Systematic and Applied Microbiology 47 (2024) 126557

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.com/locate/syapm

Helicobacter cappadocius sp. nov., from lizards: The first psychrotrophic *Helicobacter* species

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ARTICLE INFO

Keywords:
Helicobacter
Lizard
Novel species
Overall genome-related index
Psychrotrophic

ABSTRACT

It was aimed to determine the prevalence of *Helicobacter* in some reptilian and amphibian species in Türkiye and to describe the bacteria. For this purpose, 73 cloacal swab samples were used as material. The description of the isolates was performed by detailed phenotypic tests, whole genome analyses, and MALDI-TOF MS. As a result of the phenotypic analysis, two helical, curved Gram-negative, motile isolates were recovered. It was determined through the analysis of 16S rRNA gene sequences that two isolates belonged to the genus *Helicobacter*. These isolates were found to be in a distinct group from other *Helicobacter* species. However, the 16S rRNA sequence did not match any identified species, with the closest match being *Helicobacter mustelae* strain R85-13-6^T, which had an identity level of 96.2 %. Additionally, it was found that strains faydin-H75^T and faydin-H76 had a 99.3 % identity level for their 16S rRNA genes. After conducting dDDH and ANI analyses, it was found that strains faydin-H75^T and their close neighbors *H.anseris* ATCC BAA-1299^T shared 13.5 % and 68.8 % similarity, respectively. The genome size of the strains was 1.7 Mb while G + C contents were 33.5 %. Metagenomic analyses using IMNGS and Protologger tools revealed the presence of faydin-H75^T in various lizard species with high similarity, confirming its broad distribution and host specificity. The results indicated that these two strains represent a novel species, for which we propose the name *Helicobacter cappadocius* with faydin-H75^T (=NCTC014972 = LMG 33382 = DSM117062) as the respective type strain. The current novel species is the first *Helicobacter* species to exhibit a psychrotrophic feature.

Introduction

Helicobacter species are Gram-negative, microaerobic, non-spore-forming, helical, and curved rods. The majority of *Helicobacter* species are motile using flagellum with/without a protein sheath (On et al., 2017). The genus *Helicobacter*, which was first formed with *H. pylori* and *H. mustelae* (Goodwin et al., 1989), currently includes validly published 54 species (as of the date, the manuscript was written). The type strain of the genus is *H. pylori*. (Parte et al., 2020). Since constructing the genus *Helicobacter* within the class *Epsilonproteobacteria*, novel *Helicobacter* species from different animal species including cats, cheetahs, chickens, dogs, horses, marmosets, mice, pigs, wild birds, whales, and dolphins have been identified (Beisele et al., 2011; Shen et al., 2016, 2020; On et al., 2017; Gruntar et al., 2020; Parte et al., 2020; Lopez-Cantillo et al., 2023). Also, very recently, in Japan, *Helicobacter* strains isolated from the blood of a human with diffuse large B-cell lymphoma were described as *H. kumamotonensis* (Kawamura et al., 2023).

While many species in the genus *Helicobacter* have been isolated from mammalians and avian species, (Ochoa and Collado, 2021), the investigations conducted on the presence of *Helicobacter* spp. in reptiles

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https://doi.org/10.1016/j.syapm.2024.126557
Received 22 April 2024; Received in revised form 4 October 2024; Accepted 8 October 2024
Available online 14 October 2024

which are ectothermic animals are very scarce (Gilbert et al., 2014, 2017). Helicobacters are part of the gastrointestinal microbiota of reptilians, and the presence of Helicobacters in reptilians has been demonstrated by cultural and molecular analyzes of samples obtained from healthy (Lawson and Owen, 2007; Gilbert et al., 2014; Rudolf et al., 2018; Bunker et al., 2022; Bunker and Weiss, 2022), infected (Schrenzel et al., 2010; Desiderio et al., 2021; Gilbert et al., 2014; Conley et al., 2021), and dead animals.

Although studies (Gilbert et al., 2017; Chan et al., 2021) on isolating and characterizing *Helicobacter* spp. from reptilians have been carried out, there is no validly published *Helicobacter* species belonging to these animals (Parte et al., 2020).

Considering that there is no data on the prevalence and zoonotic potential of helicobacters in various reptilian and amphibian species in Türkiye, we aimed to investigate the fecal carriage of helicobacters in these animals. In this context, two *Helicobacter* isolates obtained from the cloacal swab samples from lizards (*Apathya cappadocica* and *Ophisops elegans*) were considered representative of novel species in the genus *Helicobacter* based on pairwise 16S rRNA gene sequence analysis. Therefore, a polyphasic characterization of two strains from lizards to confirm their taxonomic lineage within the genus *Helicobacter* was provided.

Materials and methods

Cloacal samples

A total of 73 cloacal swab samples from the various reptilian and amphibian species were analyzed. The swab samples were taken from the animals that lived in different locations (Table S1) in Türkiye between April 2022 and June 2022. For newts, seasonally formed water pools containing plants were screened with arthrrap. The individuals were kept in glass jars until the swab samples were taken. While samples were taken from both sexes from *Triturus anatolicus*, only males of the *Ommatotriton nesterovi* were sampled due to the narrow cloacal opening. For reptiles, transect walks were performed to sample the areas during the day. Stones, logs, and other materials were removed and searched for fossorial species. The animals were hand-captured one by one and swabs were taken from the cloacal opening. After that, all individuals were released to the same locations. One swab sample belonging to each reptilian and amphibian species was placed in the sterile falcon tube using a sterile swab and transferred to the laboratory immediately under cold conditions. All swab samples were stored at −80 °C in Çankırı, Türkiye (40°37′05.9″N 33°37′22.3″E), and later, transferred for processing to Kayseri, Türkiye (38°42′24.0″N 35°32′25.1″E).

Culture

Each of the swab samples was homogenized with 250 µl Brain Heart Infusion Broth (BHI) (Oxoid, CM1135). Two different methods were used for isolation of the *Helicobacter* spp.;

(i) Direct inoculation: Fifty µl of the homogenized feces sample was directly inoculated onto Columbia Blood Agar Base (Neogen NCM0031C, USA) supplemented with 7 % defibrinated horse blood (HBA) and Dent supplement (SR0147E, Thermo Fisher Scientific). The plates were incubated at 37 °C in a microaerobic atmosphere (AnaeroPack™-MicroAero system, Mitsubishi Gas Chemical Co Inc.) for 3–5 days.

(ii) Enrichment and filtration: Two hundred µl of the homogenized feces sample was inoculated into 10 mL BHI broth added with Dent supplement. The inoculated media were incubated at 37 °C in a microaerobic condition (AnaeroPack™-MicroAero system, Mitsubishi Gas Chemical Co Inc.) for 2 days. After incubation 300 µl of each suspended sample was spread on a 47 mm, 0.65 µm pore size sterile filter (Sartorius AG, Goettingen, Germany) previously placed on the non-selective blood agar (Columbia Blood Agar Base) supplemented with 7 % HBA using the modified filter technique (Steele and McDermott, 1984). The plates were incubated upright at 37 °C for 45 min in a humid microaerobic atmosphere (AnaeroPack™-MicroAero system, Mitsubishi Gas Chemical Co Inc.). After incubation, the filter was removed and the agar surface streaked with a loop. The inoculated plates were then incubated again under the same conditions as described above. The grown colonies (small, 2–3 mm in size, smooth, greyish on the agar plates) were evaluated for representatives of the microaerophilic, helical, curved, Gram-negative, motile, and oxidase, and catalase-positive microorganisms. The colonies were sub-cultured on Columbia blood agar base supplemented with 7 % defibrinated horse blood. The pure cultures obtained from the colonies were stored in BHI broth supplemented with 15 % glycerol at −84 °C to use in further molecular analyses and phenotypic tests (Zanoni et al., 2007; Baele et al., 2008; On et al., 2017).

Molecular identification

For the genomic DNA extraction from the isolates obtained from reptiles, a commercial microbial DNA isolation kit (DNeasy UltraClean Microbial Kit, Qiagen, Hilden, Germany) was used and the protocols regarding DNA isolation were performed following the manufacturer's instructions.

Helicobacter genus-specific PCR

In the molecular confirmation of the obtained isolates, *Helicobacter* genus-specific PCR used of primers 16SF-CTATGACGGGTATCCGGC and 16SR-CTCACGACACGAGCTGAC was performed as described previously (Al-Soud et al., 2003).

16S rRNA phylogenetic analysis and comparative genome analyses

The amplification and sequencing of the 16S rRNA gene were performed using universal primers 27F and 1492R (Lane, 1991). The similarity index of the 16S rRNA genes from the strains was determined by comparing them against the GenBank database of 16S ribosomal RNA sequences (Bacteria and Archaea). Additionally, 16S rRNA phylogenetic analyses were conducted through the GGDC web server, utilizing the DSMZ phylogenomics pipeline adapted for single genes, as previously described (Meier-Kolthoff et al., 2013a, 2013b; Meier-Kolthoff and Göker, 2019). To initiate the process, a multiple sequence alignment was generated using MUSCLE (Edgar, 2004). Subsequently, both Maximum Likelihood (ML) and Maximum Parsimony (MP) trees were inferred from the alignment. RAxML and TNT were employed for ML and MP tree construction, respectively, following the procedures outlined previously (Goloboff et al., 2008; Stamatakis, 2014). For the ML tree, rapid bootstrapping, in conjunction with the autoMRE bootstopping criterion, was applied, followed by a search for the best tree. In this context, 1000 bootstrapping replicates were utilized. As for the MP tree, tree bisection-and-reconnection branch swapping, along with ten random sequence addition replicates, was employed, with 1000 bootstrapping replicates according to the methodology presented previously (Pattengale et al., 2010).

For genomic analysis, the sequencing library was prepared utilizing the Nextera XT DNA Library Preparation Kit, and sequencing was conducted on the Illumina NovaSeq 6000 platform in 2x150-bp paired-end (PE) sequencing mode with a 1000-cycle HiSeq reagent kit. The high-quality reads were assembled into contigs through de novo assembly using the SPAdes assembler 3.13.0 for strains faydin-H75^T and faydin-H76. Draft genome sequence data were submitted to GenBank, and contigs longer than 1000 bp were annotated using the NCBI prokaryotic genome annotation pipeline (PGAP) (Bankevich et al., 2012; Tatusova et al., 2016).

According to the minimal standards for describing new species belonging to the families *Helicobacteraceae* (On et al., 2017), to perform

the digital DNA-DNA hybridization (dDDH) analyses together with a comprehensive phylogenomic analysis, the type strain genomes server pipeline (TYGS, https://tygs.dsmz.de/) was used to compare the draft genomes of strains faydin-H75^T and faydin-H76 with the genomes of the type strains deposited in the DSMZ database. The dDDH analysis was based on Formula 3, which is recommended for whole-genome sequence-based comparisons and calculates the intergenomic distance using high-scoring segment pairs (HSPs) to estimate the DNA-DNA hybridization value. The average nucleotide identity values based on BLASTN (ANIb) and MUMMER (ANIm) algorithms were calculated for our strains together with their phylogenetic neighbours to determine overall genome relatedness indices using the JSpecies software tool available at https://jspecies.ribohost.com/jspeciesws/ (Richter and Rosselló-Móra, 2009). Additionally, OrthoANI values were calculated using the OrthoANI calculator (https://www.ezbiocloud.net/tools/orthoani) to further validate the ANI results (Yoon et al., 2017). The phylogenetic tree based on the whole genome sequences of *Helicobacter* type strains and *Helicobacter* spp. strains recovered from reptile were constructed by the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) bacterial genome tree pipeline (Olson et al., 2023).

The analyses of antibiotic resistance genes and virulence genes were conducted using the Resistance Gene Identifier (RGI) (https://card.mcmaster.ca/analyze/rgi) and the virulence factor database (VFDB) database (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi) (Alcock et al., 2020; Liu et al., 2022). Also, the antiSMASH servers (https://antismash.secondarymetabolites.org/) (Blin et al., 2023) were employed to identify the bioactive secondary metabolite gene cluster. Additionally, Prophages were predicated in strains faydin-H75^T genomes using the PHASTEST (PHAge Search Tool with Enhanced Sequence Translation) web server (https://phastest.ca/). The intact, questionable, and incomplete prophage sequences were defined by score values of $>$ 90, 70 to 90, and $<$ 70, respectively (Wishart et al., 2023).

A study was conducted to compare protein families in the genomes of *Helicobacter* type strains, including *H. cappadocius* faydin-H75^T and faydin-H76, along with eight *Helicobacter* spp strains found in reptiles living in cold environments. The analysis used genus-specific protein families (PLfams) and the protein family sorter tool. The genomes were obtained through the BV-BRC server. Then, the assigned protein family groups were used to determine unique metabolic pathways shared by the members of each species using the pathway comparison tool based on RASTtk annotations (Brettin et al., 2015).

Ecological distribution

The ecological traits of the strain were examined utilizing the Protologger web tool (Hitch et al., 2021), available at https://www.protologger.de/. Within Protologger, the 16S rRNA gene sequences were compared against a repository of 19,000 amplicon datasets, while the genomes were evaluated against 49,094 high-quality metagenome-assembled genomes (MAGs). Protologger calculates abundances based on sequence similarities and alignments within its database and typically investigates abundance at the genus level. To support species-level identification, we conducted additional BLAST analyses of 16S rRNA sequences and whole genome sequencing (WGS) data from GenBank, confirming the presence and specificity of the strain faydin-H75^T across various metagenomic datasets.

The 16S rRNA sequence of the strain faydin-H75^T was subjected to BLAST analysis against amplicon-based metagenomic SRA data of lizards available in GenBank. Additionally, WGS-based metagenomic SRA data of lizards from GenBank were uploaded to the Galaxy platform, and the SRA files were trimmed using Trimmomatic. The trimmed reads were aligned to the genome of faydin-H75^T using BWA-MEM2 with default settings. The resulting alignment files were then subjected to BLASTn with 95 % similarity and 70 % length thresholds to determine the abundance of faydin-H75^T in the WGS data. To obtain the relative abundance of the analyzed genomes (% of reads), the number of mapped reads was divided by the total number of reads in each metagenome and then divided again by the size (bp) of the total length of the genome.

Additionally, the prevalence of the strains across various sample metagenomes, including those from reptiles, was analyzed using the Integrated Microbial Next Generation Sequencing (IMNGS) platform available at https://www.imngs.org/ (Lagkouvardos et al., 2016).

Furthermore, a BLAST search was conducted by querying the 16S rRNA gene sequences of the strains against the GenBank database to identify sequences with high similarity. The matching sequences from both culture-dependent and culture-independent studies were subsequently retrieved. A phylogenetic tree was constructed using the maximum likelihood (ML) approach with MEGA X software (Kumar et al., 2018). Evolutionary distances were calculated employing Kimura's two-parameter model (Nishimaki and Sato, 2019). The ML algorithm incorporated a discrete gamma distribution (+G) and allowed for some sites to be evolutionarily invariable (+I). Node reliability was assessed through bootstrap analysis, involving 1000 resamplings (Felsenstein, 1985).

Phenotypic tests

Detailed phenotypic tests were performed to characterize *Helicobacter* isolates recovered from the cloacal samples of the reptiles. The isolates were incubated for 2–3 days before each biochemical test. The phenotypic tests were carried out twice at different times on freshly prepared media using standard methods as previously described (On and Holmes, 1991a,1991b,1992; On et al., 2017). For the Gram-staining, it was used the Gram-Stain kit (bioMérieux, Marcy l'Etoile, France). The cell morphology of faydin-H75^T was examined at Erciyes University, Technology Research, and Application Center, using Zeiss Gemini FESEM 500 scanning electron microscope (SEM) (Germany) equipped with a STEM detector. Bacterial movement was investigated via the hanging drop technique. Oxidase activity was determined with oxidase test sticks (Bactident Oxidase, 1.00181.0002, Merck), and a catalase test was performed using 3 % hydrogen peroxide solution and evaluated by observation of bubble formation within 5 s. An API Campy kit (BioMérieux, Marcy l'Etoile, France) was employed following the manufacturers' instructions to determine further enzymatic activities of the faydin-H75^T and faydin-H76 isolates.

The growth characteristics on SBA supplemented with 1 % (w/v) glycine (Merck), 2 % (w/v) NaCl or 3.5 % (w/v) NaCl, and on MacConkey (Merck), and Mueller-Hinton agar media were determined according to the procedures as described previously [3,36–39].After incubation for 3–5 days, the growth characteristics of the isolates under anaerobic (AnaeroPack system, Mitsubishi Gas Chemical Co Inc.) and aerobic atmospheres at 37℃, and under the microaerobic conditions (AnaeroPack™-MicroAero system, Mitsubishi Gas Chemical Co Inc.) at 10℃, 15℃, 20℃, 25℃, 30℃, 37℃, 42℃, and 45℃ were evaluated. In addition to the tests aforementioned above, α-hemolysis on 7 % SBA, and resistance to nalidixic acid (30 μg, Oxiod) and cephalothin (30 μg, Oxoid) were determined. *Helicobacter pylori* CCUG 39500^T was used as a control strain throughout the study (Dewhirst et al., 2000; On et al., 2017).

Chemotaxonomic analysis

The faydin-H75^T and faydin-H76 strains were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells (WC-MALDI-TOF MS). The MALDI-TOF MS results and mass spectrum profiles (MSP) created from the spectra of each strain were obtained by the Centre for Implementation and Research of the Plant Health Clinic of Hatay Mustafa Kemal University, Türkiye (Version 3.1; Microflex LT; Bruker Daltonics GmbH, Germany). Mass spectra were generated with a Microflex mass spectrophotometer and assessed using the Biotyper Real-Time Classification software with flexControl software (version 3.4) (Bruker Daltonics). A logarithmic score between 0 and 3 to describe the extent of peak matching was assigned. A score between

Table 1
Phenotypic characteristics differentiating *Helicobacter cappadocius* sp. nov. from other validated species of the genus *Helicobacter*.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Bacterial Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	(+)	+	+	+
Urease	+	+	+	+	+	−	+	+	+	+	−	−	−	−	+	−	−	−	+	+
Alkaline phosphatase	+	+	+	+	−	+	−	+	ND	+	−	−	+	+	−	+	(−)	+	+	−
Hippurate hydrolysis	−	−	+	−	ND	ND	ND	−	−	−	ND	ND	ND	−	−	ND	−	−	−	−
Nitrate reduction	+	−	+	+	−	+	−	+	+	+	−	+	−	−	−	+	+	+	+	−
Indoxyl acetate hydrolysis	+	−	−	+	+	−	+	−	−	+	+	+	−	+	−	−	(−)	−	−	−
Gamma-Glutamyl transpeptidase	+	+	+	−	−	−	+	+	ND	+	−	−	−	ND	+	−	ND	V	−	+
Reduction of Triphenyltetrazolium chloride (TTC)	+	−	+	+	ND	ND	ND	+	ND	+	ND	−	+	V	ND	ND	+	V	ND	−
H_2S production	−	−	ND	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	−	ND	−	ND	−	ND	ND
Growth at/in/under																				
25 °C (microaerobic)	+	−	−	−	ND	ND	−	−	−	−	ND	−	−	−	−	−	−	−	−	−
37 °C (microaerobic)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42 °C (microaerobic)	−	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−
Anaerobic (37 °C)	+	−	ND	+	−	ND	−	+	ND	ND	−	−	−	−	−	+	−	+	+	ND
Aerobic (37 °C)	−	−	ND	−	−	ND	ND	−	ND	ND	−	−	−	−	−	−	−	−	−	ND
2 % NaCl	−	−	ND	+	ND	ND	−	−	−	−	ND	−	ND	−	−	−	−	−	−	+
3.5 % NaCl	−	−	ND	−	ND	ND	−	−	−	−	ND	−	ND	−	−	−	ND	ND	ND	ND
Glycine (1 %)	+	−	ND	+	+	−	−	−	+	−	+	+	−	−	−	+	−	−	+	−
Resistance to Nalidixic acid (30 μg)	−	+	ND	−	−	−	−	I	+	+	−	+	−	−	I	I	−	+	I	−
Resistance to Cephalothin (30 μg)	+	−	ND	+	+	+	+	+	+	−	I	+	+	(−)	−	+	+	+	+	−
Periplasmic fibers	−	−	−	−	ND	−	+	+	+	−	ND	−	−	−	−	−	−	−	+	−
Number of flagella	1	2–5	6–8	2	2	2	7–10	11	3–14	10–20	2	1–2	2	2	2	1	1–2	2	6–12	2–6
Distribution of flagella	Mp	Mp	Bp	St	St	Mp	Bp	Bp	Bp	Bp	St	Bp	Bp	Bp	Bp	Mp	Bp	Bp	Bp	Bp

Characteristic	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Bacterial Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
Catalase	+	+	+	+	(+)	(−)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	−	−	+	−	−	+	+	−	−	+	−	−	−	+	−	+	+	+	−
Alkaline phosphatase	V	−	+	+	V	−	−	ND	+	+	−	−	+	+	+	−	+	−	−	+
Hippurate hydrolysis	ND	ND	−	−	−	−	+	−	−	−	ND	ND	−	−	−	ND	ND	ND	−	−
Nitrate reduction	−	−	+	+	−	+	+	+	+	+	−	−	−	−	+	−	−	−	−	(+)
Indoxyl acetate hydrolysis	−	−	−	+	+	−	−	+	−	−	+	−	−	−	−	+	−	−	−	ND
Gamma-Glutamyl transpeptidase	+	−	−	ND	ND	ND	+	ND	+	+	−	−	−	V	+	−	−	−	+	−
Reduction of Triphenyltetrazolium chloride (TTC)	ND	ND	−	−	+	+	ND	−	−	V	ND	ND	−	−	+	ND	ND	−	+	ND
H_2S production	ND	ND	−	ND	ND	−	ND	+	ND	−	ND	ND	−	−	ND	ND	ND	ND	ND	−
Growth at/in/under																				
25°C (microaerobic)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
37°C (microaerobic)	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42°C (microaerobic)	−	+	−	−	(+)	−	−	−	+	+	+	+	+	+	−	+	+	+	−	+
Anaerobic (37°C)	−	ND	−	−	−	+	+	+	+	W	−	−	+	−	ND	−	−	−	ND	−
Aerobic (37°C)	−	ND	−	−	−	−	−	−	−	−	−	−	−	−	ND	−	−	−	ND	−
2% NaCl	ND	ND	−	−	−	−	−	ND	−	−	ND	ND	−	−	−	ND	ND	ND	−	−
3.5 % NaCl	ND	ND	−	−	−	−	ND	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	ND	−
Glycine (1%)	−	+	−	−	−	−	−	+	−	−	+	−	+	−	−	+	+	+	−	−
Resistance to Nalidixic acid (30 μg)	−	−	+	−	−	−	ND	+	−	−	−	+	+	ND	−	+	+	+	−	(−)
Resistance to Cephalothin (30 μg)	+	−	+	−	−	+	ND	+	+	+	+	+	+	ND	−	+	+	+	−	+
Periplasmic fibers	+	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−
Number of flagella	6–12	1	1	14–20	2	2	4–10	2	1–2	2	4–8	1	2	2	8–10	1–2	2	2	5–10	2
Distribution of flagella	Bp	Mp	Mp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Mp	St	Bp	Bp	Bp	Bp	Bp	Bp	Bp

Characteristic	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55

(*continued on next page*)

Table 1 (*continued*)

Characteristic	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
Bacterial Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	(+)	+	+	+	+	+	+	−	+	+	+
Urease	−	+	+	−	−	(+)	−	−	+	+	+	−	−	(+)	+
Alkaline phosphatase	+	+	+	+	−	+	−	−	V	+	(−)	+	−	−	+
Hippurate hydrolysis	ND	−	−	−	−	−	−	ND	−	−	−	−	−	−	−
Nitrate reduction	(−)	−	+	+	+	−	+	−	−	−	+	−	+	−	+
Indoxyl acetate hydrolysis	−	−	+	−	−	(−)	−	−	(−)	−	ND	+	−	+	−
Gamma-Glutamyl transpeptidase	+	+	+	ND	ND	ND	−	+	+	+	+	−	−	−	+
Reduction of Triphenyltetrazolium chloride (TTC)	ND	ND	+	ND	ND	V	ND	ND	−	+	ND	−	ND	−	+
H_2S production	ND	−	−	−	ND	−	ND	ND	ND	ND	ND	−	−	−	ND
Growth at/in/under															
25°C (microaerobic)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
37°C (microaerobic)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42°C (microaerobic)	−	−	(+)	+	+	+	+	+	−	−	+	+	+	+	−
Anaerobic (37°C)	−	−	+	+	−	−	+	−	+	+	ND	+	−	−	ND
Aerobic (37°C)	−	−	−	+	−	−	−	−	ND	−	ND	−	−	−	ND
2% NaCl	ND	−	−	ND	−	−	ND	ND	−	−	ND	−	−	ND	ND
3.5 % NaCl	ND	−	−	ND	−	−	ND	ND	−	−	ND	−	−	ND	ND
Glycine (1%)	−	−	−	+	−	−	+	+	−	−	−	−	+	+	−
Resistance to Nalidixic acid (30 μg)	+	+	−	−	−	+	+	+	+	ND	+	+	−	V	−
Resistance to Cephalothin (30 μg)	+	+	+	+	+	−	+	+	−	ND	+	+	+	+	−
Periplasmic fibers	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−
Number of flagella	7–14	10–14	4–8	2	1	4–8	2	6–12	10–23	4–10	5–7	2	2	1	5–10
Distribution of flagella	Bp	Bp	Pt	Bp	Mp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Bp	Mp	Bp

1. Helicobacter cappadocius sp.nov. (n = 2); **2**. H. acinonychis; **3**. H. ailurogastricus; **4**. Helicobacter anatolicus; **5**. H.anseris; **6**. H. apri; **7**. H. aurati; **8**. H. baculiformis; **9**. H. bilis; **10**. H. bizzozeronii; **11**. H. brantae; **12**. H. canadensis; 13. H. canicola**; 14**. H. canis; **15**. H. cetorum; **16**. H. cholecystus; **17**. H. cinaedi; **18**. Helicobacter colisuis 2022 **19**. H. cynogastricus; **20**. C. delphinicola; **21**. H. didelphidarum; **22**. H. enhydrae; **23**. H. equorum; **24**. H. felis; **25**. H. fennelliae; **26**. H. ganmani; **27**. H. heilmannii; **28**. H. hepaticus; **29**. H. himalayensis; **30**.Helicobacter ibis, **31**. H. jaachi; **32**. H. japonicus; **33**. Helicobacter kayseriensis, **34**. Helicobacter kumamotonensis Kawamura et al. 2023**, 35**. H. labacensis; **36**. H. macacae; **37**. H. marmotae; 38. H. mastomyrinus; **39**. H. mehlei; **40**. H. mesocricetorum; **41**. H. monodelphidis; **42**. H. muridarum; **43**. H. mustelae; **44**. H. pametensis; **45**. H. pullorum; **46**. H. pylori. **47**. H. rodentium; **48**. H. saguini; **49**. H. salomonis; **50**. H. suis; **51**. H. trogontum; **52**. Helicobacter turcicus, **53**. H. typhlonius**; 54**. H. valdiviensis; **55**. H. vulpis.
Data for reference taxa were obtained from previous species description studies [On et al., 2017; Gruntar et al., 2020; Shen et al., 2020; Aydin et al., 2022; Lopez-Cantillo et al., 2023; Kawamura et al., 2023]. **+:** 90–100 %, **(+):** 75–89 %, **V:** 26–74 %, **(−):** 11–25 %, −: 0–10 %, **I**: Intermediate Resistance, **ND:** Not Determined, **Bp:** Bipolar, **Mp:** Monopolar, **Pt:** Peritrichous, **St**: Subterminal, **W**: Weak growth.

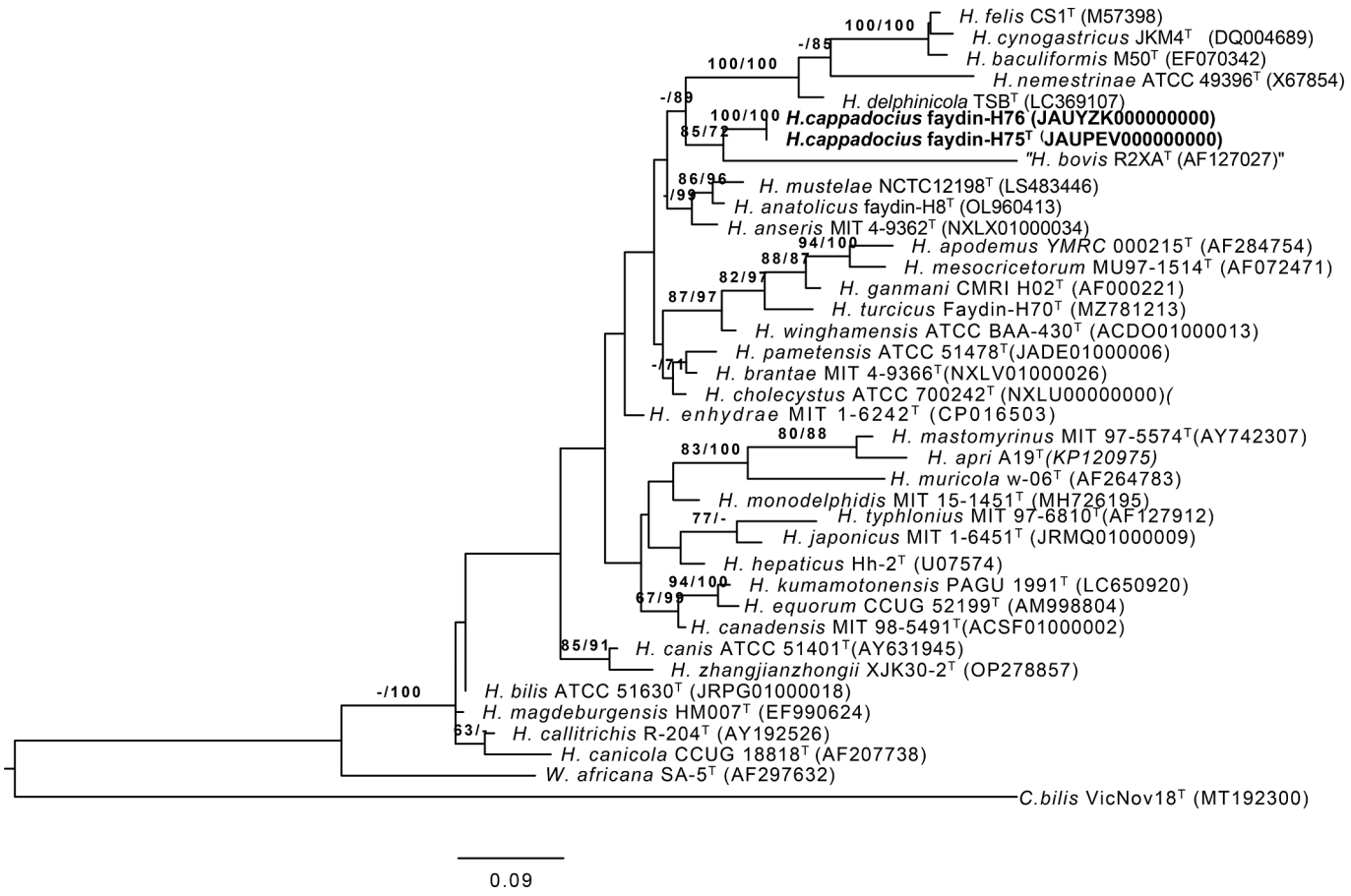

Fig. 1. The maximum likelihood tree inferred under the per site. The numbers above the branches are support values when GTR + GAMMA model and rooted by midpoint-rooting. The larger than 60 % from ML (left) and MP (right) bootstrapping branches are scaled in terms of the expected number of substitutions.

0 and 1.699 indicates a situation when there is no reliable identification; a score between 1.700 and 1.999 defines probable genus identification; a score between 2.000 and 2.299 defines a secure genus identification and probable species identification; a score between 2.300 and 3.000 indicate highly probable species identification.

Results and Discussion

The isolation and description

As a result of both the direct inoculation and the enrichment/filtration methods used in the cultural examination, two (2.73 %) of the 73 reptilian and amphibian cloacal swab samples analyzed were found to be positive in terms of microaerophilic, helical, curved, Gram-negative, and motile microorganisms. As a result of *Helicobacter* genus-specific PCR, from two isolates obtained a 780 bp band and were confirmed as *Helicobacter* spp.

When the published literature on the presence of *Helicobacter* spp. in reptiles is evaluated, it seems that there are a limited number of studies conducted. Lawson and Owen (2007) named a *Campylobacter*-like bacterium isolated from a snake (*Heterodon nasicus*) *Helicobacter serpensis* using various phenotypic and genotypic methods. However, the description of this snake-borne bacterium as a species within the genus *Helicobacter* was based solely on a poster presentation, and no full article or detailed information about this species could be accessed. Stacy and Wellehan (2010) detected the presence of *Helicobacter* sp. in a pancake tortoise (*Malacochersus tornieri*) with septicemia by the molecular method. In a comprehensive study conducted by Gilbert et al. (2014), the prevalence of *Helicobacter* spp. in various reptile species was found to be 4.8 % by cultural examination and 39.1 % by PCR. In addition, the researchers reported that based on 16S rRNA phylogeny, *Helicobacter* lineages recovered from reptiles constructed a distinct cluster from *Helicobacter* species from mammals and birds. Afterward, Gilbert et al. (2017) reported that eight *Helicobacter* spp. isolates obtained in their previous study could be putative novel *Helicobacter* species of them by the whole genome sequencing results. Rudolf et al. (2018) determined the rate of *Helicobacter* spp. in mouth and cloacal swab samples of wild and captive crocodiles to be 5 % and 31.6 %, respectively, via molecular analysis. Desiderio et al. (2021) detected the presence of *Helicobacter* sp. by molecular method from three turtles showing the disease symptoms. On the other hand, Conley et al. (2021) reported that *Helicobacter* sp. was responsible for Grand Cayman blue iguanas (*Cyclura lewisi*) deaths. Chan et al. 2021 identified *Helicobacter* isolates recovered from endangered Blue Iguanas (*Cyclura lewisi*) as *Helicobacter cyclurae*. In addition, the recent analysis of the cloacal microbiome in several lizard species has revealed that *Helicobacteriaceae* is the next most abundant taxon in the microbial community. However, the method used in these studies limits the presentation of detailed information about *Helicobacter* species; only data on the genus *Helicobacter* were provided without species-level details (Bunker et al., 2022; Bunker and Weiss, 2022).

As can be seen from the detailed literature data mentioned above, although studies on the existence and isolation of *Helicobacter* spp. from reptiles have been carried out, there is no helicobacter species described according to the taxonomic rules from these animal groups. In our study, the prevalence rate (2.7 %) we obtained by cultural examination appears to be relatively lower compared to the results of the studies that used molecular and cultural methods conducted on various reptiles mentioned above. It is thought that this situation depends on various factors such as the diversity of the animal groups examined, the number of samples analyzed, whether the analyzed animals are healthy,

Table 2
The dDDH, ANIb, ANIm, and difference in G + C% values among the genomes of strain *Helicobacter cappadocius* faydin-H75^T, and their related type strains in the *Helicobacter* genus.

Query strain	Subject strain	dDDH (d_4, in %)*	dDDH (d_6, in %)**	OrthoANI %	ANIb %	ANIm%	G + C content difference (in %)
H. cappadocius faydin-H75^T	*H. cappadocius* faydin-H76	100	100	99.9	100	99.9	0
H. cappadocius faydin-H75^T	*H. anseris* ATCC BAA-1299^T	18.5	13.5	69.5	68.8	84.2	1.4
H. cappadocius faydin-H75^T	*H. cholecystus* ATCC 700242^T	20	13.3	68.4	67.8	86.5	1.5
H. cappadocius faydin-H75^T	*H. mustelae* NCTC 12198^T	19.6	13.3	68	67.1	86.5	8.9
H. cappadocius faydin-H75^T	*H. enhydrae* MIT 01-6242^T	18.7	13.3	68	66.8	89.7	7.2
H. cappadocius faydin-H75^T	*H. japonicus* MIT 01-6451^T	21.4	13.2	68.5	67.8	88.7	2.2
H. cappadocius faydin-H75^T	*H. pametensis* ATCC 51478^T	21.3	13.2	67.9	67.2	88.4	6.5
H. cappadocius faydin-H75^T	*H. bilis* ATCC 51630^T	25.1	13.1	68.3	67.5	88.7	1.3

*dDDH formula 2:identities / High Scoring Pairs (HSP) length, ** dDDH formula: 3: identities / total length (Recommended for *Helicobacter* Genus)(On et al., 2017).

infected, or dead, and the detection method of the *Helicobacter*.

As evident from the published literature data provided above, some studies have been conducted on the presence and isolation of *Helicobacter* spp. from reptiles. However, despite these efforts, no *Helicobacter* species have been formally described according to taxonomic rules within these animal groups. Notably, while *Helicobacter serpensis* (Lawson and Owen, 2007) from snakes and *Helicobacter cyclurae* (Chan et al., 2021) from iguanas have been reported, both species are not currently approved and are absent from databases such as LPSN (https://lpsn.dsmz.de/genus/helicobacter) and NCBI (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi).

In our study, we observed a relatively lower prevalence rate (2.7 %) compared to previous studies on the various reptilian and amphibian species. Several factors may contribute to this discrepancy, including the diversity of animal groups examined, the number of samples analyzed, the health status of the animals (whether healthy, infected, or deceased), and the detection method used for helicobacters.

Considering growth tests at various temperatures, it appears that although the optimal growth temperature of the 54 validly published *Helicobacter* species, is 37 °C, not all species are capable of growth below 30 °C (Table 1). However, it has been reported that *Helicobacter* spp. of reptilian origin grow at 25 °C (Lawson and Owen, 2007; Gilbert et al., 2017) and 28 °C (Chan et al., 2021). In our study, it was determined that *Helicobacter cappadocius* also grows below 20 °C (the temperature range for the growth of *Helicobacter cappadocius* is between 14 °C and 41 °C). This psychrotrophic characteristic is reported for the first time. Therefore, *Helicobacter cappadocius* sp.nov. which is capable of growing below 20 °C, is the first *Helicobacter* species with psychrotrophic properties. It is believed that the growth ability of *Helicobacter* sp. faydin H-75^T at temperatures below 20 °C is associated with the body temperature of the reptiles from which the *Helicobacter* was isolated. This trait of the bacterium is thought to have been acquired over a long period after the colonization of the reptile digestive system.

Phylogenetic analysis based on 16S rRNA gene sequences

The nearly full-length 16S rRNA gene sequences of strains faydin-H75^T and faydin-H76, comprising 1367 bp and 1363 bp, respectively, underwent comprehensive analysis. In the course of pairwise sequence analysis for the 16S rRNA gene, it was observed that strain faydin-H75^T exhibited the highest identity level of 96.2 % with *H. mustelae* strain R85-13-6^T. Furthermore, the 16S rRNA gene sequence identity between strains faydin-H75^T and faydin-H76 was determined to be 99.9 %. Considering that their 16S rRNA gene sequence identity levels fell below the established threshold of 98.7 % for species demarcation, both isolates are identified as representatives of one novel *Helicobacter* species (Chun et al., 2018). The nucleotide matrix consisted of 38 operational taxonomic units and 1824 characters, with 642 variable and 327 parsimony-informative positions. The base-frequency check revealed no compositional bias ($p = 1.00$, $\alpha = 0.05$). Maximum Likelihood (ML) analysis, employing the GTR + GAMMA model, produced the highest log likelihood of −9631.52, with an estimated alpha parameter of 0.15. Despite non-convergence in ML bootstrapping, 650 replicates were performed, resulting in an average support of 55.86 %. In contrast, Maximum Parsimony (MP) analysis yielded the best score of 1517, accompanied by a consistency index of 0.6 and a retention index of 0.61, leading to the identification of single best trees. The MP bootstrapping average support was determined to be 85.8 %. The phylogenetic trees based on the 16S rRNA gene sequence analysis imply that the strains might be representatives of one novel species in the genus *Helicobacter*, as the strains formed distinct branches well-separated from their close neighbors (Fig. 1).

General taxonomic genome features

The genomes of the strains faydin-H75^T, and faydin-H76 were submitted to the NCBI GenBank database under accession numbers JAUPEV000000000 and JAUYZK000000000, respectively. The genome sizes of the strains are around 1.7 Mb and the GC content was determined as 33.5 % in both strains (Table S2). The genome relatedness indices of the strains were analyzed using three algorithms, namely dDDH, ANIb, and ANIm. The values obtained were compared with the closest species *H. anseris* ATCC BAA-1299^T. The results showed that the range of values for dDDH, OrthoANI, ANIb, and ANIm were 13.5 %, 69.5 %, 68.8 %, and 84.2 %, respectively (Table 2). These values were found to be significantly lower than the established cut-off values for each algorithm. The cut-off values for ANIb and ANIm were 95 %, while for dDDH, it was 70 %. Genome-based phylogenetic analyses were conducted on TYGS. Phylogeny approach, inferring a balanced minimum evolution tree with branch support through the FASTME 2.1.4 algorithm (Figure S1). In the whole-genome-based tree, strain faydin-H75^T showed same cluster with *H. anatolicus* faydin-H8 strain^T isolated from crow. The inconsistencies between the phylogenetic trees based on 16S rRNA gene sequences and the phylogenomic trees constructed from whole-genome sequence analyses reveal that 16S rRNA gene sequence analyses are insufficient to understand phylogeny and evolution of the members of the genus *Helicobacter*. In the phylogenetic analysis performed using strains isolated from reptiles and *Helicobacter* type strains, it was seen that the group isolated from lizards was located together with faydin-H75^T, and faydin-H76, and the group isolated from lizards was separated from other species (Fig. 2).

Genome analysis

A total of 54 virulence genes were identified in the genomes of the strains faydin-H75^T and faydin-H76 by VFDB (Table 3). These virulence genes encode various virulence factors, including acid tolerance, adhesion, immune evasion, motility, endotoxin, enzyme, lipid metabolism, and quorum sensing. The presence of these virulence factors suggests that these strains have the potential to cause disease. However, further studies are needed to determine the actual virulence of these strains. These studies may include to investigate the disease-causing

Fig. 2. The phylogenetic tree based on single-copy genes found in the the whole genome sequences of faydin-H75^T and faydin-H76, *Helicobacter* type strains and *Helicobacter* spp. strains recovered from reptile were constructed by the −BV-BRC bacterial genome tree pipeline.

mechanisms of these strains in vivo models.

Whole-genome analysis of the faydin-H75^T and faydin-H76 strains revealed the presence of six antimicrobial resistance genes. These genes encode various mechanisms of resistance, including efflux pumps and target site alteration, which confer resistance to a range of antibiotics, including elfamycin, cephalosporin, cephamycin, penam, fusidan, fluoroquinolone, tetracycline, and nitroimidazole. The identification of these resistance genes is concerning, as it suggests that the corresponding strains may be resistant to some commonly used antibiotics. This could complicate the treatment of infections caused by these strains and necessitate the use of alternative antibiotics, which may have broader side effect profiles or be more expensive.

In our study, the antiSMASH analysis identified a unique phosphonate biosynthetic gene cluster in both strains, faydin-H75^T and faydin-H76, which showed a 6 % similarity to the known polysaccharide B biosynthetic gene cluster from Bacteroides fragilis (MIBiG accession number BGC0001411). This 6 % similarity indicates that the identified gene cluster shares a portion of its gene content and organization with the polysaccharide B gene cluster from *Bacteroides fragilis*, suggesting a novel pathway with some conserved elements. The synteny of the identified phosphonate biosynthetic gene cluster in our strains includes genes involved in key enzymatic steps of the biosynthesis pathway. These genes are syntenic with known phosphonate gene clusters from other microbial genomes. Specifically, the gene cluster in our strains

Table 3
Virulence and antimicrobial resistance genes found in the genome of *Helicobacter cappadocius* sp. nov. faydin-H75^T and faydin-H76 The virulence and antimicrobial resistance gene profiles were analyzed for both strains, faydin-H75^T and faydin-H76. Since the profiles were found to be identical.

Virulence Genes*

Virulence Factor Class	Virulence Factor	Virulence Genes
Acid resistance	Urease	ureA, ureB, ureE, ureF, ureG, ureH, ureI
Adherence	LPS	wbpI
	The tad locus (Haemophilus)	tadA
Immune evasion	Lipooligosaccharide (LOS)	gmhA
Immune modulator	Neutrophil-activating protein (HP-NAP)	napA
Motility	Flagella	flaA, flaB, flaG, flgB, flgC, flgD, flgE_1, flgE_2, flgG_1, flgG_2, flgH, flgI, flgK, flgL, flhA, flhB_1, flhB_2, flhF, fliA, fliD, fliE, fliF, fliG, fliH, fliI, fliL, fliM, fliN, fliP, fliQ, fliR, fliS, fliY, motA, motB, pflA
Motility and export apparatus	Flagella(*Campylobacter*)	flgR, flhG
Endotoxin	LPS(*Bordetella*)	bplB
Enzyme	Streptococcal enolase (*Streptococcus*)	eno
Glycosylation system	O-linked flagellar glycosylation (Campylobacter)	pseF
Lipid and fatty acid metabolism	Pantothenate synthesis (Mycobacterium)	panD
Quorum sensing	Autoinducer-2(Vibrio)	luxS

Antimicrobial Resistance Genes*

RGI criteria	ARO Term	SNP	Detection Criteria	Drug Class	Resistance Mechanism	% Identity of Region	% Length of Reference Sequence
Loose	Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin	R234F	protein variant model	elfamycin	antibiotic target alteration	73.87	97.56
Loose	Helicobacter pylori pbp1 mutants conferring resistance to amoxicillin	S414N, T593S	protein variant model	cephalosporin, cephamycin, penam	antibiotic target alteration	62.05	100.15
Loose	*Staphylococcus aureus* fusA with mutation conferring resistance to fusidic acid	V90I	protein variant model	fusidane	antibiotic target alteration	61.04	99.86
Loose	*Helicobacter pylori* pbp2 mutants conferring resistance to amoxicillin	E572G	protein variant model	cephalosporin, cephamycin, penam	antibiotic target alteration	57.53	102.72
Loose	hp1181		protein homolog model	Fluoroquinolone, tetracycline, nitroimidazole	antibiotic efflux	54.46	100.90
Loose	*Clostridioides difficile* gyrA conferring resistance to fluoroquinolones	P116A	protein variant model	fluoroquinolone	antibiotic target alteration	53.77	102.23

showed structural similarity in gene organization and functional annotation with clusters found in Bacteroides fragilis and other related species. The conserved arrangement of genes includes those responsible for initial substrate activation, intermediate transformation, and final product formation, highlighting their functional roles within the biosynthesis pathway.

The phage region identified in strains faydin-H75^T and faydin-H76 was analyzed using the PHASTEST web server, revealing a 70 % questionable score, which indicates a possible prophage sequence (Figure S2). This region includes 10 coding sequences (CDS) predicted to encode phage structural proteins (head, tail, plate, fiber) and hypothetical proteins. Further analysis showed that this phage region shares similarities with those in other *Helicobacter* species, suggesting a conserved phage structure within the genus, although specific genes within this region vary among different *Helicobacter* species. Comparison with existing databases revealed that several genes are also present in other *Helicobacter* species, indicating potential horizontal gene transfer or a conserved ancestral origin.

Genus-specific protein family (PLfam) analysis across *Helicobacter* species, including *H. cappadocius* strains faydin-H75^T and faydin-H76, revealed a total of 23,775 protein families. Among these, 274 families are part of the core genome shared by all *Helicobacter* species (Table S3).

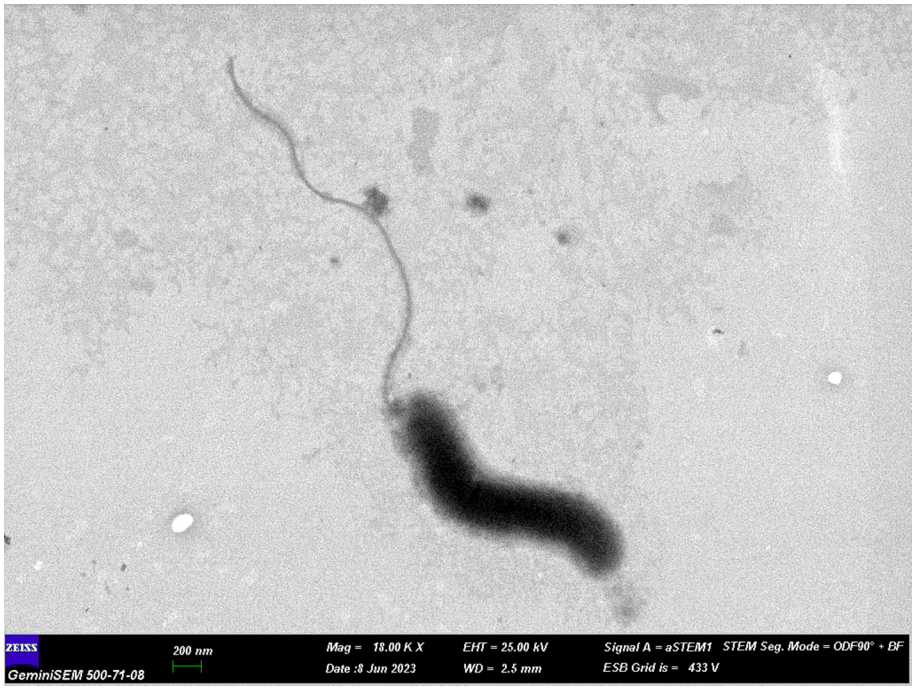

Fig. 3. TEM micrograph of *Helicobacter cappadocius* sp.nov. faydin-H75^T. The spiral bacterium has a single polar flagellum.

In contrast, 167 unique protein families were identified in the *H. cappadocius* strains, representing the accessory genome specific to this novel species (Table S4). These unique protein families are thought to contribute to various biological processes, though their exact functions remain to be elucidated. Some of the protein families identified in *H. cappadocius* are associated with general metabolic pathways such as retinol metabolism, cytochrome P450 systems, and fatty acid biosynthesis. Additionally, many of the protein families in the accessory genome are classified as 'hypothetical proteins,' indicating that their functions are yet to be defined. The presence of these unique protein families suggests potential involvement in metabolic pathways, including methane biosynthesis, glycolysis/gluconeogenesis, and the degradation of environmental toxins such as tetrachloroethene and 1,4-dichlorobenzene.

A separate study compared protein families across the genomes of various *Helicobacter* type strains, including *H. cappadocius* strains faydin-H75^T and faydin-H76, along with eight *Helicobacter* spp. strains isolated from reptiles grown at 25 °C. It was found that a specific amidase family protein (bll0246) was present in the genomes of the eight reptile-associated *Helicobacter* species, including *H. cappadocius*. However, this protein was absent in other *Helicobacter* species that do not grow at 25 °C. The amidase gene, previously characterized as a putative tricarballylate catabolism gene (tcuC) by Gilbert et al. (2017), is associated with catalytic activity (Gene Ontology GO:0003824) and plays a key role in catalyzing biochemical reactions at physiological temperatures. Its presence in the genomes of *Helicobacter* species isolated from cold-blooded reptiles suggests a critical function in enabling growth at 25 °C.

While automated annotation tools are useful for pathway identification, they have limitations, particularly when applied to novel or poorly characterized species (Huang et al., 2018). Therefore, further experimental studies are essential to fully understand the functions of these protein families. Gene expression analyses, enzyme activity assays, and pathway reconstructions should be employed to confirm the roles of these protein families, particularly those involved in xenobiotic degradation, amino acid metabolism, and energy production (Bileschi et al., 2022). Unraveling the functions of these unique proteins in *H. cappadocius* will be crucial for understanding the bacterium's environmental adaptations and metabolic capabilities.

Ecological distribution

All approved members of the *Helicobacter* genus have been isolated from mammalian and avian species (Parte et al., 2020). However, there are also studies on the colonization of reptiles with helicobacters (Gilbert et al., 2014). In the present study, the ecological preferences and distribution of strain faydin-H75^T were examined using the Protologger web tool. Analysis of the 16S rRNA genes showed that their presence in the metagenomes of chicken, pig, and mouse was low, at 1 %, 1 %, and 1.7 %, respectively. For insects, plants, humans, sludge, wastewater, freshwater, corals, cattle, marine environments, marine sediments, rhizospheres, and soil, the distribution was even lower, falling below 1 % (Figure S3). For a more comprehensive analysis, the IMNGS tool was used to analyze the presence of strains across different metagenomes, including those derived from reptilian genomes. High frequencies of 16S rRNA sequences from these strains were found in the metagenomes of species such as Lilford's wall lizard (*Podarcis lilfordi*), Italian wall lizard (*Podarcis sicula*), and Crocodile lizard (*Shinisaurus crocodilurus*), as shown in Table S5. Additionally, the BLAST analysis of the 16S rRNA sequence of faydin-H75^T against amplicon-based metagenomic SRA data from GenBank showed high similarity with multiple lizard species, indicating a broad distribution of the strain across different hosts (Table S6). The metagenome data from lizard species such as *Podarcis lilfordi, Podarcis virescens, Podarcis sicula, Podarcis guadarramae lusitanicus*, and *Takydromus septentrionalis* showed over 99.5 % similarity. The WGS-based metagenomic analysis confirmed the presence of strain faydin-H75^T in various lizard samples with varying levels of abundance. The highest relative abundance was observed in the *Lacerta Agilis* (Sand lizard) sample with 0.049 %, followed by *Phrynocephalus theobaldi* (Theobald's toad-head agama) samples with 0.031 % and 0.026 % (Table S6). These results support the robustness of the methodologies used and the distinct classification of the strain faydin-H75^T, contributing valuable insights into its ecological distribution and host specificity.

Subsequent studies on ecological distribution indicated that the strains documented in the GenBank database shared up to 96 % sequence similarity with various documented cultured (both type and non-type strains) and uncultured *Helicobacterium* representatives, yet

Table 4
Descriptions of *Helicobacter cappadocius* sp. nov. faydin-H75$^{T.}$

Guiding Code for Nomenclature	ICPN
Nature of the type material	Strain
Genus name	*Helicobacter*
Species name	*Helicobacter cappadocius*
Specific epithet	*cappadocius*
Species status	sp. nov.
Species etymology	*Helicobacter cappadocius* sp. nov. cappadocius; cap.pa.do'ci.us. L. masc. adj. cappadocius, Cappadocian, refers to the lizard species from which the type strain was isolated.
Description of the new taxon and diagnostic traits	The colony morphologies of faydin-H75^{T} and faydin-H76 strains on SBA were alpha-hemolytic, non-pigmented, grey in color, circular with convex elevation, and smooth with entire margins. In Gram-staining, the bacterial cells were observed to be Gram-negative and helical. Electron microscopic images of the strain faydin-H75^{T} revealed that bacterial cells had an average length of 2–2.2 μm and an average width of 0.45–0.5 μm, and possessed a single polar flagellum. The flagellum size of the bacterium is 3.1–3.2 μm. In addition, no periplasmic fibers were observed. The strains were found to be positive for bacterial motility by the hanging drop technique. The bacterial cells transformed into coccoid forms in aging cultures (>2 days). The isolates were detected to be positive oxidase, catalase and urease activities. While the isolates grew in presence of 1 % glycine and with 1 % NaCl, it was not observed the growth in presence of 2 % NaCl. The growth was observed on Mueller-Hinton agar. They did not grow on MacConkey agar and Nutrient agar. While the isolates did not grow under aerobic conditions at 37 °C, growth was observed in an anaerobic atmosphere. Both faydin-H75^{T} and faydin-H76 isolates grew in a microaerobic environment between 14 °C and 41 °C. The isolates were detected to be resistant to cephalothin but not nalidixic acid. API Campy test kit confirmed positive results for the urease, nitrate, indoxyl acetate, alkaline phosphatase activities, gammaglutamyl transferase, triphenyl tetrazolium chloride reduction, pyrrolidonyl arylamidase, L-arginine arylamidase, and L-aspartate arylamidase. In addition, hippurate hydrolysis and H_2S production were not detected.
Country of origin	Turkey
Region of origin	Hatay
Date of isolation (dd/mm/yyyy)	01/08/2022
Host	*Apathya cappadocica*
Source of isolation	cloacal swab
Sampling date (dd/mm/yyyy)	16/05/2022
Latitude (xx°xx′xx″N/S)	36°40′07.0″N
Longitude (xx°xx′xx″E/W)	36°21′55.0″E
16S rRNA gene accession nr.	OR361833 for faydin-H75^{T}, OR361833 for faydin-H76
Genome accession number [RefSeq; EMBL; …]	faydin-H75^{T}; JAUPEV000000000 faydin-H76; JAUYZK000000000
Genome status	Incomplete
Genome size	1,754,341
GC %	33,5
Number of strains in study	2
Source of isolation of non-type strains	*Ophisops elegans*
Information related to the Nagoya Protocol	Turkey is not yet Party to the Nagoya-Protocol
Designation of the Type Strain	faydin-H75^{T};
Strain Collection Numbers	NCTC014972^{T}, LMG 33382^{T}, DSM117062^{T}

none exhibited complete similarity. The highest sequence matches, which were between 96 % and 99 %, occurred in samples from lizards, assorted bird species, chickens, humans, monkeys, and ferrets. Results from BLAST analysis suggest a clustering of reptilian strains (Figure S4).

Morphological and phenotypic characterization of the isolates

The colony morphologies of faydin-H75^{T} and faydin-H76 strains on SBA were alpha-hemolytic, non-pigmented, grey in color, circular with convex elevation, and smooth with entire margins. In Gram-staining, the bacterial cells were observed to be Gram-negative and helical (Figure S5). Electron microscopic images of the strain faydin-H75^{T} revealed that bacterial cells had an average length of 2–2.2 μm and an average width of 0.45–0.5 μm, and possessed a single polar flagellum. The flagellum size of the bacterium is 3.1–3.2 μm. In addition, no periplasmic fibers were observed (Fig. 3). The strains were found to be positive for bacterial motility by the hanging drop technique. The bacterial cells transformed into coccoid forms in aging cultures (>2 days). The isolates were detected to have positive oxidase, catalase, and urease activities. While the isolates grew in the presence of 1 % glycine and with 1 % NaCl, it was not observed the growth in presence of 2 % NaCl. The growth was observed on Mueller-Hinton agar. They did not grow on MacConkey agar and Nutrient agar. While the isolates did not grow under aerobic conditions at 37 °C, growth was observed in an anaerobic atmosphere. Both faydin-H75^{T} and faydin-H76 isolates grew in a microaerobic environment between 14 °C and 41 °C. The isolates were detected to be resistant to cephalothin but not nalidixic acid. API Campy test kit confirmed positive results for the urease, nitrate, indoxyl acetate, alkaline phosphatase activities, gammaglutamyl transferase, triphenyl tetrazolium chloride reduction, pyrrolidonyl arylamidase, L-arginine arylamidase, and L-aspartate arylamidase. In addition, hippurate hydrolysis and H_2S production were not detected. The phenotypic characteristics that differentiated strains faydin-H75^{T} and faydin-H76 from *Helicobacter* species are given in Table 1 (On et al., 2017; Gruntar et al., 2020; Shen et al., 2020; Aydin et al., 2022; Lopez-Cantillo et al., 2023; Kawamura et al., 2023). The complete list of phenotypic characteristics is given in the descriptions (Table 4).

Chemotaxonomic analysis

Although MALDI-TOF MS is considered as a rapid and direct method for the detection of potentially pathogenic bacteria, no reliable identification was obtained for the novel strains in the present study. The average MALDI score values recorded were 1.2 and 1.3 for strains faydin-H75^{T} and faydin-H76, respectively.

Conclusions

Helicobacter cappadocius sp. nov. cap.pa.do'ci.us; L. masc. adj. cappadocius, Cappadocian refers to the lizard species from which the type strain was isolated.

In the present study, a polyphasic characterization based on phenotypic and phylogenetic analyses as well as a comprehensive genome analysis of two *Helicobacter* strains recovered from the cloacal swab samples of two reptile species was accomplished. For more comprehensive phylogenomic characterization, whole-genome-based comparisons were performed for the strain faydin-H75^{T}. This study shows that this strain represents a novel species within the genus *Helicobacter*, for which the names *Helicobacter cappadocius* sp. nov. The type strain for the novel species are faydin-H75^{T} (=NCTC 014972 = LMG 33382 = DSM 117062). The pathogenicity of the bacterium for humans and animals is unknown.

According to our current knowledge, *Helicobacter cappadocius* sp. nov. recovered from Cappadocian Lizard (*Apathya cappadocica*) and Snake-eyed Lizard (*Ophisops elegans*) is the first *Helicobacter* species that grow below 20 °C, unlike other *Helicobacter* species. It was detected that

the temperature range for growth of *Helicobacter cappadocius* sp. nov. is between 14 °C and 41 °C. Moreover, it is thought that more comprehensive studies, including analyses such as transcriptome and proteomics, are required to reveal the mechanisms that enable growth at 14 °C of *Helicobacter cappadocius* sp.nov.

Sequence accession numbers

The 16S rRNA gene sequence data of three isolates (*Helicobacter* sp. faydin-H75^T, and *Helicobacter* sp. faydin-H76) reported here were deposited in GenBank under accession numbers as OR361833, and OR361834. The accession numbers of the draft whole-genome sequences of the strains *Helicobacter* sp. faydin-H75 *Helicobacter* sp. faydin-H76 are JAUPEV000000000 and JAUYZK000000000, respectively. The version described in this paper is the first version.

Ethical statement

This study was approved by the Local Ethics Committee for Animal Experiments (HADYEK) of Aydın Adnan Menderes University, Aydın, Türkiye. The Ethics Committee reference number is 64583101–64.

CRediT authorship contribution statement

Fuat Aydin: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Serdal Tarhane:** Resources, Methodology. **Emre Karakaya:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Seçil Abay:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tuba Kayman:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Özgür Güran:** . **Emin Bozkurt:** Resources, Methodology. **Nazan Üzüm:** Resources, Methodology. **Aziz Avci:** Resources, Methodology. **Kurtuluş Olgun:** Resources, Methodology. **Daniel Jablonski:** Resources, Methodology. **Cansu Aydin:** Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **İzzet Burçin Saticioğlu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2024.126557.

Data availability

Data will be made available on request.

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