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

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Blue iguanas (Cyclura lewisi) are endangered reptiles found only on Grand Cayman. Previously, DNA for a novel Helicobacter species GCB11 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, gyrB, and hsp60 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

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, enterohpatic 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. GCB1. 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. GCB1 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. GCB1 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 blue iguanas. Retrospective analysis from a mortality event of green iguanas a year prior and screening of healthy free-ranging blue iguanas. Recent study, two free-ranging blue iguanas located at the Grand Cayman Queen Elizabeth II Botanic Park (QEIIBP) in 2015.

**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 N₂, CO₂, and H₂ (80:10:10). Detailed biochemical characterization analysis was performed on five individual isolates using a RapID™ 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, gyrB, 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 GAG GTG 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 CTT ATT GTA AAA AGA GGN ATG GAY AA-3′) and HSP60DR (5′-CAG TAA GGT AAG GTA ACG TCC CCC TTC DAT RTC YT-3′) were used to amplify the hsp60 gene (Inglis et al., 2006); primers specific for Helicobacter species iguana isolates gyrB-igu-F (5′-GAT ACT TAT AAA GGT GGC TGG-3′) and gyrB-igu-R (5′-CAA ATT CCT TAT CAA TTC CGC A-3′) were used to amplify the gyrB 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 NexteraXT 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 | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Urease         | - | + | + | + | + | - | - | + | - | + | + | - | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Alkaline       | ± | + | + | - | U | - | - | + | + | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Gamma-glutamyl transpeptidase | + | U | - | - | + | U | - | - | U | + | - | U | U | U | U | + | - | - | - | - | - | - | U | U | U | U | + | - | + | + | - | - | - | - | - | - |
| Growth at 42°C | - | - | + | + | + | ± | + | + | + | + | + | ± | - | - | - | - | ± | - | - | + | + | + | - | ± | + | - | ± | + | - | + | + | + | - | - | - | - |
| 1 % glycine    | - | - | - | + | - | + | U | + | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Resistance to NA (30 mg) | + | + | + | - | U | - | - | + | ± | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| resistance to CE (30 mg) | + | + | + | ± | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Periplasmic fibers | - | - | + | + | + | + | + | - | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Distribution of flagella | M | M | St | Bp | Bp | Bp | Bp | Bp | M | Bp | Bp | Bp | Bp | Bp | Bp | Bp | Bp | M | Bp | Bp | Bp | Bp | Bp | Bp | Bp | Bp | Bp | M | M | Bp | Bp | Bp | Bp | Bp | M | M | Bp | Bp | Bp | Bp | M | M | Bp | Bp | Bp | Bp | M |
| Number of flagella | 1 | 2 | - | 5 | 2 | 2 | 7 | -10 | 3 | 4 | 2 | 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 | 35.4 | 38.9 | 31.7 | 38 | 34.6 | 35.2 | 35.3 | 37-38 | 31.6 | 37.7 | 44.5 | 35 | 36.7 | 35.9 | 39.9 | 41 | 37.5 | 40.6 | 39.6 | 34 | 35.3 | 34 | 42.5 | 38 | 34 | 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. cetae*; 11. *H. cholerae*; 12. *H. cinetodes*; 13. *H. delphidis*; 14. *H. equorum*; 15. *H. felis*; 16. *H. fennelliae*; 17. *H. ganimii*; 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. saganii*; 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**

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A

H. suis HS1^1 (EF204589)
H. helminth AS81^1 (HM625820)
H. bacilliformis M50^1 (EF070342)
H. bizzozeronii CCUG 35545^1 (NR_026372.1)
H. cynogastricus KKM4^1 (DQ004689)
H. solomonii 56878 (U93951)
H. felis ATCC 49179^1 (M57398)
H. cetorum MIT 99-5566^2 (AF292378)
H. pylori 26695 (NR_074393)
H. acinonyxis ATCC51101^1 (M88148)
H. pemetris ATCC 51478^2 (M88147)
H. cholecytus ATCC 700242^2 (NR_043108.1)
H. brantae MIT 04-9366^2 (DQ415546)
H. mustelae ATCC43772^2 (M35048.2)
H. anseris MIT 04-9362^2 (DQ415545)
H. valdiviensis WEB14^1 (KF549903)
H. bilis MIT ATCC 51630^2 (U18766)
H. aprv A19^2 (KP120975)
H. mastomyrinus MIT 97-5574^2 (NG_042882)
H. typhlonius MIT 97-6810^2 (AF127912)
H. macacae MIT 99-5501^2 (AF333338)
H. pullorum NCTC 1282^2 (NR_043053)
H. equorum CCUG 52199^2 (DQ3007735)
H. canadensis ATCC 700768^2 (AF262037)
H. mesocricetorum ATCC700932^2 (AF072471)
H. gannomi MIT 99-5102 (AY631951)
H. rodentium ATCC700285^3 (U96296)
H. trogontum ATCC 700114^2 (AY686609)
H. hepaticus ATCC 51488^2 (U075742)
H. muridarum CCUG 29262^2 (M80825)
H. japonicus MIT 01-6451^2 (EF373968)
H. cinaedi CCUG 18818^2 (AF348748)
H. canis ATCC 51401^2 (AY631945)
H. jaebei MIT 09-6949^2 (KP701326)
H. monodelphis MIT 15-1451^2 (MH726195)
H. didelphidum MIT 17-337^2 (MH726196)
H. marmotae MIT 98-6070^2 (AF333441)
H. himalayensis Y51^2 (KJ167974)
Helicobacter sp. 13500482-2 (KJ081210)
Helicobacter sp. 11503491-1 (KJ081206)
Helicobacter sp. 12502634-8 (KJ081209)
Helicobacter sp. 11502596-1 (KJ081204)
Helicobacter sp. 12502232-10 (KJ081207)
Helicobacter sp. 12502265-12 (KJ081208)
H. cyclurae MIT 16-1353^2 (MW147609)
H. cyclurae MIT 16-1351 (MW147611)
H. cyclurae MIT 16-1358 (MW147610)
Helicobacter sp. 11502629-2 (KJ081205)
Helicobacter sp. GC011 (MG910456)
H. aurati MIT 97-5075^1 (NR_025124)
H. fernelliae ATCC 35683^1 (AF328745.1)

Squamate (1)
Blue iguana (3)
Chelonian (2)
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Figure 1

Continued

B

C. jejuni 6871 (AY628400.1)
Helicobacter sp. 11052629-2 (MLAO00000000)
Helicobacter sp. 13500401-1 (MLAO00000000)
Chelonian (2)

H. monodelphis MIT 15-1451* (NYH01000000)
H. gannani CMR1002* (KMM66537.1)

H. canadensis MIT 98-5491* (AY787946)
H. pullorum NCTC 12826 (AJS581999)
H. muridorum CCUG 29262* (AJS582000)
H. aurati MIT 97-5075* (KT697621.1)
H. didelphidarum MIT 17-337* (NXLQ01000000)
H. bilis ATCC 51630* (AJS582111)
H. trogontum ATCC 700114* (AJS582021)

H. pylori ATCC 43504* (AY787944)
H. felis ATCC 49179* (AJS58228.1)
H. suis HS1* (EF204597.1)
H. saguini MIT 97-6194-5* (CPO28939)

H. cycluroe MIT 16-1353* (NYH00000000)
H. cycluroe MIT 16-1345
H. cycluroe MIT 16-1351
Blue iguana (3)

H. mustelae ATCC 43772* (AJS58219.1)
H. macaca MIT 99-5501* (AJS58218)
H. canis ATCC 51401* (AJS58218)

H. fennelliae ATCC 35684* (AY787947)
H. apri A19* (KPI64511)

H. equorum E92* (DO888713)
H. ciaoedi CCUG 18818* (AJS58216)

H. typhlonius MIT 97-6810* (LN907958)
H. hepaticus ATCC 51448* (AY787943)
H. joihi MIT 09-6949* (KP701329)
H. marmotae MIT 98-6070* (KMM66529.1)

H. anseris MIT 04-9362* (KT198998.1)

H. cholecystus ATCC 700242* (KMM66522.1)
H. brantaee MIT 04-9366* (KT697623.1)

Helicobacter sp. 13500482-2 (MLAO00000000)
Helicobacter sp. 11502596-1 (MLAO00000000)
Helicobacter sp. 12502634-8 (MLAO00000000)
Helicobacter sp. 12502232-10 (MLAQ00000000)
Helicobacter sp. 11503491-1 (MLAO00000000)
Helicobacter sp. 13500477-4 (MLAS00000000)

Squamate (1)
FIGURE 1 | Phylogenetic analysis of 16S rRNA, (A) hsp60, (B) and gyrB (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 Chelonia: 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)
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. At 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. GC1B1, 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 (hsp60) and DNA gyrase subunit B (gyrB) 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. cylindracea isolates shared 94–99% sequence identity within the species in their gyrB but only had 72% sequence identity with the gyrB gene sequence of closely related species, Helicobacter ansers (Fox et al., 2006). For hsp60 gene analysis, the three H. cylindracea 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. cylindracea 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. cylindracea 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 NYHM000000000.
### Comparison Genome ANI dDDH

| Genome                        | ANI   | dDDH |
|-------------------------------|-------|------|
| Helicobacter acinonychis ATCC 51101\[UAGA010000000\] | 69.8% | 45%  |
| Helicobacter allurogastricus ASB7\[CDMG010000000\] | 69.5% | 34.3%|
| Helicobacter anseris MIT 04-9362\[NLX010000000\] | 70.8% | 30.6%|
| Helicobacter aurati MIT 97-5075\[NLXW010000000\] | 70.2% | 48.2%|
| Helicobacter baculiformis M50\[FZMF010000000\] | 69.9% | 45.1%|
| Helicobacter bilis ATCC 51630\[JRPG000000000\] | 71.4% | 29.6%|
| Helicobacter bizzozeronii CCUG 35545\[CAGP000000000\] | 70.0% | 44.8%|
| Helicobacter brantae MIT 04-9366\[NXLV010000000\] | 70.7% | 32.5%|
| Helicobacter canadensis MIT 98-5491\[ABQS000000000\] | 70.6% | 52.9%|
| Helicobacter canis ATCC 51401\[VXKE010000000\] | 71.1% | 33.3%|
| Helicobacter cetorum\[CP003481\] | 70.6% | 37.9%|
| Helicobacter cholecystus ATCC 700242\[NXLU010000000\] | 70.0% | 49.4%|
| Helicobacter cinaedi CCUG 18818\[ABQT000000000\] | 71.0% | 25.3%|
| Helicobacter cynogastricus JKM4\[FZMQ010000000\] | 71.3% | 43.7%|
| Helicobacter didelphidarum MIT 17-337\[NXLQ010000000\] | 70.5% | 30.4%|
| Helicobacter equorum MIT 12-6600\[NXLT010000000\] | 70.6% | 31.2%|

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...
binding factor (Peb4) from *Campylobacter jejuni* ssp. *jejuni* NCTC 11168 were present in all reptile-associated helicobacter species 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 cdtnABC 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. GCBII 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
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.
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 ≥ 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 cytotoxid 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 cholecytus* (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 16-1353³ 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 NYHM00000000.

**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/, NYHM00000000; 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, 16US033594/9, 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. JP 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.

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