Two Gram-stain-negative, gently curved rod-shaped isolates (WBE14 T and WBE19), recovered from wild bird faecal samples in the city of Valdivia (Southern Chile) were subjected to a polyphasic taxonomic study. Results of a genus-specific PCR indicated that these isolates belonged to the genus Helicobacter. This was further confirmed by a phylogenetic analyses based on the 16S rRNA, 60 kDa heat-shock protein (cpn60) and gyrase subunit B (gyrB) genes, where both strains formed a novel phylogenetic line within this genus. The 16S rRNA gene sequence similarity of strain WBE14 T to the type strains of all other species of the genus Helicobacter examined ranged from 89.4 to 97.0 %; Helicobacter brantae and Helicobacter pametensis were the most closely related species. However, on the basis of the protein-coding genes Helicobacter pullorum and Helicobacter canadensis are the most closely related species. These data, together with their different morphological and biochemical characteristics, revealed that these strains represent a novel species, for which the name Helicobacter valdiviensis sp. nov. is proposed, with the type strain WBE14 T (= CECT 8410 T = LMG 27920 T).

In the present study, two novel isolates (WBE14 T and WBE19) recovered from wild bird faecal samples were subjected to a polyphasic examination including a phylogenetic analysis of the 16SrRNA, 60 kDa heat-shock protein (cpn60) and gyrase subunit B (gyrB) genes and morphological, physiological and biochemical characterization, in order to determine their taxonomic position. Based on the reported findings, we propose and describe these strains as representatives of a novel species within the genus Helicobacter.

During a study on the prevalence and diversity of campylobacteria in wild bird faecal samples carried out in 2013 in the city of Valdivia, Southern Chile (L. Collado, unpublished data), two isolates (WBE14 T and WBE19), suspected of belonging to an unknown taxon, were recovered. The samples corresponded to voided faecal samples excreted into urban public parks. Although the specific type of bird corresponding to each specimen remains unidentified, at the time of sampling black-faced ibis (Theristicus melanopis), southern lapwing (Vanellus chilensis) and chimango caracara (Milvago chimango) were the main species observed at these sites. Faecal samples were collected using sterile cotton-tipped swabs that were immediately placed into 8 ml Bolton broth (Oxoid). Incubation was performed at 37 °C for 48 h under micro-aerobic conditions (CampyGen, Oxoid). After enrichment, an aliquot (400 μl) was transferred onto the surface of a 0.45 μm membrane filter (Millipore) which was placed on a Petri dish containing

The genus Helicobacter was proposed by Goodwin et al. (1989) to accommodate two species of the genus Campylobacter. Campylobacter pylori (now Helicobacter pylori, the type species of the genus) and Campylobacter mustelae (now Helicobacter mustelae). At the time of writing (August 2013), the genus comprised 33 species with validly published names, namely H. pylori, H. mustelae, H. felis, H. fennellaeae, H. cinaedi, H. nemestrinae, H. muridarum, H. acinonychis, H. canis, H. hepaticus, H. pametensis, H. pullorum, H. bilis, H. bizzozeronii, H. trogontum, H. cholecystus, H. rodentium, H. salomonis, H. canadensis, H. mesocricetorum, H. aurati, H. typhlonius, H. ganmani, H. cetorum, H. marmotae, H. anseris, H. brantae, H. cynogastricus, H. mastomyrinos, H. equorum, H. baculiformis, H. suis and H. heilmannii (http://www.bacterio.net/h/helicobacter.html). Although by far the most studied species has been H. pylori, other helicobacters have become increasingly important over recent years as emergent pathogens and as potential zoonotic agents associated with inflammatory bowel diseases, hepatobiliary diseases, coronary artery diseases, gastritis and pyoderma gangrenosum-like ulcers (On et al., 2005; Haesebrouck et al., 2009; Rossi & Hanninen, 2012).
Blood Agar Base (Merck) supplemented with 5 % defbrinated sheep blood (Quad Five) and it was allowed to filter passively under ambient conditions for 30 min. After filtration, the filters were removed with sterile forceps and discarded. The inoculated plates were incubated under the aforementioned conditions. The novel strains were initially identified as Campylobacter-like organisms (CLOs) on the basis of their morphology and micro-aerobic growth. However, these isolates showed a novel BsrI restriction pattern (282/247/240/223 bp) with the RFLP identification method described by Marshall et al. (1999). Therefore, they were selected for polyphasic characterization to establish their taxonomic classification.

Genomic DNA of strains WBE14T and WBE19 was extracted using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer’s instructions. Genus-specific PCRs for the genera Campylobacter, Helicobacter and Arcobacter were applied as described by Linton et al. (1996), Germani et al. (1997) and Harmon & Wesley, (1997), respectively. For both strains only a 399 bp band specific to helicobacters was amplified. A nearly complete 16S rRNA gene sequence (>1340 bp) as well as 555 bp of the cpn60 gene of strains WBE14T and WBE19 were amplified as described by Vandamme et al. (2006) and Hill et al. (2006), respectively. Additionally, the gyrB and urease genes were also evaluated with the protocols described by Hannula & Hänninen (2007) and O’Rourke et al. (2004), respectively. Both DNA strands of the PCR products (16S rRNA, cpn60 and gyrB genes) were directly sequenced with an ABI 3730 XL automatic DNA sequencer (ABI) by a commercial sequencing facility (Macrogen, Seoul, Korea). Alignment of the sequences was performed with the CLUSTAL W program (Thompson et al., 1994). Phylogenetic trees were reconstructed with the MEGA 5 software (Tamura et al., 2011), by using the neighbour-joining method (Saitou & Nei, 1987) with Kimura’s two-parameter calculation model (Kimura, 1980) and the stability of the groupings was estimated by bootstrap analysis (500 replications). Similarity values between the 16S rRNA gene sequence of strain WBE14T and the type strains of all members of the genus Helicobacter with validly published names were calculated with the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012), obtaining a range of 89.4–97.0 %. The phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) clearly indicated that strains WBE14T and WBE19 represented a single species within the genus Helicobacter, most closely related to H. brantae (97.0 % similarity) and H. pametensis (96.7 % similarity), which, interestingly, were also originally isolated from wild bird faeces (Dewhirst et al., 1994; Fox et al., 2006).

The taxonomic situation of the novel strains within the genus Helicobacter and the demonstration of their representing a novel lineage were confirmed by the cpn60 and gyrB phylogenetic trees (Figs 2 and 3). However, on the basis of these protein-coding genes, H. pullorum and H. canadensis are the most closely related species. These two species are members of the genus Helicobacter with an unsheathed-flagellum, which have been isolated from stool samples of humans with gastroenteritis as well as from faecal samples of birds (On et al., 2005).

The urease genes could not be amplified with the O’Rourke protocol. This could seem strange because, as shown below, the strain WBE14T showed urease activity. However, other urease-positive members of the genus Helicobacter, such as Helicobacter cynogastricus, did not produce the expected amplicon with this method either (Van den Bulck et al., 2006).

Phenotypic characterization of strains WBE14T and WBE19 was performed according to the recommendations of Dewhirst et al. (2000). Growth of strains WBE14T and WBE19 was determined on nutrient broth no. 2 (Oxoid) supplemented with 5 % defbrinated sheep blood (Quad Five) and 2 % agar (Merck). Micro-aerobic growth (using CampyGen, Oxoid) was evaluated at 25 °C, 37 °C and 42 °C for 48–72 h. Aerobic and anaerobic growth (using AnaeroGen, Oxoid) were evaluated at 37 °C for 72 h. Indoxyl acetate hydrolysis was determined as described by Mills & Gherna (1987). Urease activity was tested with the method of Owen et al. (1985). Catalase activity was evaluated by adding a 3 % H2O2 solution and observing the reaction within 5 s. Oxidase activity was determined with Bactident Oxidase strips (Merck). The urease activity and a set of additional phenotypic tests (reduction of nitrates, esterase activity, hydrolysis of hippurate, γ-glutamyltransferase activity, reduction of triphenyl-tetrazolium chloride (TTC), alkaline phosphatase activity, production of H2S, assimilation of glucose and pyrrolidonyl-, L-arginine- and L-aspartate-arylamidase activities) were evaluated by using the API Campy identification system (bioMérieux) according to the manufacturer’s instructions. Motility was observed after 48 h in cultures by examining wet mounts in nutrient broth no. 2 (Oxoid) by phase-contrast microscopy. The morphology, cell size and presence of flagella of strains were determined with a transmission electron microscope (TECNAI 12, Philips) after negative staining with 1 % uranyl acetate for 1 min. (Fig. S1, available in the online Supplementary Material). The results of all mentioned tests are listed in the species description below. Table 1 shows the most important phenotypic characteristics differentiating the novel strains from the other species of the genus Helicobacter.

In conclusion, the present study demonstrates the existence of a novel species of the genus Helicobacter, for which the name Helicobacter valdiviensis sp. nov. is proposed. Further studies are necessary to define the host range of this novel species and to determine its clinical relevance and zoonotic potential.

**Description of Helicobacter valdiviensis sp. nov.**

_Helicobacter valdiviensis_ (val.di.vi.en’sis. N. L. masc. adj. valdiviensis belonging to Valdivia, a Chilean city, where the organism was first isolated).

Cells are Gram-stain-negative gently curved rods, non-encapsulated, non-spore-forming and are 0.3–0.5 μm wide.
and 1.2–3 μm long. Coccoid cells were observed in old cultures. They are motile with a single, monopolar, unsheathed flagellum. Cells do not possess a periplasmic fibril. Colonies on blood agar incubated in micro-aerobic conditions at 37 °C for 48 h are 2 mm in diameter, translucent, circular with entire margins and convex. No swarming on solid media was noted. Pigments are not produced. Grows on blood agar at 37 °C and at 42 °C under micro-aerobic conditions (does not require atmospheric hydrogen). No growth was observed at 37 °C on aerobiosis and in anaerobic conditions or at 25 °C under micro-aerobic conditions. No haemolysis is seen on blood agar. Has oxidase and catalase activity and hydrolyses indoxyl acetate. No c-glutamyl transferase, hippuricase, pyrrolidonyl arylamidase, alkaline phosphatase, L-arginine or L-aspartate arylamidase activity is detected. Urease is variable and nitrate and triphenyl tetrazolium chloride are not reduced. Neither hydrogen sulphide nor acid are produced from glucose fermentation. Cells are resistant to cephalothin but showed variable susceptibility to nalidixic acid. Growth is obtained on media containing 1 % glycine. The clinical significance of H. valdiviensis is unknown.

Fig. 1. Neighbour-joining tree based on 16S rRNA sequences showing the phylogenetic position of Helicobacter valdiviensis sp. nov. within the genus Helicobacter. Bootstrap values (>70 %) based on 500 replications are shown at nodes. Bar, two substitutions per 100 nt.
Fig. 2. Neighbour-joining tree based on cpn60 sequences showing the phylogenetic position of *Helicobacter valdiviensis* sp. nov. within the genus *Helicobacter*. Bootstrap values (>70%) based on 500 replications are shown at nodes. Bar, five substitutions per 100 nt.

Fig. 3. Neighbour-joining tree based on gyrB sequences showing the phylogenetic position of *Helicobacter valdiviensis* sp. nov. within the genus *Helicobacter*. Bootstrap values (>70%) based on 500 replications are shown at nodes. Bar, five substitutions per 100 nt.
### Table 1. Phenotypic characteristics that differentiate *Helicobacter valdiviensis* sp. nov. from other 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  | 21  | 22  | 23  | 24  | 25  | 26  | 27  | 28  | 29  | 30  | 31  | 32  | 33  | 34  |
|-----------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Catalase production               | +   | +   | +   | +   | +   | +   | +   | −   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Nitrate reduction                 | −   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Urease                            | −   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Alkaline phosphatase hydrolysis   | −   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| γ-Glutamyl transpeptidase         | −   | +   | +   | +   | −   | −   | −   | N  | D  | +   | +   | +   | +   | −   | −   | −   | −   | −   | −   | −   | +   | −   | −   | +   | +   | −   | −   | −   | −   | −   | −   | +   | +   |
| Indoxyl acetate hydrolysis        | +   | (−) | +   | (−) | −   | −   | (−) | +   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   |
| Growth at 42 °C                   | +   | (−) | ±   | (−) | ±   | −   | +   | (−) | +   | +   | +   | ±   | +   | +   | +   | −   | +   | +   | −   | +   | +   | +   | −   | +   | +   | −   | −   | −   | −   | −   | −   | −   | −   |
| Growth on 1% glycine              | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | +   | ±   | −   | +   | (−) | ND  | +   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   |
| Susceptibility to                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Nalidixic acid                    | V   | S   | R   | S   | R   | S   | R   | S   | R   | R   | R   | R   | S   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   |
| Cephalotin                        | R   | R   | S   | R   | S   | R   | S   | R   | S   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   | R   |
| Periplasmic fibres                | −   | −   | +   | −   | −   | −   | +   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   | −   |
| Number of flagella                | 1–4 | 4–8 | 14–12 | 10–14 | 12–5 | 2 | 2 | 2 | 2 | 1 | 3–14 | 10–20 | 5–7 | 1 | 2 | 10–23 | 2 | 1–27–10 | 2 | 2 | 2 | 2 | 2 | 6–12 | 2 | 1 | 11 | 4–10 | 4–10 |
| Sheathed flagella                 | −   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |

*Weak reaction detected (in two out of seven strains).
†Positive reaction for WBE14 and negative for WBE19 (identical results obtained by API Campy and the method of Owen et al., 1985).
‡Some strains giving a negative result have been described (Dewhirst et al., 2000).
§WBE14 is resistant and WBE19 is susceptible.
||The tests were assessed in plates with 32 mg of the respective antibiotic l−1.
The type strain is WB14T (=CECT 8410T=LMG 27920T), isolated from a wild bird faecal sample in Valdivia, Chile.

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References


