference strain and Finnish flexispira strains were in the same branch of the phylogenetic tree and had highly similar sequences, supporting the results of the present study. The sequences of taxa 2 and 3 strains had 88.9, 96 and 95.4% similarity to *H. trogontum*, *H. bilis* and the taxon 8 reference strain, respectively. However, the respective amino acid sequences of taxa 2 and 3 differed from the other flexispiras by only one amino acid, indicating close interrelatedness. Strain FL56 had only 90.3–91.2% sequence similarity to the *H. bilis* cluster (Mikkonen *et al.*, 2004), supporting the results of 16S rRNA and *ureB* gene analysis. *H. canis* and *H. cinaedi* clustered with other enterohepatic species and were separated from *H. bilis* and *H. trogontum*.

Conclusions

Some confusion exists in the taxonomy and naming of organisms with flexispira morphology. *H. trogontum* is a named species associated with rat, sheep and pig, and Kirkbride *et al.* (1986) and Bryner *et al.* (1987) have shown that this species is strongly associated with ovine abortions. Our present and previous studies have revealed that a group of flexispira strains isolated from faecal samples of humans, dogs, cats and sheep as well as from the porcine stomach had highly similar phenotypic and genotypic characteristics based on their 16S rRNA, *ureB*, *cdtB* and HSP60 gene sequences, had high whole genomic relatedness in DNA–DNA hybridizations and differed from other named *Helicobacter* species. These facts lead us to conclude that reference strains of taxa 2, 3 and 8 and Finnish canine and feline flexispira strains constitute a single species, *H. bilis*. The methods in our studies fulfil the minimum requirements presented by Dewhirst *et al.* (2000b) for description of a novel *Helicobacter* species, as well as the later recommendations of the ad hoc committee for the revaluation of the definition of the bacterial species (Stackebrandt *et al.*, 2002). Strain FL56 had different characteristics from other flexispiras and its position

requires further studies. Bacteria resembling *H. bilis* have been isolated from blood cultures of human patients in association of bacteraemia (Tee *et al.*, 1998; Sorlin *et al.*, 1999; Weir *et al.*, 1999), and similar DNA has been detected in bile and tissue samples of patients with various hepatobiliary diseases (Fox *et al.*, 1998; Matsukura *et al.*, 2002). These studies suggest that the clinical significance of *H. bilis* in gastrointestinal diseases should be further evaluated using adequate diagnostic methods. Based on its wide spectrum of hosts, *H. bilis* has a capacity for zoonotic transmission. Some case studies have shown potential transmission from dogs to humans (Romero *et al.*, 1988). Our results provide diagnostic tools for further studies on the role of *H. bilis* in various diseases of animals and humans and confirm the power of the polyphasic approach in the taxonomy of species with a complex evolutionary background. More strains are required for studies on the taxonomy of strains FL56 and *Helicobacter* sp. taxon 7.

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