frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 14 September 2021
doi: 10.3389/fevo.2021.676682

Helicobacter cyclurae sp. Nov., Isolated From Endangered Blue Iguanas (*Cyclura lewisi*)

*Nathan Chan[1], Zeli Shen[1], Anthony Mannion[1], Susanna Kurnick[1], Ioana S. Popescu[2], Frederic J. Burton[3], Paul P. Calle[4] and James G. Fox[1]**

[1] Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA, United States, [2] Island Veterinary Services, George Town, Cayman Islands, [3] Department of the Environment, George Town, Cayman Islands, [4] Wildlife Conservation Society, Zoological Health Program, Bronx, NY, United States

Blue iguanas (*Cyclura lewisi*) are endangered reptiles found only on Grand Cayman. Previously, DNA for a novel *Helicobacter* species GCBI1 was detected in sick and dead iguanas. In the current study, fecal and cloacal swab samples were obtained from 25 iguanas. Through molecular and microbiological techniques, a novel *Helicobacter* species was cultured from feces and characterized, for whom we propose the name *Helicobacter cyclurae*. This novel helicobacter had a prevalence of 56% by PCR and 20% by culture in samples analyzed. The type strain MIT 16-1353 was catalase, oxidase, and gamma-glutamyl transpeptidase positive. By electron microscopy, *H. cyclurae* has a curved rod morphology and a single sheathed polar flagellum. Phylogenetic analysis using 16S rRNA, *gyr*B, and *hsp*60 indicated that these strains were most closely related to *Helicobacter* sp. 12502256-12 previously isolated from lizards. *H. cyclurae* has a 1.91-Mb genome with a GC content of 33.37%. There were 1,969 genes with four notable virulence genes: high temperature requirement-A protein-secreted serine protease, gamma-glutamyl transpeptidase, fibronectin/fibrinogen binding protein, and neutrophil-activating protein. Whole-genome phylogeny, average nucleotide identity, and digital DNA–DNA hybridization analysis confirmed that *H. cyclurae* is a novel species, and the first helicobacter cultured and characterized from blue iguanas.

Keywords: *Helicobacter*, *Cyclura lewisi*, blue iguana, novel species, Grand Cayman, endangered, reptiles

OPEN ACCESS

Edited by:
Enrique P. Lessa, Universidad de la República, Uruguay

Reviewed by:
Gregorio Iraola, Institut Pasteur de Montevideo, Uruguay
Rodney L. Honeycutt, Pepperdine University, United States

****Correspondence:***
James G. Fox
jgfox@mit.edu

Specialty section:
This article was submitted to Phylogenetics, Phylogenomics, and Systematics, a section of the journal Frontiers in Ecology and Evolution

Received: *08 March 2021*
Accepted: *05 August 2021*
Published: *14 September 2021*

Citation:
Chan N, Shen Z, Mannion A, Kurnick S, Popescu IS, Burton FJ, Calle PP and Fox JG (2021) Helicobacter cyclurae sp. Nov., Isolated From Endangered Blue Iguanas (Cyclura lewisi). Front. Ecol. Evol. 9:676682. doi: 10.3389/fevo.2021.676682

INTRODUCTION

The Cayman Islands are a group of Caribbean islands composed of the Grand Cayman, Little Cayman, and Cayman Brac. These islands are inhabited with diverse fauna and flora including numerous orchids and animals such as the Cayman Brac Parrot and dwarf boa. The Grand Cayman iguana, *Cyclura lewisi*, a blue colored reptile is indigenous only to Grand Cayman. It is currently listed as Endangered under the US Endangered Species Act. They have been threatened to the point of extinction when in the past their numbers dwindled to fewer than 20 animals in the wild due to predation from feral species, vehicular accidents, and habitat loss (Goodman and Burton, 2005). Due to the collective efforts of the Wildlife Conservation Society and local partners in Grand Cayman, the number of this species in the wild has drastically improved.

Helicobacter is a genus of bacteria that are known to cause diseases in humans and a variety of animal species. Specifically, enterohepatic *Helicobacter* species (EHS) colonize the gastrointestinal tract and are associated with gastroenteritis and hepatitis in humans and animals. Importantly, several of these *Helicobacter* species cause bacteremia and systemic diseases (Fox, 2002; Stacy and Wellehan, 2010; Shen et al., 2017). Reptiles are also colonized by *Helicobacter* spp., with one report finding a prevalence of 4.8 and 39.1% by culture and PCR, respectively, and more specifically 7.4 and 30.7% for animals of the suborder *Lacertilia* (Gilbert et al., 2014). In a recent study, two free-ranging blue iguanas located at the Grand Cayman Queen Elizabeth II Botanic Park (QEIIBP) in 2015 developed hind limb paralysis and septicemia with spiral-shaped bacteria noted on blood smears (Conley et al., 2021). PCR results, using specific *Helicobacter* species primers, from the blood and tissue of these animals were positive for a novel *Helicobacter* species, provisionally designated *Helicobacter* sp. GCBI1. The novel helicobacter, by 16S rRNA analysis, is 95.1% similar to a helicobacter identified in a Greek tortoise, and phylogenetic analysis of *Helicobacter* sp. GCBI1 suggested that it is an EHS as it grouped with other EHS in the chelonian clade, *Helicobacter* spp. taxon 2 (Gilbert et al., 2014). Examination and clinical testing of affected animals along with responsive treatment for helicobacter infection was suggestive that *Helicobacter* sp. GCBI1 was the causative agent of disease in Grand Cayman blue iguanas. Retrospective analysis from a mortality event of green iguanas a year prior and screening of healthy free-ranging green iguanas from QEIIBP also found this helicobacter by qPCR (Conley et al., 2021). In this paper, we describe, by utilizing molecular and microbiological methodology, the isolation of a novel *Helicobacter* species, *H. cyclurae*, that is distinct from previously described *Helicobacter* sp. GCBI1, from feces and cloacal swabs in Grand Cayman blue iguanas.

MATERIALS AND METHODS

Animals

A total of 25 blue iguanas were screened for helicobacter. For 17 animals, fecal samples were collected; for eight animals, feces were not available, so eight cloacal swab samples were collected instead. Collected samples were placed in freeze media (20% glycerol in Brucella broth) and kept at −80°C prior to processing.

Bacterial Isolation and Characterization

Feces were homogenized in freeze media; the freeze media containing fecal material and cloacal swab suspensions were directly placed on CVA (cefoperazone, vancomycin, and amphotericin) plates or filtered through 0.65-μm filters onto tryptic soy agar plates with 5% sheep blood agar plates (Remel Laboratories, Lenexus, KS) and incubated under microaerobic conditions at 37°C with a gas mixture of N_2, CO_2, and H_2 (80:10:10). Detailed biochemical characterization analysis was performed on five individual isolates using a RapID^{TM} NH System (Remel Laboratories, Lenexus, KS). Biochemical characterization of urease, catalase, and oxidase production; sensitivity to nalidixic acid and cephalothin; and growth in the presence of 1% glycine were analyzed as previously described by our laboratory (Shen et al., 2005, 2017) and following the guideline of minimal standards for describing new species belonging to the families *Campylobacteraceae* and *Helicobacteraceae* (On et al., 2017; Shen et al., 2020).

DNA Extraction, PCR, and Sequence Analysis for 16S rRNA, *gyr*B, and *hsp60* Genes

A High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN) was used for bacterial DNA extraction, and the QIAamp DNA Stool Mini Kit (Qiagen, Germantown, MD) was used for fecal DNA extraction. The nearly full 16S rRNA sequence of five strains was amplified with primer 9F (5′-GAG TTT GAT YCT GGC TCA G-3′) and 1541R (5′-AAG GAGGTG WTC CAR CC-3′). *Helicobacter* genus-specific primers which amplify a 1.2-kb product from 16S rRNA gene were used for PCR for fecal and cloacal swab samples (Fox et al., 1998). Primers HSP60AF (5′-GCT AAT CCT ATT GAA GTG AAA AGA GGN ATG GAY AA-3′) and HSP60DR (5′-CAC TAA GGT AGT TAA AGC TTC CCC TTC DAT RTC YT-3′) were used to amplify the *hsp*60 gene (Inglis et al., 2006); primers specific for *Helicobacter* species iguana isolates gyrB-igu-F (5′-GAT ACT TAT AAA GTT TCC GG-3′) and gyrB-igu-R (5′-CAA ATT CCT TAT CAA TTC CGC A-3′) were used to amplify the *gyr*B gene. PCR amplifications were performed using the Expand High Fidelity PCR System (Roche Molecular Biochemicals, Indianapolis, IN). The following conditions were used for amplification: 35 cycles of denaturation at 94°C for 1 min, annealing at 55–58°C for 1 min, and elongation at 72°C for 1.5 min, followed by an elongation step of 7 min at 72°C. The PCR products from bacterial isolates and fecal samples were directly sequenced, using a commercial sequencing facility. Sequences were compared directly with the NCBI GenBank nucleotide database by BLAST search. Phylogenetic trees were constructed by the neighbor-joining method with the Lasergene software package (DNASTAR Madison WI) based on the comparison of genes from other *Helicobacter* species. Bootstrap values (>75%) were based on 1,000 replications.

Draft Genome Sequencing and Comparative Analysis

Genomic DNA from the type strain MIT 16-1353 isolated from the feces of a blue iguana was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicenter) following the manufacturer's protocol for bacterial cell samples. DNA libraries were prepared using NextraXT for sequencing of 2 × 150 paired-end reads by Illumina MiSeq. Raw sequenced reads were decontaminated of adapter sequences and quality trimmed to a Phred quality score (Q) ≥ 10 using BBDuk from the BBMap package version 37.17 (BBMap, SourceForge, Fairfax, VA). Decontaminated reads were then assembled into contigs with SPAdes followed by genome annotation with RAST, with both services hosted by PATRIC (Wattam et al., 2017). Through OrthoFinder (Emms and Kelly, 2019),

TABLE 1 | Phenotypic characteristics that differentiate *H. cyclurae* from other EHS and selected gastric *Helicobacter* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	U	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	−	+	+	(+)	+	+	+	(+)	(−)	+	+	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+	+	+
Nitrate reduction	−	−	−	+	−	+	−	±	−	−	+	+	−	+	+	−	+	+	+	−	−	−	−	+	(−)	−	+	+	+	−	+	−	−	+	+	−
Indoxyl acetate hydrolysis	−	−	+	−	+	−	+	+	+	−	−	(−)	−	−	+	+	−	+	−	+	−	+	−	U	−	−	+	−	−	(−)	−	−	−	U	−	+
Urease	−	+	+	−	+	+	−	−	−	+	−	−	+	−	+	−	−	+	−	+	−	−	+	−	−	+	+	−	−	(+)	−	−	+	+	−	(±)
Alkaline phosphatase	±	+	−	+	−	U	−	−	+	−	+	(−)	±	+	+	±	−	U	+	−	−	−	+	+	+	−	+	+	−	+	−	−	+	(−)	−	−
Gamma-glutamyl transpeptidase	+	U	−	−	+	U	−	−	U	+	−	U	+	−	U	U	U	U	+	−	−	−	−	−	+	U	U	U	U	+	−	+	+	+	−	−
Growth at 42°C	−	(−)	+	+	+	±	+	+	+	+	+	±	−	−	±	(−)	−	−	+	+	+	+	+	+	+	−	−	±	+	+	(−)	+	+	−	+	+	+
1 % glycine	−	−	+	−	−	+	+	−	−	U	+	−	−	−	−	−	−	+	−	+	−	+	+	−	−	−	−	+	−	−	+	+	−	−	+	+
Resistance to NA (30 mg)	+	+	−	−	−	+	−	±	−	±	I	−	−	+	−	−	−	+	−	−	+	+	+	−	+	−	−	−	+	(+)	+	+	U	+	−	(±)
resistance to CE (30 mg)	+	−	+	+	+	+	+	+	(−)	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	−	+	+	−	(−)	+	+	U	+	+	+
Periplasmic fibers	−	−	−	−	+	+	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−	−
Distribution of flagella	M	M	St	Bp	Bp	Bp	St	Bp	Bp	Bp	M	Bp	Bp	M	Bp	Bp	Bp	Bp	Bp	Bp	M	Bp	Bp	Bp	Bp	Bp	Pt	Bp	M	M	Bp	Bp	Bp	Bp	Bp	M
Number of flagella	1	2—5	2	2	7—10	3–4	2	2	2	2	2	1–2	6–12	1	14–20	2	2	2	1–2	7–14	1	2	2	2	7–14	10–14	4–8	2	1	4–8	2	6–12	4–10	5–7	1–2	1
DNA G+C content (mol%)	33.3	30	30	40.2	35.5	34.8	39.1	33.7	48	35.6	35.2	37–38	31.6	37.7	44.5	35	36.7	35.9	39.9	41	37.5	40.6	39.6	34	35.33	34	42.5	38	34–35	35–37	37	34.6	40	33.2	38.8	31.8

1, H. cyclurae; 2, H. acinonychis; 3, H. anseris; 4, H. apri; 5, H. aurati; 6, H. bilis; 7, H. brantae; 8, H. canadensis; 9, H. canis; 10, H. cetorum; 11, H. cholecystus; 12, H. cinaedi; 13, H. didelphidarum; 14, H. equorum; 15, H. felis; 16, H. fennelliae; 17, H. ganmani; 18, H. hepaticus; 19, H. himalayensis; 20, H. jaachi; 21, H. japonicus; 22, H. macacae; 23, H. marmotae; 24, H. mesocricetorum; 25, H. monodelphidis; 26, H. muridarum; 27, H. mustelae; 28, H. pametensis; 29, H. pullorum; 30, H. pylori; 31, H. rodentium; 32, H. saguini; 33, H. suis; 34, H. trogontum; 35, H. typhlonius; 36, H. valdiviensis; + = all strains examined give a positive result. - = all strains examined give a negative result. (+) = 80–94% strains positive; ± = 33–66 % strains positive; (-) = 7–33 % strains positive; NA: nalidixic acid; CE: cephalothin; I, intermediate resistance; Bp, bipolar; M, monopolar; St, subterminal; Pt, peritrichous; U, unknown (On et al., 2017; Shen et al., 2020).

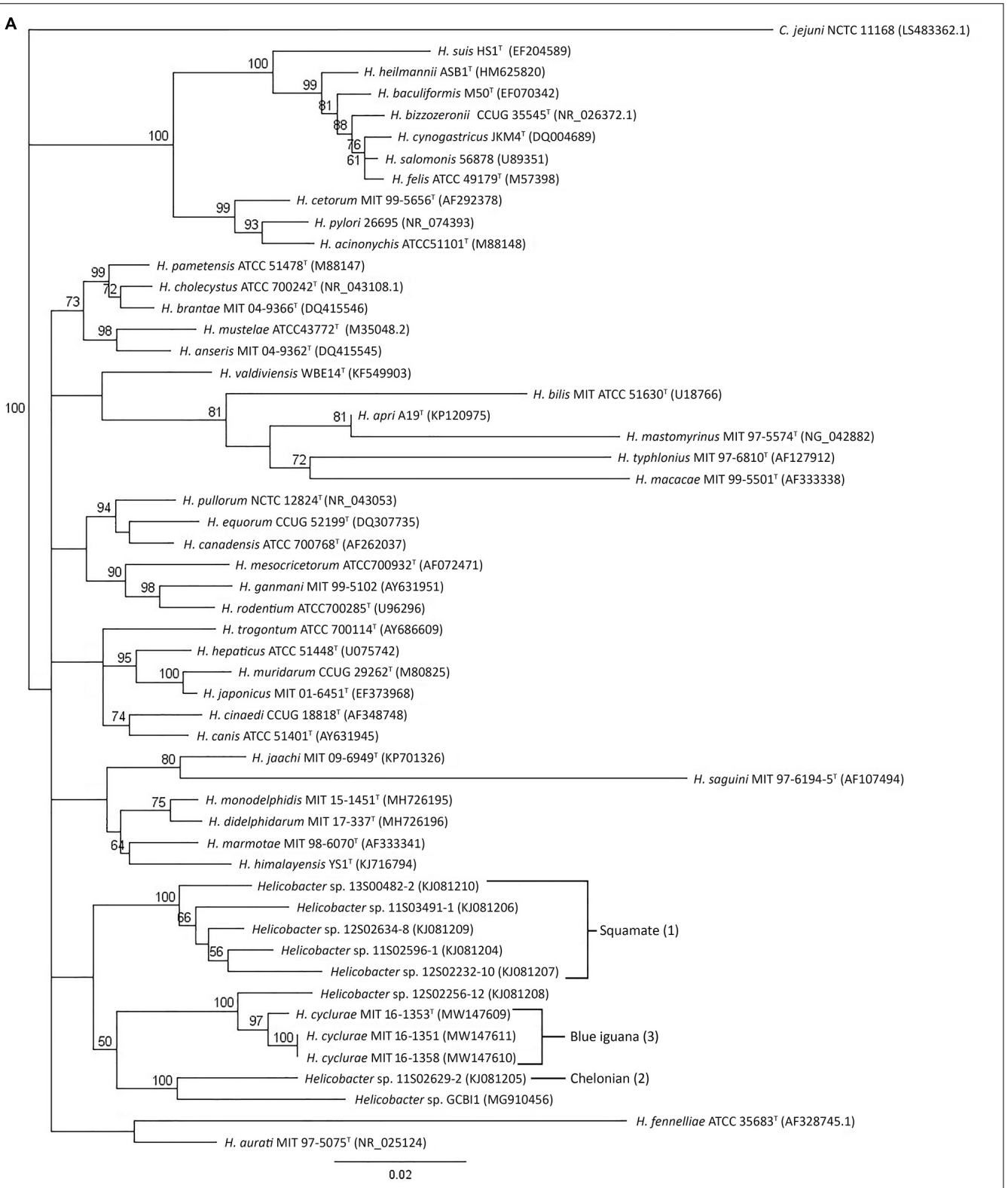

FIGURE 1 | Continued

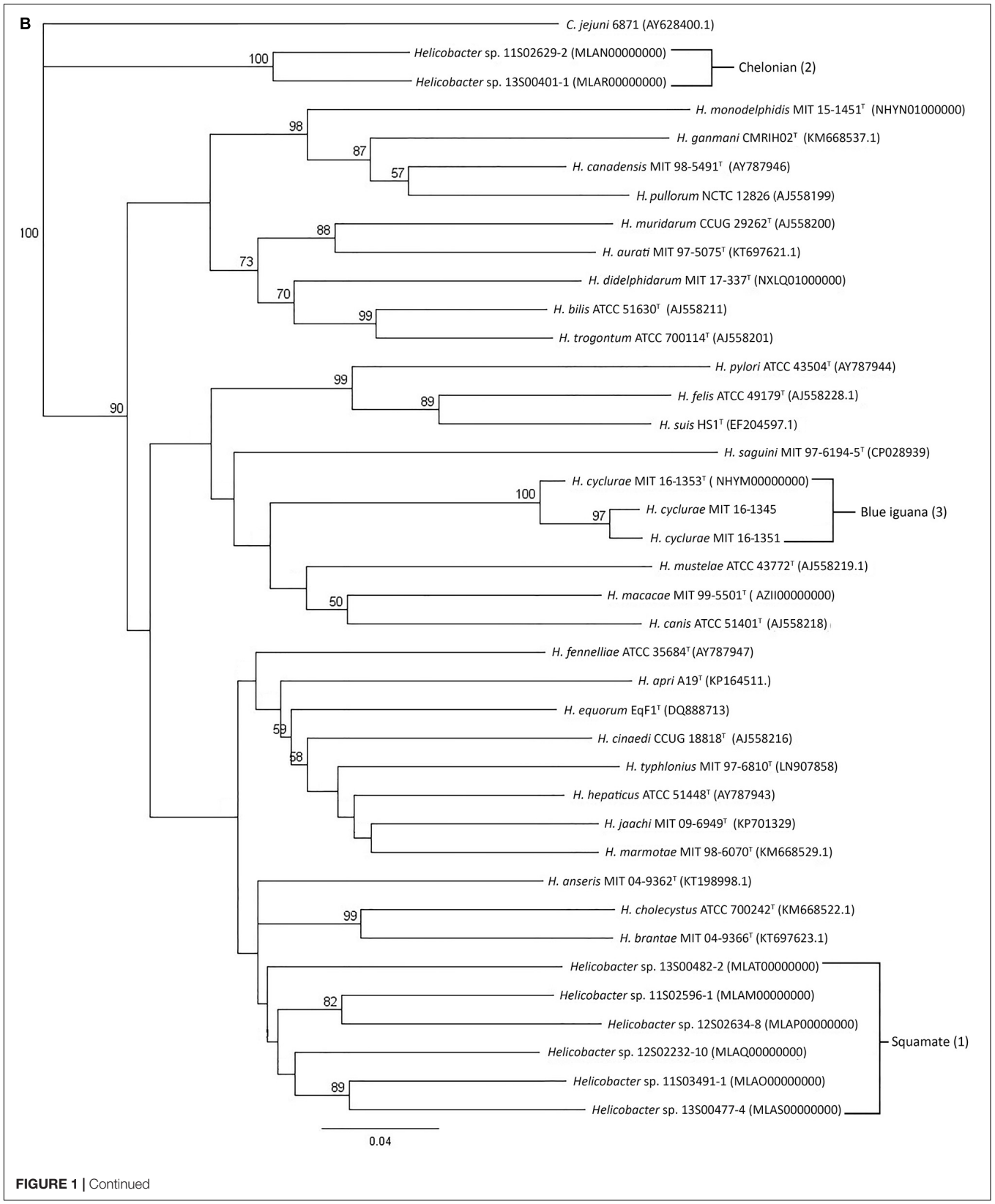

FIGURE 1 | Continued

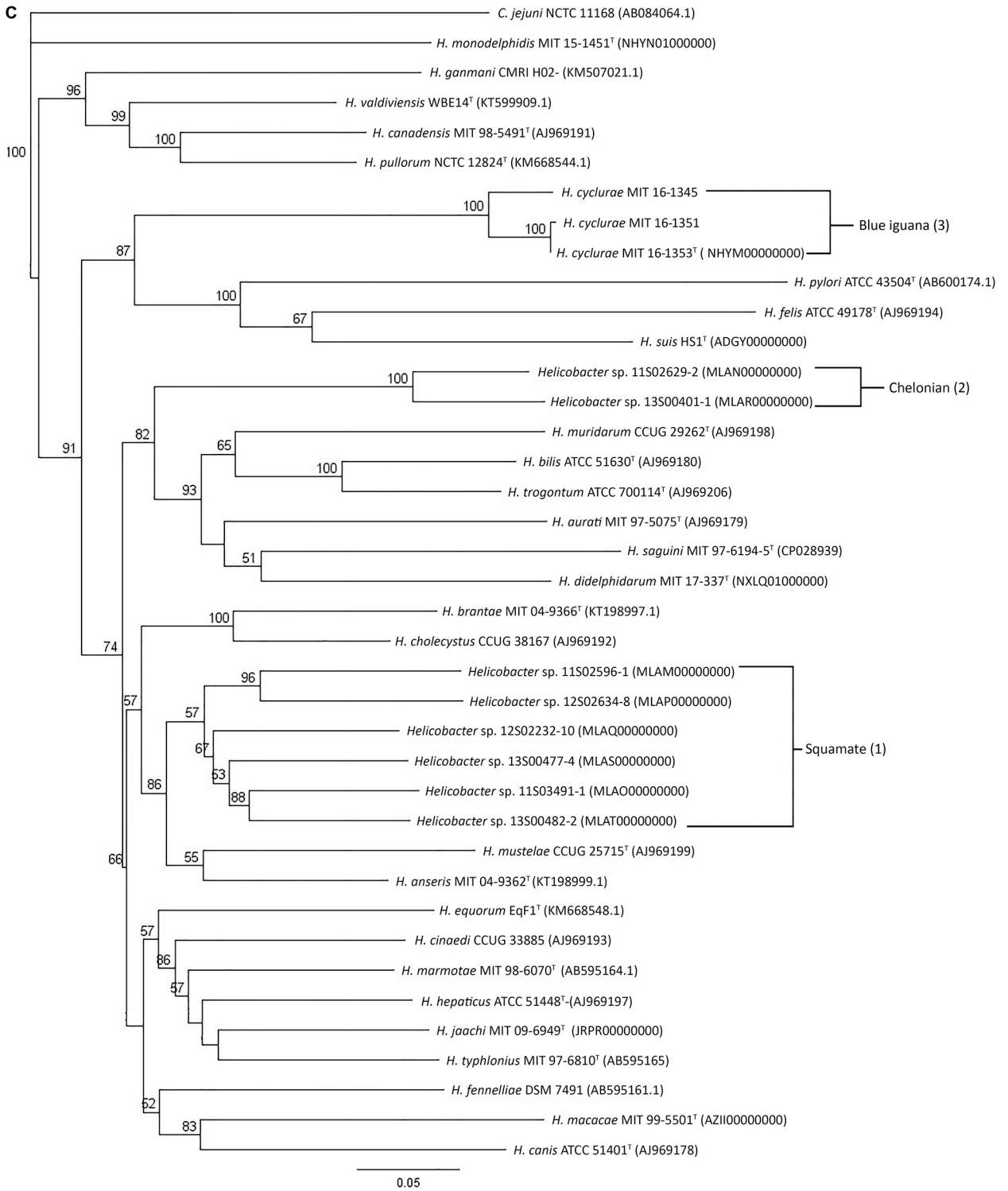

FIGURE 1 | Phylogenetic analysis of 16S rRNA, **(A)** *hsp*60, **(B)** and *gyr*B **(C)** gene sequences. Neighbor-joining trees were based on the comparison of genes from different *Helicobacter* species. *C. jejuni* is used as an outgroup. Bootstrap values (>50%) based on 1,000 replications are indicated. GenBank accession numbers (in parentheses) are provided for each strain. The scale bar indicates branch length as number of substitutions per nucleotide. (1) Reptiles in the order of Chelonian: turtles and tortoises. (2) Reptiles in the order of Squamata: snakes and lizards. (3) Blue iguana: belongs to the order of Squamata.

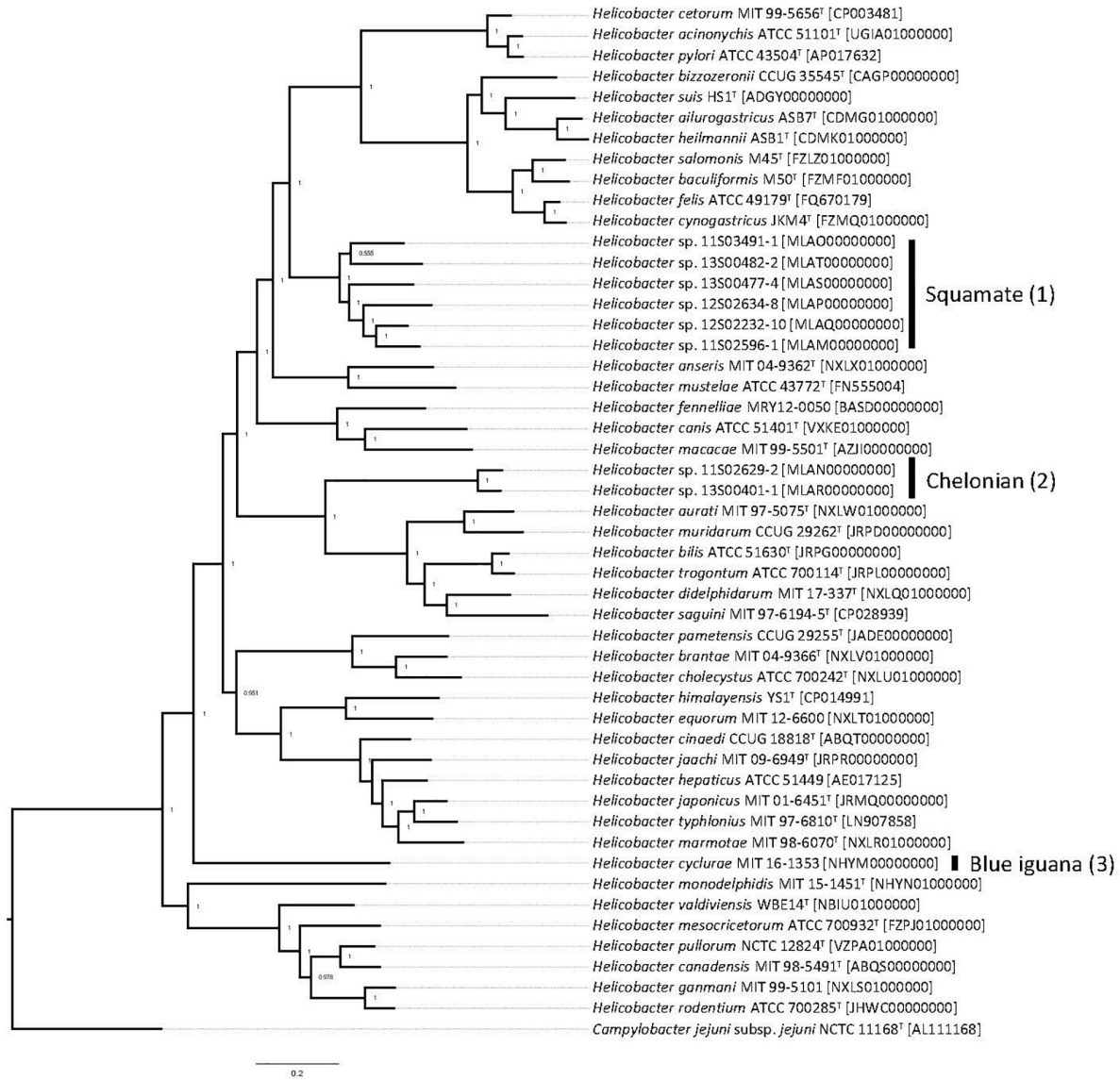

FIGURE 2 | Pan-genomic phylogenetic tree. Genomes for *Helicobacter* species were accessed from public databases. Type strains were used when available, and GenBank accession numbers for each genome are shown in brackets. The pan-genomic phylogenetic tree was created from the multi-sequence alignment of concatenated core gene sequences. Local support values, which indicate the reliability of each branch in the tree (0–1, lowest to highest support), were calculated by FastTree by resampling the site likelihoods 1,000 times using the Shimodaira–Hasegawa test. The scale bar indicates branch length as number of substitutions per amino acid. (1) Reptiles in the order of Chelonian: turtles and tortoises. (2) Reptiles in the order of Squamata: snakes and lizards. (3) Blue iguana: belongs to the order of Squamata.

concatenated core gene sequences were determined followed by MAFFT for multi-sequence alignment and FastTree to infer approximately maximum-likelihood phylogenetic trees. Orthogroups, determined using OrthoFinder, were further analyzed to identify shared and unique gene between isolate MIT 16-1353 from the blue iguana and the genomes of other helicobacters isolated from reptile hosts. Average nucleotide identities (ANI) were calculated with pyani (Pritchard et al., 2016). Genomes with an ANI >95% were considered the same species. Digital DNA–DNA hybridization (dDDH) percentages were calculated using the Genome-to-Genome Distance Calculator (GGDC) (Meier-Kolthoff et al., 2013). Genomes with a dDDH >70% were considered the same species. Virulence factor gene homologs were identified by BLASTP analysis of genome annotation against the virulence factor database (VFDB) (Liu et al., 2019). Homolog genes were considered to have sequence identity ≥50% and sequence coverage ≥ 95%.

Electron Microscopy

Isolate MIT 16-1353 was examined by electron microscopy. Cells grown on blood agar plate for 48 h were gently suspended in PBS at a concentration of about 10^8 cells/ml. The sample was negatively stained with 1% (w/v) phosphotungstic acid (pH 6.5)

TABLE 2 | Profile of the *H. cyclurae* draft genome compared with *H. pylori* and *H. hepaticus*.

	***H. pylori* 26695**	***H. cyclurae* MIT 16-1353**	***H. hepaticus* ATCC 51449**
Genome size (Mb)	1.67	1.91	1.8
GC content (%)	38.9	33.3	35.9
No. of genes	1,674	1,969	1,849
Selected virulence genes[a]	*cagA*, *vacA*, *ggt*, *nap*, T4SS, *htrA*, *ure*	*flpA*, *ggt*, *htrA*, *napA*, Peb4	*cdt*, T6SS, *ure*
GenBank	AE000511	NHYM00000000	AE017125

[a]*vacA, vacuolating cytotoxin A; cagA, cytotoxin-associated gene; ggt, gamma-glutamyl transpeptidase; nap, neutrophil-activating protein; T4(6)SS, type IV(VI) secretion system; htrA, high-temperature requirement-A protein-secreted serine protease; ure, urease; flpA, fibronectin-binding protein; cdt, cytolethal distending toxin; Peb4, major antigenic peptide PEB-cell binding factor.*

for 20–30 s. Specimens were examined with a JEOL model 2100F transmission electron microscope.

RESULTS

Helicobacter Isolation and Characterization

Helicobacter-like organisms were isolated from 4 of the 17 fecal samples and one of the eight cloacal swab samples. These helicobacter isolates were compared with other *Helicobacter* species to determine differences in biochemical characteristics (**Table 1**). They were gram negative and grew at 28°C and 37°C but not at 42°C or with 1% glycine. The bacterium appeared on 5% sheep blood agar plates as a single colony, 2–3 mm in diameter, mucoid, and clear or grayish in color. These isolates of the *Helicobacter* species had strong catalase, oxidase, and gamma-glutamyl transpeptidase activity and displayed resistance to nalidixic acid and cephalothin. There was no nitrate reduction or urease activity nor hydrolyzing activity of indoxyl acetate in these isolates; variation on alkaline phosphatase was noted among isolates.

PCR and Phylogenetic Analysis

Ten fecal samples and four cloacal swab samples from 25 animals were helicobacter PCR positive using genus 16S rRNA-specific primers. *Helicobacter* species isolated from four fecal samples and one cloacal swab sample (see above) had over 99% 16S rRNA sequences similarity with each other. Its 16S rRNA sequence clustered with other *Helicobacter* species that have been isolated from lizards. The most closely related 16S rRNA sequence was *Helicobacter* sp. 12S02256-12 (KJ081208) with 98% similarity (**Figure 1A**; Gilbert et al., 2014). Sequences from PCR products of nine fecal or cloacal swab samples which were culture-negative had similar sequences with the novel cultured *Helicobacter* sp. The 16S rRNA sequences of this novel *Helicobacter* species were different from the *Helicobacter* sp. GCBI1, which was identified by PCR in the blood and tissues of blue iguanas; these two *Helicobacters* only shared 96% sequence identity (**Figure 1A**; Conley et al., 2021).

The housekeeping gene sequences for heat shock protein 60 (*hsp*60) and DNA gyrase subunit B (*gyr*B) have been used for classification of phylogenetic relationships among *Helicobacter* species (Mikkonen et al., 2004; Hannula and Hanninen, 2007). Phylogenetic trees based on the partial *gyrB* and *hsp60* gene sequences of the novel *Helicobacter* species are presented in **Figures 1B,C**. The three *H. cyclurae* isolates shared 94–99% sequence identity within the species in their *gyr*B but only had 72% sequence identity with the *gyrB* gene sequence of closely related species, *Helicobacter anseris* (Fox et al., 2006). For *hsp60* gene analysis, the three *H. cyclurae* isolates shared 96–97% sequence identity and had 78% sequence identity with the *hsp60* gene of the most closely related species, *Helicobacter brantae* (Fox et al., 2006).

Whole-Genome Analysis

Whole-genome sequencing of the type strain, MIT 16-1353, indicated that *H. cyclurae* has a genome size of 1.91 Mb with a GC content of 33.3% (**Figure 2**) and contains 1,969 genes (**Table 2**). A whole-genome phylogenetic tree constructed from the multi-sequence alignment of concatenated core genes confirms the iguana helicobacter is distinct from other known *Helicobacter* species, including the species isolated from reptiles described by Gilbert et al. (2017). Instead, *H. cyclurae* was most closely placed with *Helicobacter monodelphidis* MIT 15–1451 isolated from captive opossums with cloacal prolapse (Shen et al., 2020). ANI and dDDH comparisons between the iguana isolate genome and other *Helicobacter* species were below 95 and 70%, respectively, supporting the finding that the iguana isolate is a novel *Helicobacter* species (**Table 3**). The type strain, MIT 16-1353, has been submitted to GenBank under accession number NHYM00000000.

In agreement with the whole-genome phylogenetic relationship, *H. cyclurae* shared more orthogroups (i.e., homologous genes) with the *Helicobacter* species isolated from chelonians (*Helicobacter* spp. 11S02629-2 and 13S00401-1) compared to squamates (*Helicobacter* spp. 13S00482-2, 11S03491-1, 13S00477-4, 12S02634-8, 12S02232-10, and 11S02596-1) (**Figure 3**). More than 25% of orthogroups for *H. cyclurae* were not found in these other reptile-associated helicobacter genomes. The unique genes harbored by *H. cyclurae* included metabolic and functional pathways for amino acid synthesis, transporters, and oxidative phosphorylation (**Figure 4**). However, the *H. cyclurae* genome was deficient for several genes related to lipid and lipopolysaccharide biosynthesis as well as other metabolic and functional pathways compared to the unique orthogroups belonging to the chelonian and squamate helicobacters (**Figure 4**). These included glutamyl-tRNA amidotransferase subunit A, putative methyltransferase *YcgJ*, and the putative tricarballylate catabolism locus *tcuRABC*, which

TABLE 3 | ANI and dDDH values for *Helicobacter cyclurae* MIT 16-1353 [NHYM00000000] vs. other *Helicobacter* species genomes.

Comparison Genome	ANI	dDDH
Helicobacter acinonychis ATCC 51101^T [UGIA01000000]	69.8%	45%
Helicobacter ailurogastricus ASB7^T [CDMG01000000]	69.5%	34.3%
Helicobacter anseris MIT 04-9362^T [NXLX01000000]	70.8%	30.6%
Helicobacter aurati MIT 97-5075^T [NXLW01000000]	70.2%	48.2%
Helicobacter baculiformis M50^T [FZMF01000000]	69.9%	45.1%
Helicobacter bilis ATCC 51630^T [JRPG00000000]	71.4%	29.6%
Helicobacter bizzozeronii CCUG 35545^T [CAGP00000000]	70.0%	44.8%
Helicobacter brantae MIT 04-9366^T [NXLV01000000]	70.7%	32.5%
Helicobacter canadensis MIT 98-5491^T [ABQS00000000]	70.6%	52.9%
Helicobacter canis ATCC 51401^T [VXKE01000000]	71.1%	33.3%
Helicobacter cetorum MIT 99-5656^T [CP003481]	70.6%	37.9%
Helicobacter cholecystus ATCC 700242^T [NXLU01000000]	70.0%	49.4%
Helicobacter cinaedi CCUG 18818^T [ABQT00000000]	71.0%	25.3%
Helicobacter cynogastricus JKM4^T [FZMQ01000000]	71.3%	43.7%
Helicobacter didelphidarum MIT 17-337^T [NXLQ01000000]	70.5%	30.4%
Helicobacter equorum MIT 12-6600 [NXLT01000000]	70.6%	31.2%
Helicobacter felis ATCC 49179^T [FQ670179]	69.3%	44%
Helicobacter fennelliae MRY12-0050 [BASD00000000]	71.1%	41.7%
Helicobacter ganmani MIT 99-5101 [NXLS01000000]	71.0%	33.7%
Helicobacter heilmannii ASB1^T [CDMK01000000]	69.3%	44%
Helicobacter hepaticus ATCC 51449 [AE017125]	71.0%	20.5%
Helicobacter himalayensis YS1^T [CP014991]	71.2%	29.9%
Helicobacter jaachi MIT 09-6949^T [JRPR00000000]	70.7%	23.4%
Helicobacter japonicus MIT 01-6451^T [JRMQ00000000]	71.4%	33.5%
Helicobacter macacae MIT 99-5501^T [AZJI00000000]	71.8%	32.7%
Helicobacter marmotae MIT 98-6070^T [NXLR01000000]	70.8%	32.1%
Helicobacter mesocricetorum ATCC 700932^T [FZPJ01000000]	70.7%	41.7%
Helicobacter monodelphidis MIT 15-1451^T [NHYN01000000]	70.4%	33.8%
Helicobacter muridarum CCUG 29262^T [JRPD00000000]	71.0%	38.7%
Helicobacter mustelae ATCC 43772^T [FN555004]	70.6%	53.4%
Helicobacter pametensis CCUG 29255^T [JADE00000000]	71.4%	35.9%
Helicobacter pullorum NCTC 12824^T [VZPA01000000]	70.9%	21.4%
Helicobacter pylori ATCC 43504^T [AP017632]	70.2%	46%
Helicobacter rodentium ATCC 700285^T [JHWC00000000]	70.7%	26.4%
Helicobacter saguini MIT 97-6194-5^T [CP028939]	71.0%	25.9%
Helicobacter salomonis M45^T [FZLZ01000000]	69.8%	44.7%
Helicobacter sp. 11S02596-1 [MLAM00000000]	72.4%	32.7%
Helicobacter sp. 11S02629-2 [MLAN00000000]	72.3%	57.1%
Helicobacter sp. 11S03491-1 [MLAO00000000]	72.0%	35.6%
Helicobacter sp. 12S02232-10 [MLAQ00000000]	72.2%	43.1%
Helicobacter sp. 12S02634-8 [MLAP00000000]	72.3%	37.4%
Helicobacter sp. 13S00401-1 [MLAR00000000]	72.5%	47.7%
Helicobacter sp. 13S00477-4 [MLAS00000000]	72.1%	32.3%
Helicobacter sp. 13S00482-2 [MLAT00000000]	72.4%	37%
Helicobacter suis HS1^T [ADGY00000000]	70.1%	42.2%
Helicobacter trogontum ATCC 700114^T [JRPL00000000]	71.1%	43.7%
Helicobacter typhlonius MIT 97-6810^T [LN907858]	71.4%	36.6%
Helicobacter valdiviensis WBE14^T [NBIU01000000]	70.5%	33.7%
Campylobacter jejuni ssp. *jejuni* NCTC 11168^T [AL111168]	69.9%	24.1%

are present in the other reptile genomes described by Gilbert et al. (2017).

Virulence factor profiles were distinct between *H. cyclurae* and other reptile-associated helicobacters. All helicobacter genomes, including *H. cyclurae*, encoded high-temperature requirement-A protein-secreted serine protease (*htrA*), gamma-glutamyl transpeptidase (*ggt*), and neutrophil activating protein (*napA*). Homologous sequences to the major antigenic peptide PEB-cell

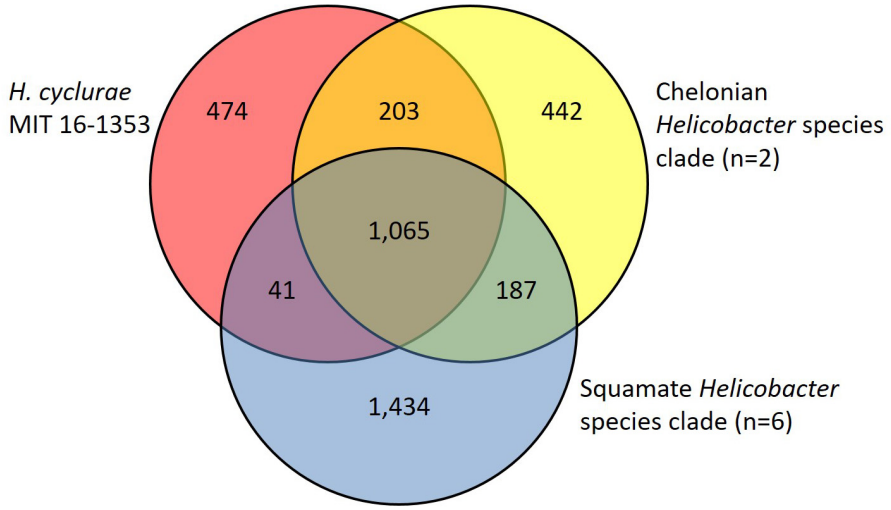

FIGURE 3 | Venn diagram showing shared and unique orthogroups among genomes from *Helicobacter* species isolated from reptilian hosts.

binding factor (Peb4) from *Campylobacter jejuni* ssp. *jejuni* NCTC 11168 were present in all reptile-associated helicobacters except strain 13S00401-1 isolated from a chelonian host. Unlike most other reptile-associated helicobacter genomes, *H. cyclurae* did not harbor a homologous sequence to *dupA*, *hopZ*, or campylobacter invasion antigen B (*ciaB*) gene. Similar to *Helicobacter* sp. 11S02629-2 (chelonian), *H. cyclurae* has a homologous sequence to the *C. jejuni* virulence factor fibronectin-binding protein gene (*flpA*). Interestingly, both chelonian-associated helicobacters contain the *cdtABC* operon for cytolethal distending toxin, while *H. cyclurae* or the helicobacters from squamate hosts did not. *H. cyclurae* and both chelonian isolates did not have genes for the alpha and beta urease subunits, which were present in the squamates-associated helicobacter genomes. Together, these results support that *H. cyclurae* has unique genetic, metabolic, and virulence profiles compared to helicobacter isolated from other reptile hosts.

Electron Microscopy

In order to visualize and describe the morphology of *H. cyclurae*, electron microscopy was performed. *H. cyclurae* MIT 16-1353 is 2–3 μm in length and 0.3–0.5 μm in width and are curved with a smooth surface and a single sheathed polar flagellum (**Figure 5**).

DISCUSSION

In this study, we isolated the first novel *Helicobacter* species, *H. cyclurae*, from fecal samples and cloacal swabs of blue iguanas inhabiting Grand Cayman. The novel helicobacter was characterized by biochemical and whole-genome sequencing; 16S rRNA, *gyrB*, and *hsp60* gene sequencing; and ultrastructural characterization, following the guidelines described by On et al. (2017) pertaining to the minimal standards for describing new *Helicobacter* spp. (Shen et al., 2020). According to these guidelines, these phenotypic and genotypic characterizations are necessary to effectively and unambiguously distinguish a new taxon from extant species and subspecies and determine phylogeny in the genus. This was particularly important for supporting that *H. cyclurae* is a novel species, considering helicobacters have been previously isolated from reptilian hosts (Gilbert et al., 2014). A single phenotypic and/or genotypic method is insufficient to make accurate taxonomic determinations, and as demonstrated in our recent publication describing two novel *Helicobacter* species isolated from opossums, the aggregate of phenotypic and genotypic data is appropriate instead (Shen et al., 2020).

Biochemically and phenotypically, *H. cyclurae* was compared to other *Helicobacter* species listed in **Table 1**. All of the isolates were oxidase and catalase positive, had gamma-glutamyl transpeptidase activity, did not have urease activity nor hydrolyze indoxyl acetate, did not reduce nitrate to nitrite, and did not grow in 1% glycine or at 42°C. Phylogenetic analysis of the 16S RNA gene indicated this novel helicobacter is most closely related to a helicobacter sequenced from a *Lacertilia*, *Helicobacter* sp. 12S02256-12 (Gilbert et al., 2014). *H. cyclurae* is different from *Helicobacter* sp. GCBI1 identified by PCR in a study of septic blue iguanas, which was most closely related to *Helicobacter* sp. 11S02629-2 isolated from tortoises (Gilbert et al., 2014).

Interestingly, we observed that the *Helicobacter* species from chelonians, squamates, and blue iguanas each formed distinct clusters in our phylogenetic analyses; however, the phylogenetic topology differed depending on whether 16S rRNA, *hsp60*, and *gyrB* genes were used. This is an example of how a single gene is insufficient to ensure accurate species classification and phylogenetic placement, which has been previously appreciated for causing discordant phylogenies for *Helicobacter* species (Dewhirst et al., 2005). Therefore, to complement our single-gene phylogenetic analyses, we also performed whole-genome sequence analysis, which is the most robust determination of phylogenetic and taxonomic

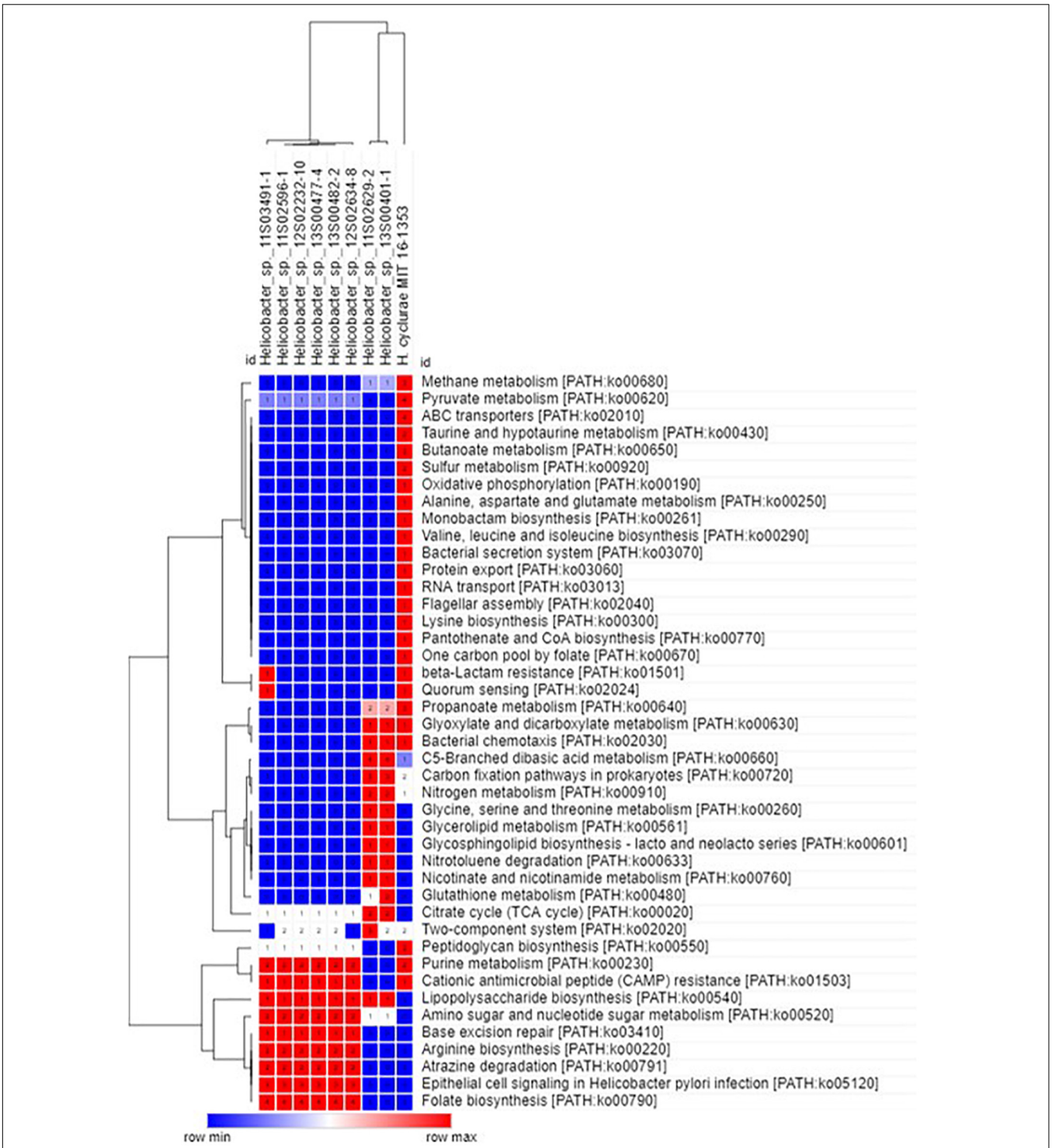

FIGURE 4 | Hierarchal clustering heatmap of unique orthogroups and associated KEGG metabolic/functional pathways from *Helicobacter* species isolated from reptilian hosts.

classification for prokaryotes. Whole-genome phylogenetic analysis of core genes indicated that *H. cyclurae* was most similar to *H. monodelphidis* MIT 15-1451 isolated from captive opossums with cloacal prolapse (Shen et al., 2020). Together, these analyses show how the phylogenetic placement of *Helicobacter* species does not appear to be dependent on the host and that *H. cyclurae* is phylogenetically divergent from other helicobacters isolated from reptiles.

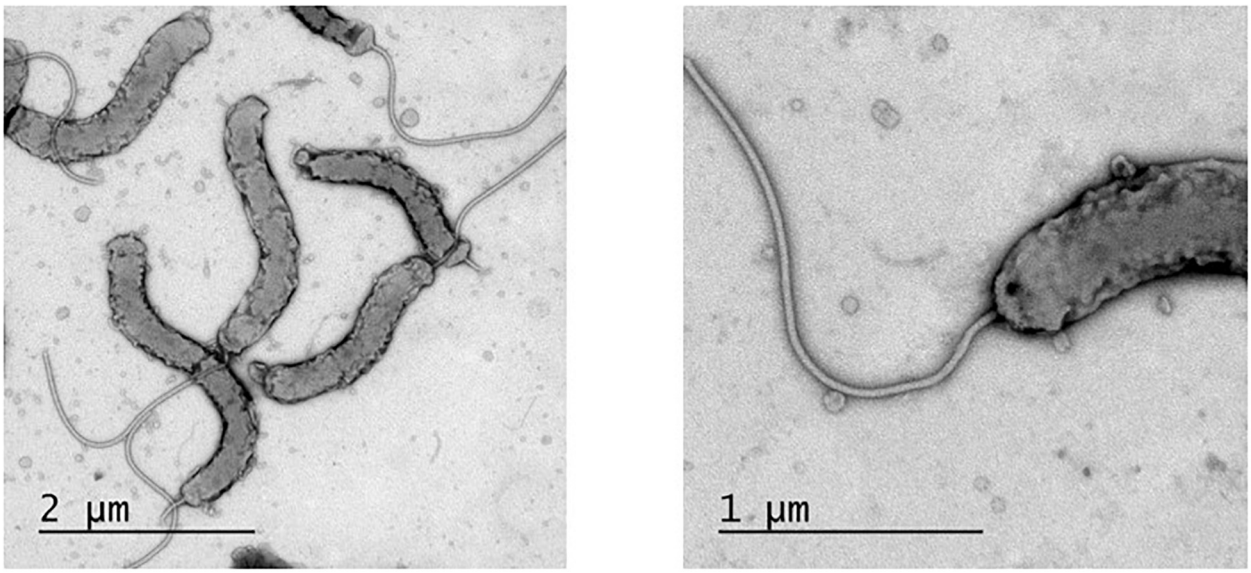

FIGURE 5 | Transmission electron micrograph of *Helicobacter cyclurae* MIT 16-1353. Measured 2–3 µm in length and 0.3–0.5 µm in width and with a single polar sheathed flagellum.

Conventional DNA–DNA hybridization (DDH) has been used to differentiate known vs. novel bacterial species, including *Helicobacter* species, based on a threshold of 70% similarity (i.e., species that are $\geq$ 70% similar are the same species). ANI and dDDH are *in silico* nucleotide-level similarity indexes analogous to conventional DDH that use whole-genome sequence data instead. ANI and dDDH similarity of ∼95 and ∼70%, respectively, are the equivalent thresholds to conventional DDH. Therefore, ANI and dDDH analyses of *H. cyclurae* against *Helicobacter* species, including those from reptiles, were below these ∼95 and ∼70% similarity thresholds, supporting our other phenotypic and genotypic characterizations that *H. cyclurae* is a novel species.

Similar to *H. pylori*, this novel helicobacter does have the virulence factors high-temperature requirement-A protein-secreted serine protease (*htrA*) and gamma-glutamyl transpeptidase (*ggt*). Additionally, it has a virulence factor fibronectin/fibrinogen binding protein (*fbps*) that is not present in *H. pylori* but identified in *Helicobacter bilis*; this virulence property is capable of providing invasive properties in host cells. Unlike *Helicobacter hepaticus*, the prototypical EHS *H. cyclurae* lacks urease and cytolethal distending toxin genes. While morphology alone cannot be used to differentiate helicobacters, electron microscopy allows description of the cell morphology and size, the number and arrangement of flagella, and the presence or absence of flagellar sheaths and periplasmic fibers, all of which can vary substantially between different *Helicobacter* species. Transmission electron microscopy illustrates that *H. cyclurae* has a single polar sheathed flagellum similar to *Helicobacter cholecystus* (Owen, 1998; Whary and Fox, 2004).

The 56% prevalence of *H. cyclurae* in the blue iguanas surveyed in our study is higher than what has been previously reported in lizards (Gilbert et al., 2014). Also, our culture and PCR results indicated that *H. cyclurae* was more commonly identified in the feces and cloacal samples from clinical normal animals in our study compared with *Helicobacter* sp. GCBI1 identified in clinically ill iguanas in blood and tissue samples (Conley et al., 2021). In our previous study, clinically ill blue iguanas who developed lethargy, weakness, inappetence, and septicemia had spiral-shaped bacteria identified on peripheral blood smears (Conley et al., 2021). Through 16S rRNA gene-specific primers, *Helicobacter* spp. were detected in 11/19 blood or tissue samples of iguanas tested. Whereas PCR utilizing *Helicobacter* sp. GCBI1-specific primers identified *Helicobacter* sp. GCBI1 in 7/19 of these animals, no helicobacters were cultured from the blood and tissue samples of these animals (Conley et al., 2021). One of the animals which had *Helicobacter* sp. GCBI1 identified in its blood also had *H. cyclurae* isolated from its feces, indicating that more than one *Helicobacter* species may be present in the same iguana. It is hypothesized that *Helicobacter* GCBI1 might have originated from an invasive species, the green iguana (*Iguana iguana*), that is competing for habitat and resources with blue iguanas (Conley et al., 2021). Ongoing screening of green iguanas for these two novel *Helicobacter* species will be important in elucidating *Helicobacter* species colonization dynamics in iguanas inhabiting Grand Cayman (Popescu, 2018).

CONCLUSION

In conclusion, using the criteria for characterizing *Helicobacter* species (On et al., 2017; Shen et al., 2020), we have described the isolation of a novel helicobacter, *H. cyclurae*, from blue iguanas, an endangered species from Grand Cayman. To support the recovery effort of the blue iguana population, it is

important to identify and understand the mechanisms whereby newly recognized microorganisms contribute to diseases. Further studies are needed to identify if *H. cyclurae* can be pathogenic under certain circumstances in blue iguanas.

Description of *Helicobacter cyclurae* sp. Nov.

H. cyclurae sp. nov. (cy.clu'rae. N.L. gen. n. cyclurae of the blue iguana *Cyclura*). The organism is motile; cells are slightly curved, 2–3 μm long and 0.3–0.5 μm wide, with a monopolar sheathed flagellum. The bacteria are gram negative and non-sporulating. The organism under microaerobic conditions grows slowly at 28 and 37°C, but not at 42°C. It appears on solid agar as single colonies. The bacterium is oxidase, catalase, and gamma-glutamyl transpeptidase positive and has variable alkaline phosphatase activity, but urease, indoxyl acetate hydrolysis, and nitrate reduction are negative. It did not grow on 1% glycine and is resistant to nalidixic acid and cephalothin. The type strain MIT 16-1353^T isolated from the feces of a blue iguana in Grand Cayman has been deposited in the Belgian Coordinated Collections of Microorganisms (BCCM) as LMG 31270 and in The National Collection of Type Cultures as NCTC 14190. It has a DNA G+C content of 33.27 mol%, and its genome size is ∼1.91 Mb. The 16S rRNA and the whole-genome sequence of the type strain have been deposited in GenBank under accession numbers MW147609 and NHYM00000000.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, NHYM00000000; https://www.ncbi.nlm.nih.gov/genbank/, MW147609.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because IACUC approval for this project was not required as WCS institutional requirements for IACUC review do not include field projects that take place outside of our facilities. However, sampling, capture, and release of wild reptiles, collecting dead animals found in the environment, or sampling after euthanasia are all standard techniques and procedures for clinical and pathology examinations or investigations. The welfare of animals included in this study was considered throughout their care, with analgesics used as deemed necessary. Animals that survived infection were released back into the environment where they were found, either in the captive setting or free-ranging within the QEIIBP. No animals were housed for continued research purposes. Within the Cayman Islands regulatory framework, the project operates under the terms of a protected species permit issued to the BIRP by the National Conservation Council. Blood, tissue, and fecal samples were collected by or under the direction of licensed veterinarians. Cayman Islands CITES export permits 2014/KY/000674, 2014/KY/000687, 2014/KY/000686, 2014/KY/000690, 2014/KY/000689, 2015/KY/000777, 2015/KY/000808, 2015/KY/000809, 2016/KY/000817, 2017/KY/000874, and 2017/KY/000923; and United States CITES import permits 15US033594/9, 16US0333594/9, and 17US033594/9 were used for sample export and import.

AUTHOR CONTRIBUTIONS

JF and PC designed and supervised the study. NC, ZS, and SK processed samples for helicobacter isolation and characterization. AM performed and analyzed the whole-genome sequence. IP and FB collected and organized the iguana samples. NC, ZS, AM, and JF analyzed and interpreted the data, and drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was supported by the NIH grants T32-OD010978, P30-ES002109, and R01-OD011141 (to JF), the Wildlife Conservation Society's Zoological Health Program, the Cayman Islands Government, the National Trust for the Cayman Islands, and the Derald H Ruttenberg Memorial Fund (to PC). The funders played no role in the study or in the preparation of article or decision to publish.

ACKNOWLEDGMENTS

We thank Aharon Oren of The Hebrew University of Jerusalem for providing nomenclature expertise in naming of these novel *Helicobacter* species, and we thank Parisa Zarringhalam for assistance with manuscript preparation. We are grateful for the assistance of Veterinary Technicians from the Wildlife Conservation Society and the staff and volunteers of the Blue Iguana Recovery Program and Cayman Islands Department of the Environment and the St. Matthew's University School of Veterinary Medicine for laboratory support.

REFERENCES

Conley, K. J., Seimon, T. A., Popescu, I. S., Wellehan, J. F. X. Jr., Fox, J. G., Shen, Z., et al. (2021). Systemic *Helicobacter* infection and associated mortalities in endangered Grand Cayman blue iguanas (*Cyclura lewisi*) and introduced green *Iguanas* (*Iguana iguana*). *PLoS One* 16:e0247010. doi: 10.1371/journal.pone.0247010

Dewhirst, F. E., Shen, Z., Scimeca, M. S., Stokes, L. N., Boumenna, T., Chen, T., et al. (2005). Discordant 16S and 23S rRNA gene phylogenies for the genus *Helicobacter*: implications for phylogenetic inference and systematics. *J. Bacteriol.* 187, 6106–6118. doi: 10.1128/JB.187.17.6106-6118.2005

Emms, D. M., and Kelly, S. (2019). OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biol.* 20:238. doi: 10.1186/s13059-019-1832-y

Fox, J. G. (2002). The non-*H pylori helicobacters*: their expanding role in gastrointestinal and systemic diseases. *Gut* 50, 273–283. doi: 10.1136/gut.50.2.273

Fox, J. G., Dewhirst, F. E., Shen, Z., Feng, Y., Taylor, N. S., Paster, B. J., et al. (1998). Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* 114, 755–763. doi: 10.1016/s0016-5085(98)70589-x

Fox, J. G., Taylor, N. S., Howe, S., Tidd, M., Xu, S., Paster, B. J., et al. (2006). *Helicobacter anseris* sp. nov. and *Helicobacter brantae* sp. nov., isolated from feces of resident Canada geese in the greater Boston area. *Appl. Environ. Microbiol.* 72, 4633–4637. doi: 10.1128/AEM.02876-05

Gilbert, M. J., Duim, B., Timmerman, A. J., Zomer, A. L., and Wagenaar, J. A. (2017). Whole genome-based phylogeny of reptile-associated *Helicobacter* indicates independent niche adaptation followed by diversification in a poikilothermic host. *Sci. Rep.* 7:8387. doi: 10.1038/s41598-017-09091-7

Gilbert, M. J., Kik, M., Timmerman, A. J., Severs, T. T., Kusters, J. G., Duim, B., et al. (2014). Occurrence, diversity, and host association of intestinal *Campylobacter*, *Arcobacter*, and *Helicobacter* in reptiles. *PLoS One* 9:e101599. doi: 10.1371/journal.pone.0101599

Goodman, R., and Burton, F. (2005). Natural history notes:*Cyclura lewisi* (Grand Cayman Blue Iguana): hatchlings. *Herpetol. Rev.* 36:176.

Hannula, M., and Hanninen, M. L. (2007). Phylogenetic analysis of *Helicobacter* species based on partial gyrB gene sequences. *Int. J. Syst. Evol. Microbiol.* 57(Pt 3), 444–449. doi: 10.1099/ijs.0.64462-0

Inglis, G. D., McConville, M., and de Jong, A. (2006). Atypical *Helicobacter canadensis* strains associated with swine. *Appl. Environ. Microbiol.* 72, 4464–4471. doi: 10.1128/AEM.02843-05

Liu, B., Zheng, D., Jin, Q., Chen, L., and Yang, J. (2019). VFDB 2019: a comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Res.* 47, D687–D692. doi: 10.1093/nar/gky1080

Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P., and Goker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinformatics* 14:60. doi: 10.1186/1471-2105-14-60

Mikkonen, T. P., Karenlampi, R. I., and Hanninen, M. L. (2004). Phylogenetic analysis of gastric and enterohepatic *Helicobacter* species based on partial HSP60 gene sequences. *Int. J. Syst. Evol. Microbiol.* 54(Pt 3), 753–758. doi: 10.1099/ijs.0.02839-0

On, S. L. W., Miller, W. G., Houf, K., Fox, J. G., and Vandamme, P. (2017). Minimal standards for describing new species belonging to the families Campylobacteraceae and Helicobacteraceae: *Campylobacter*, *Arcobacter*, *Helicobacter* and *Wolinella* spp. *Int. J. Syst. Evol. Microbiol.* 67, 5296–5311. doi: 10.1099/ijsem.0.002255

Owen, R. J. (1998). *Helicobacter*–species classification and identification. *Br. Med. Bull.* 54, 17–30. doi: 10.1093/oxfordjournals.bmb.a011667

Popescu, I. (2018). *Investigations into Green Iguana (Iguana iguana) as a Potential Carrier for a Novel Strain of Helicobacter spp, Pathogenic for the Endangered Blue Iguana (Cyclura lewisi)*. Edinburgh: The University of Edinburgh.

Pritchard, L., Glover, R. H., Humphris, S., Elphinstoneb, J. G., and Toth, I. K. (2016). Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Anal. Methods* 8, 12–24. doi: 10.1039/c5ay02550h

Shen, Z., Batac, F., Mannion, A., Miller, M. A., Bakthavatchalu, V., Ho, C., et al. (2017). Novel urease-negative *Helicobacter* sp. '*H. enhydrae* sp. nov.' isolated from inflamed gastric tissue of southern sea otters. *Dis. Aquat. Organ.* 123, 1–11. doi: 10.3354/dao03082

Shen, Z., Mannion, A., Lin, M., Esmail, M., Bakthavatchalu, V., Yang, S., et al. (2020). *Helicobacter monodelphidis* sp. nov. and *Helicobacter didelphidarum* sp. nov., isolated from a colony of gray short-tailed opossums (*Monodelphis domestica*) with endemic cloacal prolapses. *Int. J. Syst. Evol. Microbiol.* 70, 6032–6043. doi: 10.1099/ijsem.0.004424

Shen, Z., Xu, S., Dewhirst, F. E., Paster, B. J., Pena, J. A., Modlin, I. M., et al. (2005). A novel enterohepatic *Helicobacter* species '*Helicobacter mastomyrinus*' isolated from the liver and intestine of rodents. *Helicobacter* 10, 59–70. doi: 10.1111/j.1523-5378.2005.00292.x

Stacy, B. A., and Wellehan, J. F. Jr. (2010). Fatal septicemia caused by *Helicobacter* infection in a pancake tortoise (*Malacochersus tornieri*). *J. Vet. Diagn. Invest.* 22, 660–662. doi: 10.1177/104063871002200431

Wattam, A. R., Davis, J. J., Assaf, R., Boisvert, S., Brettin, T., Bun, C., et al. (2017). Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. *Nucleic Acids Res.* 45, D535–D542. doi: 10.1093/nar/gkw1017

Whary, M. T., and Fox, J. G. (2004). Natural and experimental *Helicobacter* infections. *Comp. Med.* 54, 128–158.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.