Gross anatomy and histology of the alimentary system of Characidae (Teleostei: Ostariophysi: Characiformes) and potential phylogenetic information

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A compared study of the morphology of the alimentary tract and liver of seven selected species corresponding to the main clades of the Characidae family is presented herein. Three new set of characters corresponding to 1) alimentary tract gross anatomy, 2) alimentary tract histology and 3) liver gross anatomy are evaluated as potential sources of data for future phylogenetic studies of the Characidae. Not considerable interspecific variation was observed at the histological level and therefore this source is not considered to be phylogenetically informative at the taxonomic level analyzed. In contrast, liver and alimentary tract gross anatomy presented important interspecific variation while a relatively homogenous intraspecific morphology was observed. Those characters are optimized in tree-topologies from previous phylogenetic analyses and their evolution and potential relationship with ecological traits are discussed.

Un estudio comparado de la morfología del tracto alimentario y el hígado de siete especies seleccionadas correspondientes a los principales clados de la familia Characidae es presentada aquí. Tres nuevos conjuntos de caracteres correspondientes a 1) anatomía gruesa del tracto alimentario, 2) histología del tracto alimentario y 3) anatomía gruesa del hígado son evaluados aquí como potenciales fuentes de información para futuros estudios filogenéticos de los Characidae. No se observó una variación intraespecífica considerable a nivel histológico y consecuentemente esta fuente no es considerada como informativa filogenéticamente al nivel taxonómico analizado. En contraste, la anatomía gruesa del hígado y el tracto alimentario presentaron una variación interespecífica importante mientras que una relativamente homogénea variación intraespecífica fue observada. Estos caracteres son optimizados en topologías de árboles de análisis filogenéticos previos y su evolución y potencial relación con características ecológicas son discutidas.

Keywords: Alimentary tract, Comparative anatomy, Gastrointestinal tract, Morphological characters, Phylogeny.

Introduction

The Characidae, with more than 1200 species, is the most diverse Neotropical family of fishes and the fourth most diverse worldwide. Its species are found in most freshwater bodies from northern Patagonia, Argentina, to southern USA and in a vast variety of environments reflected in their great ecological and morphological diversity (Mirande, 2010; Reis *et al.*, 2003). This makes this family an excellent model for studying the evolution of these features. Although some works have deal with particular aspects of the anatomy of the alimentary tract of some species, an integrative, extensive, and comparative approach including representatives of the main clades within the family is still lacking. For example, Leknes (2005) studied the histology of the intestine in *Gymnocorymbus ternetzi* (Boulenger, 1895) and Medina Perozo *et al.* (2009) studied the

histology of the caeca of five species of Serrasalmidae and one of Characidae. Also, a histological characterization of the alimentary tract in *Paracheirodon axelrodi* (Schultz, 1956) was made by Gómez-Ramírez *et al.* (2011) and an immunohistochemical study (serotonin, cholecystokinin, somatostatin) of the digestive tract of *Oligosarcus hepsetus* (Cuvier, 1829) was published by Vieira-Lopes *et al.* (2013). In addition, the alimentary tract of a few species belonging to other families of Characiformes have been characterized, such as the anostomids *Leporinus friderici* (Bloch, 1794) and *L. taeniofasciatus* Britski, 1997 (Albrecht *et al.*, 2001), and *Leporinus obtusidens* (Valenciennes, 1837) (Ulibarrie, 1984). In the most comprehensive phylogenetic analyses of the Characidae, a relatively low number of species was analyzed (about a 15%) and there are many internal clades with very low support and morphological synapomorphies (Mirande, 2010; Mirande *et al.*, 2011, 2013). Also, some

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discordance exists between morphological approaches and the molecular based published phylogenetic analyses of the family (Calcagnotto *et al*., 2005; Javonillo *et al*., 2010; Oliveira *et al*., 2011). Additional morphological information is needed to better assess the phylogeny of the family and especially to provide synapomorphies to many nodes.

According to morphological approaches *Markiana* Eigenmann, 1903 (*e.g.* Mirande, 2010), it is related with *Astyanax* Baird & Girard, 1854, and included in an informal subfamily-level group termed "*Astyanax* clade" by Mirande (2010). According to molecular hypotheses (*e.g.* Oliveira *et al*., 2011), it is included in the Stevardiinae. Interestingly, Baicere-Silva *et al*. (2011) found, from data of sperm and spermatogenesis, similarities between *Markiana* and the Stevardiinae, supporting the molecular approach and supporting the idea that additional morphological information is needed.

The aims of this work are: a) to provide a description of the gross anatomy of the alimentary tracts and liver of seven selected species of Characidae representing the most diverse clades of Characidae (Mirande, 2009, 2010; Javonillo *et al*., 2010; Oliveira *et al*., 2011); b) to characterize the alimentary tract at the histological level of six of these species; c) to propose a set of novel characters potentially informative for future phylogenetic researches; d) to contrast new evidence with the two alternative hypotheses of *Markiana nigripinnis* (Perugia, 1891) phylogenetic position; e) to optimize the new characters proposed in topologies from previous phylogenetic analyses (Mirande, 2010; Oliveira *et al*., 2011) and f) to discuss these results with regards to their evolution and relationships with the ecology and physiology of the analyzed species.

Material and Methods

Seven species corresponding to the main clades of the Characidae *sensu* Mirande (2009, 2010) and one species of Crenuchidae, as an external group, were selected to study their alimentary system. The criteria to select those species were: presumably generalized anatomy, availability of material in collections, possibility of collecting new fresh material for better histological preparations and relatively small body size to facilitate histological study. After that selection, the species herein studied are *Aphyocharax anisitsi* Eigenmann & Kennedy, 1903 (Aphyocharacinae), *Astyanax endy* Mirande, Aguilera & Azpelicueta, 2006, *A. rutilus* (Jenyns, 1842), *Markiana nigripinnis* ("*Astyanax* clade"), *Bryconamericus thomasi* Fowler, 1940 (Stevardiinae), *Cheirodon interruptus* (Jenyns, 1842) (Cheirodontinae), *Gymnocorymbus ternetzi* (Tetragonopterinae), and *Characidium borellii* (Boulenger, 1895) (Crenuchidae).

Adult specimens of each of these species were fixed in Bouin solution after overdose with benzocaine. After 1 day, they were transferred to ethanol 70%. Appropriate actions were taken to minimize pain or discomfort of fish, and this study was conducted in accordance with international standards on animal welfare, as well as being compliant with national regulations and the "Comité Nacional de Ética en la Ciencia y la Tecnología". At least 4 individuals of both sexes of each species were dissected under a stereo microscope, except for *Markiana nigripinnis*, since there was only one male individual available. This species and *Characidium borellii* were not used for histological studies and were only examined at a gross anatomical level. Dissections consisted on a ventral opening of the abdominal cavity, body walls were removed and the esophagus was sectioned in its anterior portion in order to free the alimentary tract, liver, pancreas and gas bladder. Pictures were taken using a digital camera attached to the stereomicroscope from left right dorsal and ventral views, after and before removing the liver. The alimentary tract of four specimens of *Astyanax rutilus* were fully sectioned and prepared for histological analysis in order to assess intraespecific variability. Also, the alimentary tract of one male of each of the remaining species was processed for histological characterization, as follows. The alimentary tract was separated with the pancreas attached to it and dehydrated together through increasing concentrations of ethanol solution and then transferred to xylene and included in paraplast at 60ºC during 6 h. Then, it was fully sectioned with a microtome at 7µm, and mounted in microscope slides. Sections were stained with Masson Trichromic. Microphotographs were obtained with a digital camera coupled to a stereo microscope in order to study the histological features of the alimentary tract and pancreas of these species.

A series of characters were defined corresponding to three different sources: 1) alimentary tract gross anatomy, 2) liver lobes gross anatomy and 3) histology of the alimentary tract.

In addition to a general description of the structures herein examined, observations are expressed as phylogenetic characters with alternative character-states which permit a better comparison between species and to have some clue about their evolution when mapped on a phylogenetic hypothesis of the family. That mapping was done on the phylogenetic hypothesis by Mirande (2010) using TNT (Goloboff *et al*., 2008) and discussed with regards to its evolution and relationship with known data from ecology and physiology of these species. We could not optimize those characters in the phylogenetic hypothesis by Oliveira *et al*. (2011) because this author only analyzed three of the species selected in the present work (*A. anisitsi, G. ternetzi, M. nigripinnis*) but we tested the phylogenetic position of *M. nigripinnis* as a member of the Stevardiinae, as proposed by Baicere-Silva *et al*. (2011) and Oliveira *et al*. (2011) by comparing differences in tree lengths of morphology-based phylogenetic trees when the inclusion of *Markiana* in the Stevardiinae is forced with a constraint.

Voucher specimens are deposited at the Ichthyological collection of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN-ict), Buenos Aires.

Results

Gross anatomy of the alimentary tract. The alimentary tract of the analyzed species consists of an esophagus, continued by an anteroventrally curved stomach followed by a twisted intestine. The first intestine portion, referred to as the pyloric intestine, presents a dorso-lateral curve (1) to the right side of the stomach where it presents a variable number of caeca among the studied species (0-8). These caeca also vary in their position with respect to the stomach (at left, right, or ventral) although its distal portion was always directed to the posterior region (Table 1). This intestine portion is followed by a posterior prolongation surpassing the stomach where it presents a second curve (2) in anteroventral direction. From this point it develops in an anterodorsal direction at the left side of the stomach and may present a third curve (3) to an anterior direction. Afterwards, it presents a curve (4) in a posterodorsal direction. From curve 4, intestine projects posteriorly surpassing curve 2, in this portion it may present a slight curve (5) ventrally followed by a slight posterior curve (6) in some species (Figs. 1-2). Intestine foldings were observed under a stereo microscope by transparency of the alimentary tract wall (*e.g.* Fig. 2A). Mucosa Foldings are mainly transversal whereas those of the caeca are mainly longitudinally directed.

Intra and interspecific variability of this general pattern is discussed in the "Description of phylogenetic characters" section.

Gross Anatomy of Liver lobes. The liver was found to be ventrally to the esophagus and anterior to the stomach and pyloric intestine. It presented a series of lobes developing posteriorly at the sides of the stomach and anterior intestine portions. These lobes varied in their development and disposition among species. It was observed one dorsal lobe (dotted in bluein Figs. 3-4) and one ventral lobe (dotted in red) in the left side, one dorsal lobe at the right side (green) and a small ventral lobe (purple), present in some species (Figs. 3-4).

Pancreas gross anatomy and histology. The pancreas of the studied species was observed as longitudinal cords with ramifications following the intestine and each intestinal caeca. Histologically it presented a well-developed exocrine pancreas with serous acinous cells presenting a central spherical nucleus with one nucleolus, a basophilic basal portion, and an eosinophilic apical portion with zymogen granules. Associated with this, a pancreatic duct with a cylindrical epithelium was observed.

The endocrine portion of pancreas presented α, β and δ cells. It was associated with blood and lymphatic vessels, nerves, and nervous plexus. No differences were noticed among the selected species (Fig. 6D).

Histology of the alimentary tract. Histology of the alimentary tract of selected species presented almost no differences at the analyzed level (types of epitheliums, tunic's structure, presence of unicellular and multicellular glands, and type of musculature). The general pattern observed is described below and variations to it are mentioned.

Esophagus. The esophagus presents a mucosa with many foldings, composed by a pseudostratified epithelium, with abundant mucous cells (Fig. 5A-B). The number and size of this foldings in esophagus and also in the intestine are apparently dependent on size of the individuals rather than to species identity, with larger individuals presenting a higher number and deeper foldings. One species, *Cheirodon interruptus*, presents gustative papillae in the esophagus region (Fig. 5C). Beneath the epithelium, a layer of loose connective tissue is present, surrounded by a muscular tunica with an inner longitudinal and an external circular layer of striated muscle. Some longitudinal muscular fibers are observed in the loose connective tissue. The striated muscle continued into the stomach in the cardiac and fundic portions.

Table 1. Intestinal *caeca* in Characidae.

|	Left side	Right side	Ventral	Total
Markiana nigripinnis	1	7	0	8
Astyanax rutilus	2	6	0	8
Astyanax endy	2	6	1	8
Gymnocorymbus ternetzi	3	4	1	8
Bryconamericus thomasi	0	6	0	6
Aphyocharax anisitsi	1	4	0	6
Cheirodon interruptus	2	6	0	8
Characidium borellii	2	4	0	6
Paracheirodon axelrodi (*sensu* Gómez-Ramírez *et al.,* 2011)	0	0	0	0
Leporinus taeniofasciatus (*sensu* Albrecht *et al.,* 2001)	0	10	0	10
L. friderici (*sensu* Albrecht *et al.,* 2001)	unknown	unknown	unknown	12
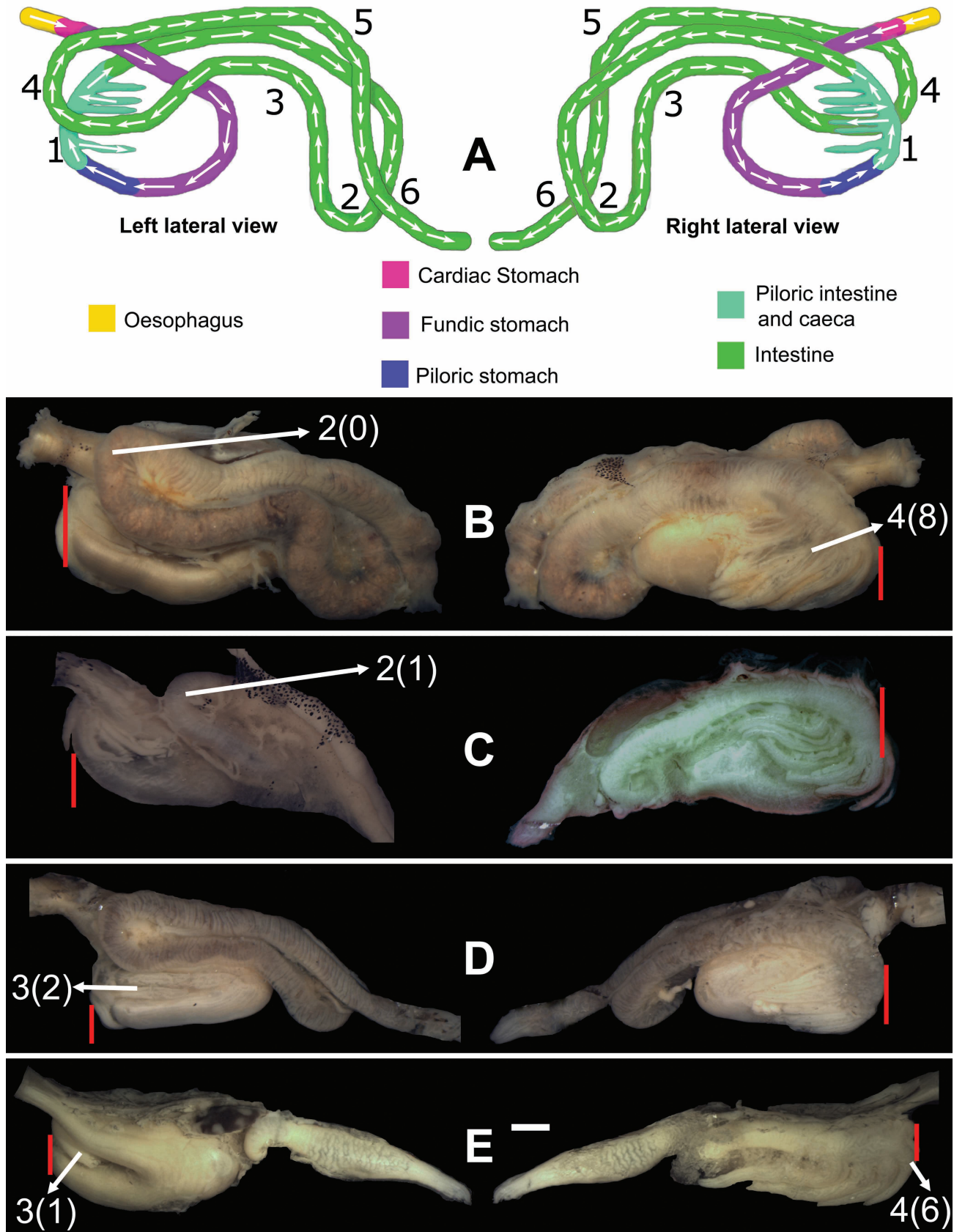
Fig. 1. Alimentary tract of selected species of the Characidae family: **A** - Schematic representation of the alimentary tract; **B** - *Astyanax endy*; **C** - *Astyanax rutilus*; **D** - *Cheirodon interruptus*; **E** - *Aphyocharax anisitsi*. Characters are indicated with numbers and character states between parentheses. Red bar indicates the anterior distal margin of the stomach. Bar=1mm.
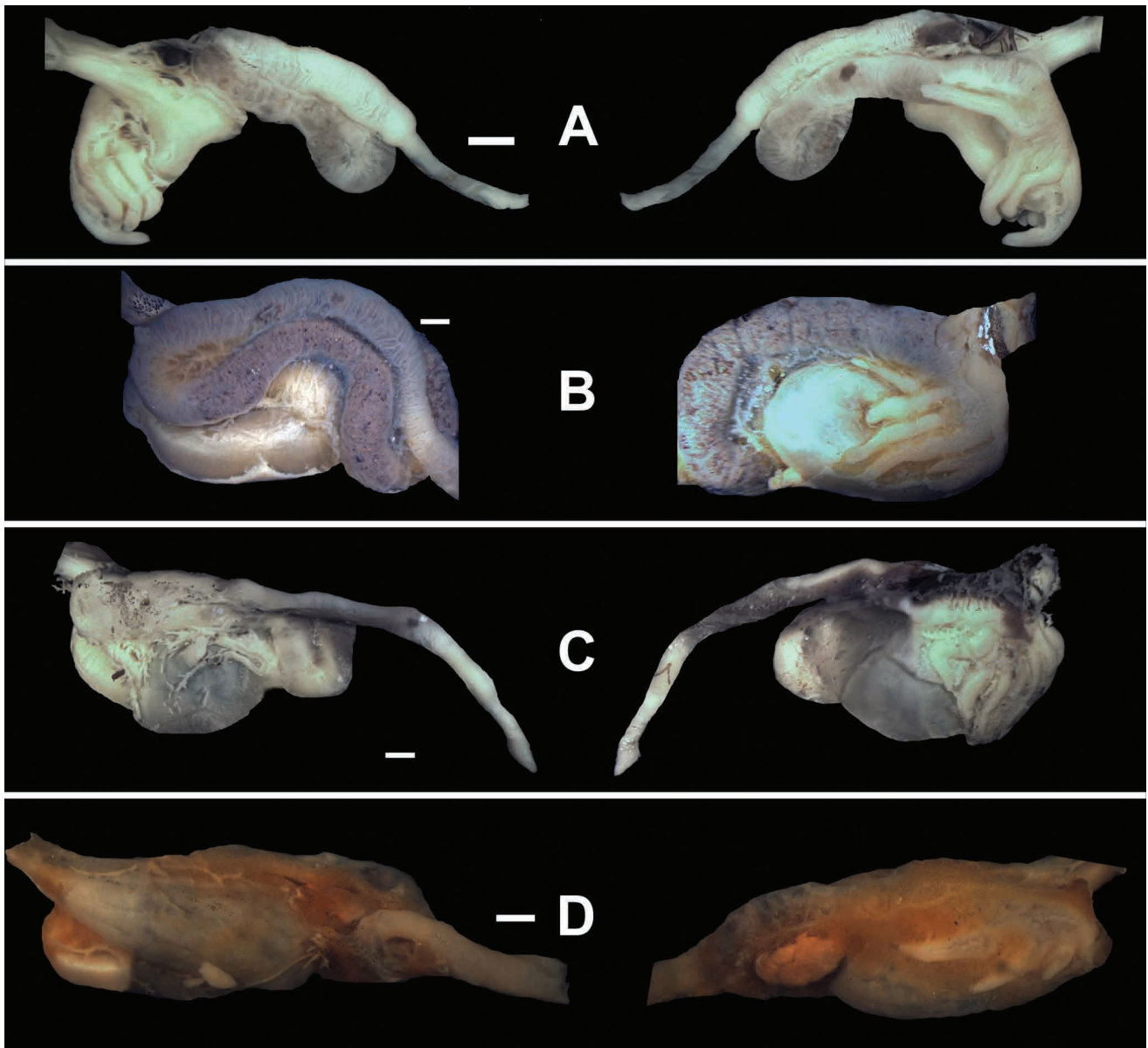
Fig. 2. Alimentary tract of selected species of the Characidae family: **A** - *Gymnocorymbus ternetzi*; **B** - *Bryconamericus thomasi*; **C** - *Markiana nigripinnis*; **D** - *Characidium borellii*. Characters are indicated with numbers and character states between parentheses. Red bar indicates the anterior distal margin of the stomach. Bar=1mm.

Stomach. The cardiac stomach is relatively short and presents a simple epithelium as a layer of secreting mucus cells, which also cover the other regions of the stomach. Beneath it, a thin layer of dense connective tissue is observed (Fig. 5E). The fundic stomach follows the cardiac stomach and presents tubular-acinous glands (Fig. 5F) in its first portion. In the following portion, ramified tubular glands are observed surrounded by loose connective tissue and dense septa of connective tissue (Fig. 5G). The final portion of the stomach, the pyloric stomach, is characterized by a great development of the smooth muscle and lack of acinous or tubular glands (Fig. 5H). This portion presents numerous foldings of the mucosa which are accompanied by dense connective tissue. Some loose connective tissue

may be observed between the dense connective tissue layer and the smooth muscle. At the end of the pyloric stomach, a pyloric valve is present separating it from the first intestinal portion, the pyloric intestine (Fig. 6A).

Intestine. The pyloric intestine portion presents a variable number of caeca which are histologically similar to the rest of the intestine (Fig. 6B-C). This is characterized by a simple columnar epithelium with a striated border and mucus secreting goblet cells. A dense connective tissue is observed beneath it (Fig. 6B) surrounded by a thin layer of smooth muscle. The mucosa presents numerous foldings in this region. The peritoneum is composed of a thin connective tissue and a cubical mesothelium.
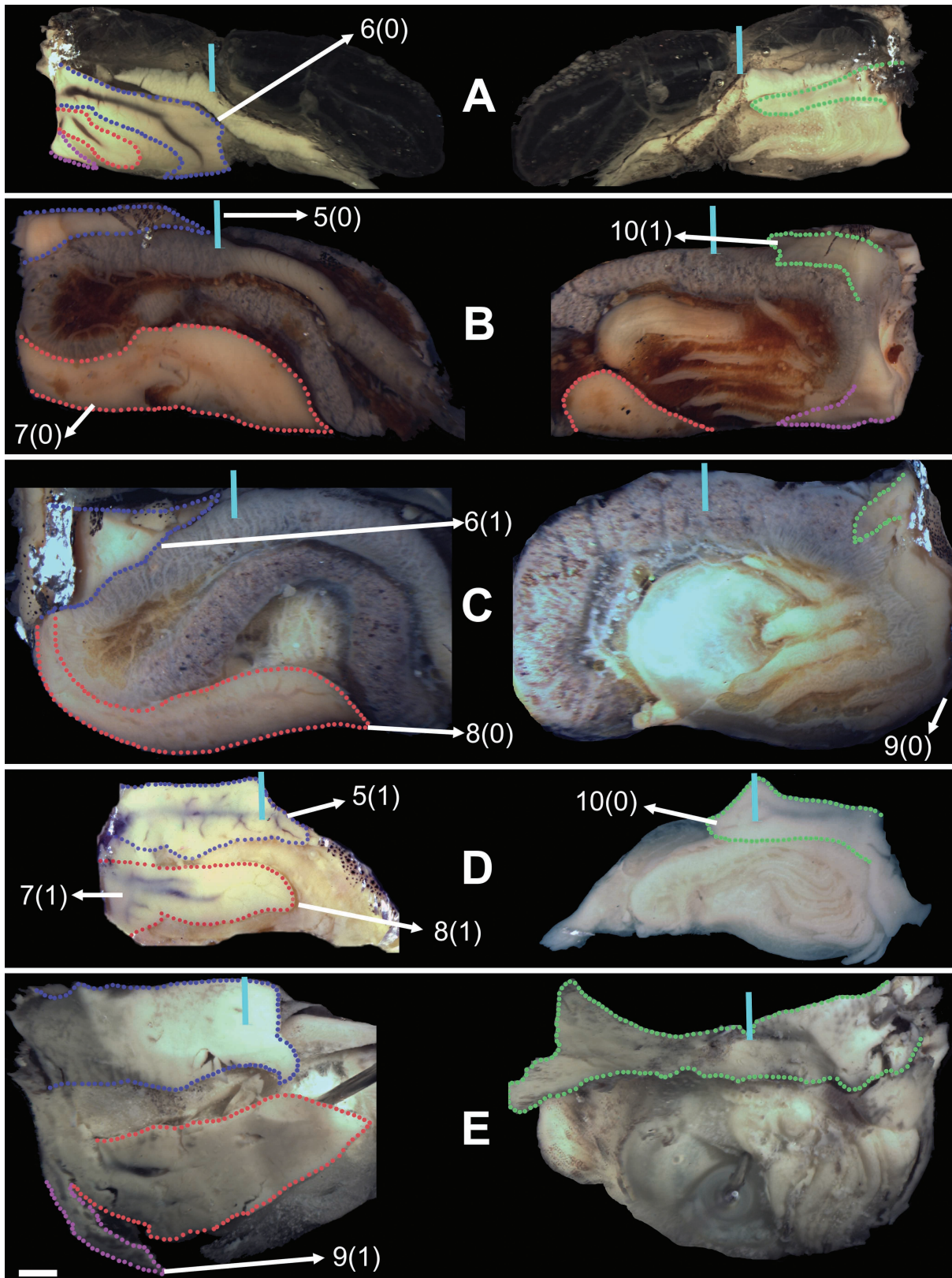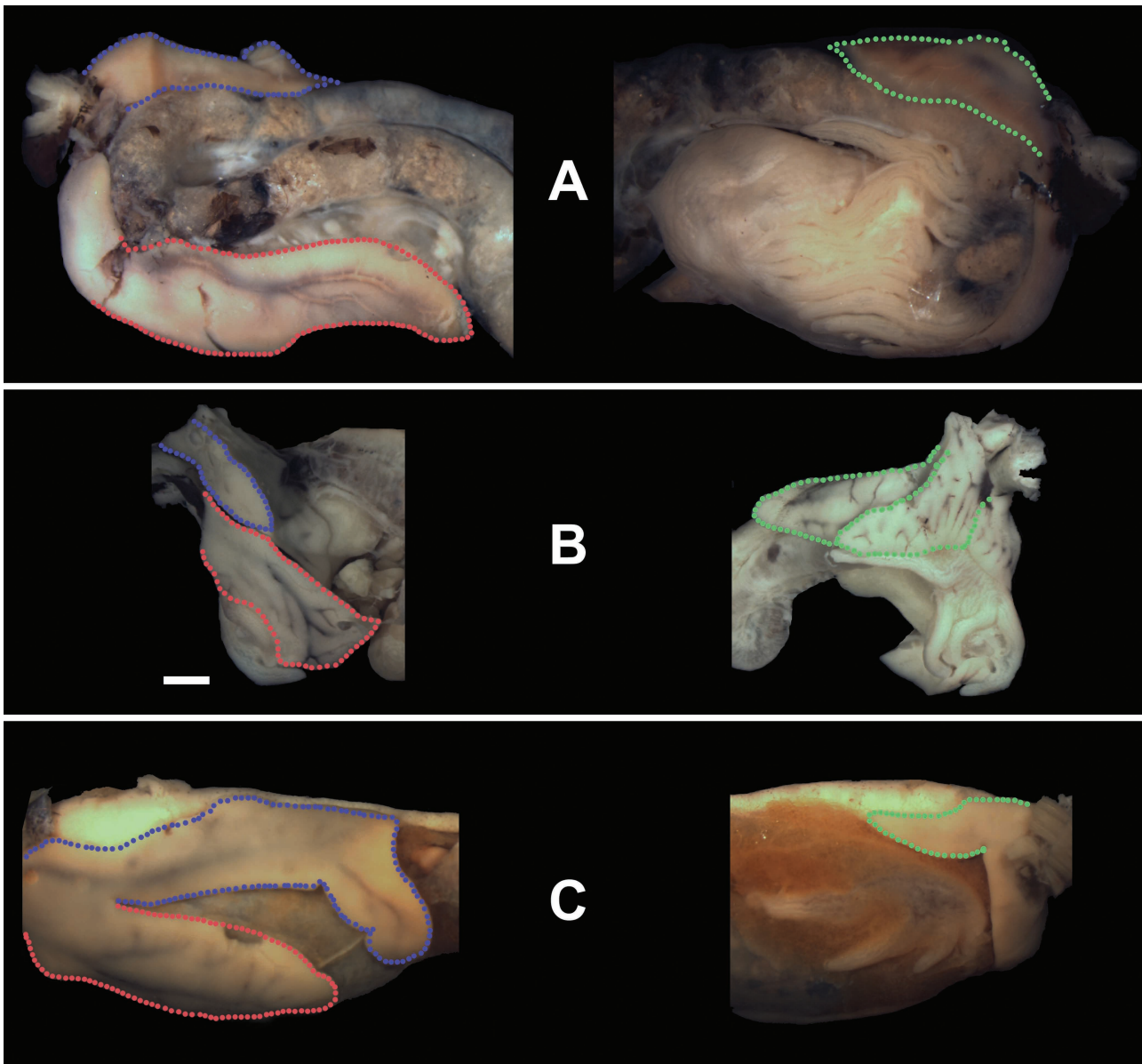
Fig. 3. Liver lobes of selected species of Characidae family. Lateral view. Anterior to left at the left side of the figure. Anterior to right at the right side of the figure. Proposed primary homologue lobes are delimited by the same color. Light blue bar indicates approximately position of anterior distal margin of gas bladder posterior chamber. Characters are indicated with numbers and character states between parentheses. **A -** *Aphyocharax anisitsi*; **B -** *Cheirodon interruptus*; **C -** *Bryconamericus thomasi*; **D -** *Astyanax rutilus*; **E -** *Markiana nigripinnis*. Bar=1mm.

Fig. 4. Liver lobes of selected species of Characidae. Lateral view anterior to left at the left side of the figure. Anterior to right at the right side of the figure. Proposed primary homologue lobes are delimited by the same color. Light blue bar indicates approximately position of anterior distal margin of gas bladder posterior chamber. **A -** *Astyanax endy*; **B -** *Gymnocorymbus ternetzi*; **C -** *Characidium borellii*. Bar=1mm.

Description of phylogenetic characters. Most characters presented here correspond to the hepatic morphology (5), followed by those related to the alimentary tract gross morphology (4) and 1 character from histology of the alimentary tract based on the interspecific variability observed in this study. Taken together, in this work we propose 11 new characters for future phylogenetic analyses.

Stomach. 1. Anteroventral curve: (0) absent; (1) present.

In all analyzed species, except from *G. ternetzi* (0), the stomach presented an anteroventral curve (1) with an approximately J shape. This is also the case of *Leporinus friderici* and *L. taeniofasciatus* (*sensu* Albrecht *et al.*, 2001).

Intestine. 2. Curve 4 of alimentary tract: (0) reaching anteriormost region of stomach; (1) reaching middle length of stomach (2) reaching posteriormost region of stomach or not reaching stomach.

State 0 present in *Astyanax endy*, *Bryconamericus thomasi*, *Cheirodon interruptus*, and *Markianna nigripinnis*. Also in *Leporinus friderici* and *L. taeniofasciatus* (*sensu* Albrecht *et al.*, 2001). State 1 present in *Astyanax rutilus* and *Gymnocorymbus ternetzi*. State 2 present in *Aphyocharax anisitsi* and *Characidium borellii*. State 2 is characterized by a reduction in intestine relative size and curves 2, 4, and 5 (Fig. 1) are adjacent, fused in one curve, and curves 3 and 6 are absent.
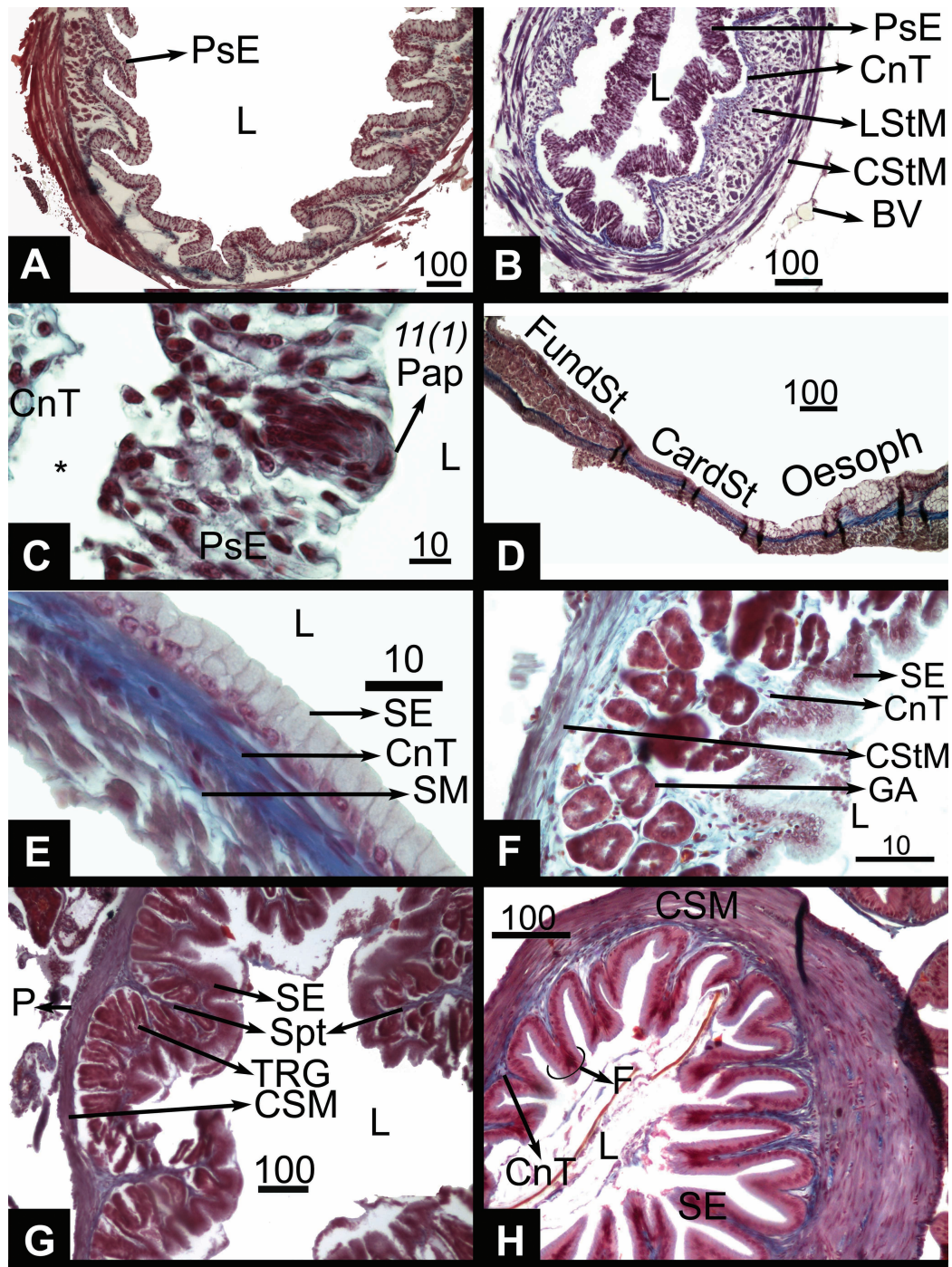
Fig. 5. Histological sections of the alimentary tract of selected species of the Characidae family: **A -** *Astyanax rutilus* esophagus in transversal section showing a folded esophagus mucosa; **B -** *Cheirodon interruptus* esophagus in transversal section showing less conspicuous foldings and a proportionally greater development of the musculature; **C -** Gustative papillae detail of *Cheirodon interruptus*. Characters are indicated with numbers and character states between parentheses; **D -** Transition esophagus-stomach of *Gymnocorymbus ternetzi* in longitudinal section; **E -** Cardiac stomach of *G. ternetzi* in longitudinal section; **F -** Anterior portion of the fundic stomach of *Aphyocharax anisitsi* in transversal section with acinous glands; **G -** Posterior portion of the fundic stomach of *A. anisitsi* in transversal section showing developed connective tissue septa and tubular ramified glands. **H -** Pyloric portion of *G. ternetzi* intestine in transversal section. Abbreviations=**BV**: Blood vessel; **CardSt**: cardiac stomach; **CnT**: connective tissue; **CSM**: Circular smooth muscle; **CStM:** Circular striated muscle; **F**: folding; **FundSt**: fundic stomach; **GA**: Acinous Gland; **L**: lumen; **LStM**: Longitudinal striated muscle; **Oesoph**: Oesophagus. **P**: peritoneum; **Pap**: papillae; **PsE**: Pseudoestratified epithelium; **SE**: Simple Epithelium; **SM**: Striated muscle; **Spt**: Septum; **TRG**: Tubular Ramified Gland; *****: space generated by a technical artifact. Bars units=μm.
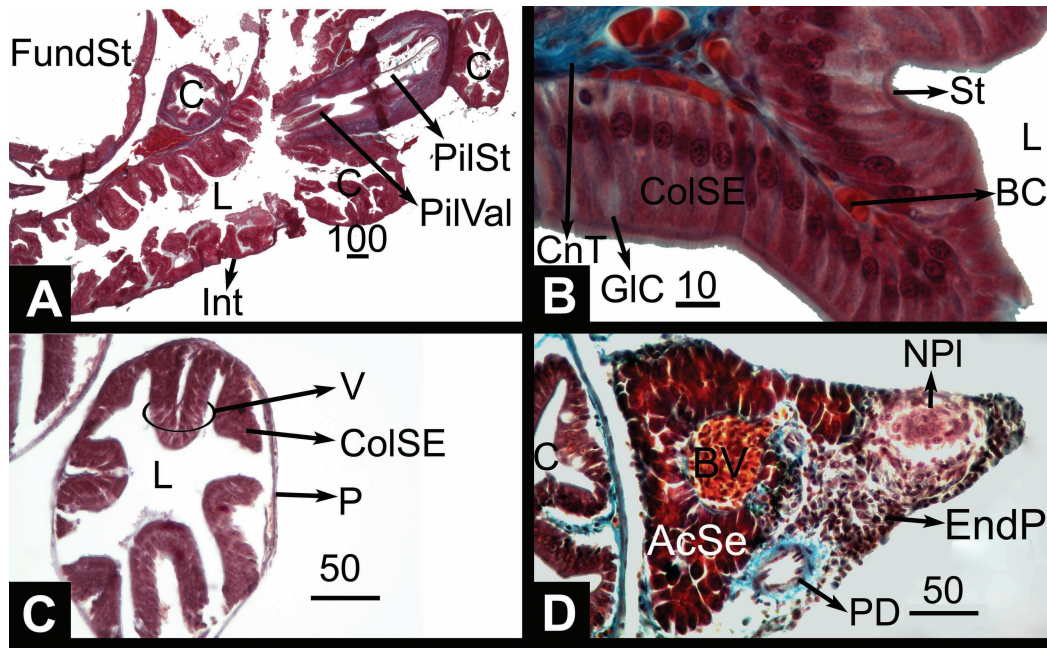
Fig. 6. Histological sections of the alimentary tract of selected species of the Characidae family: **A -** Transition stomach-intestine of *G. ternetzi* in transversal section; **B -** Detail of an intestinal folding in *A. anisitsi*; **C -** Transversal section of a intestinal caeca of *Cheirodon interruptus*; **D -** *A. anisitsi* pancreas. Abbreviations= **AcSe**: Serous Acinus **BC**: Blood Capillary; **C**: Ceca; **ColSE**: Columnar Simple Epithelium; **CnT**: Connective Tissue; **EndP**: Endocrine Pancreas; **FundSt**: Fundic Stomach, **GlC**: Globlet Cell; **Int**: Intestine; **L**: Lumen; **NPl**: Nervous Plexus; **P**: Peritoneum; **PD**: Pancreatic duct; **PilSt**: Pyloric Stomach; **PilVal**: Pyloric Valve; **St**: Apical Striations; **V**: Folding. Bars units=μm.

3. Intestinal caeca, number at the left side of stomach: (0) none; (1) 1; (2) 2; (3) 3.

State 0 present in *Byconamericus thomasi*. State 1 present in *Aphyocharax anisitsi* and *Markiana nigripinnis*. State 2 present in *Astyanax endy*, *A. rutilus*, *Characidium borellii*, and *Cheirodon interruptus*. State 3 present in *Gymnocorymbus ternetzi*.

4. Intestinal caeca, total number: (0) 6; (1) 8. State 0 is present in *Aphyocharax anisitsi*, *Bryconamericus thomasi* and *Characidium borellii*. State 1is present in *Astyanax endy*, *A. rutilus*, *Cheirodon interruptus*, *Gymnocorymbus ternetzi* and *Markiana nigripinnis*.

Liver. 5. Left dorsal lobe of liver: (0) approximately reaching vertical through anterior margin of gas bladder posterior chamber; (1) surpassing vertical through anterior margin of gas bladder posterior chamber.

State 0 in *Astyanax endy*, *Bryconamericus thomasi*, and *Cheirodon interruptus*. State 1 in *Aphyocharax anisitsi*, *Astyanax rutilus*, *Characidium borellii*, *Gymnocorymbus ternetzi,* and *Markiana nigripinnis*.

6. Form of left dorsal lobe of liver: (0) ventrally curved, (1) approximately straight.

State 0 was observed in *Aphyocharax anisitsi* and *Characidium borellii* and state 1 in the remaining analyzed species.

7. Left ventral lobe of liver: (0) developed ventrally with respect to stomach; (1) submedially located.

State 0 was observed in *Astyanax endy*, *Bryconamericus thomasi*, *Characidium borellii*, and *Cheirodon interruptus*. State 1 in the remaining analyzed species: *Aphyocharax anisitsi*, *Astyanax rutilus*, *Gymnocorymbus ternetzi*, and *Markianna nigripinnis*.

8. Left ventral lobe of liver: (0) distal portion reaching curve 2 of the intestine; (1) less developed and distal portion not reaching curve 2 of the intestine.

State 0 in *Astyanax endy*, *Bryconamericus thomasi*, *Gymnocorymbus ternetzi*, and *Markiana nigripinnis*. State 1 in *Aphyocharax anisitsi*, *Astyanax rutilus*, *Characidium borellii*, and *Cheirodon interruptus*.

9. Ventral lobe of liver: (0) absent or reduced; (1) present.

State 0 in *Aphyocharax anisitsi*, *Astyanax endy*, *Cheirodon interruptus*, and *Markiana nigripinnis*. State1 in *Astyanax rutilus*, *Bryconamericus thomasi*, *Characidium borellii*, and *Gymnocorymbus ternetzi*.

10. Distal margin of right dorsal lobe of liver: (0) surpassing vertical through anterior margin of gas bladder posterior chamber; (1) not surpassing vertical through anterior margin of gas bladder posterior chamber.

State 0 in *Astyanax rutilus*, *Gymnocorymbus ternetzi*, and *Markiana nigripinnis*. State 1 in *Aphyocharax anisitsi*,

Astyanax endy, *Bryconamericus thomasi*, *Characidium borellii*, and *Cheirodon interruptus*.

Histology. 11. Taste buds in esophagus: (0) present; (1) absent.

State 0 in *Cheirodon interruptus*. Taste buds are absent or not distinguishable in the remaining analyzed species (state 1). In the anostomids *L. friderici* and *L. taeniofasciatus*, taste buds are also absent (Albrecht *et al*., 2001).

Characters optimization. Characters above defined were optimized in the phylogenetic hypothesis proposed by Mirande (2009, 2010) (Fig. 7) and the following transformations were observed:

Char. 1 (1 steps): Root: 1; *Gymnocorymbus ternetzi*: 1>0.

Char. 2 (4 steps): Root: [0,12]; *Characidium borellii*: [0,12]>2; Node 11: [0,12]>0,1; *Gymnocorymbus ternetzi*: [0,12]>1; *Astyanax endy*: [0,1]>0; Node 10: [0,1]>0; *Astyanax rutilus*: [0,1]>1; *Aphyocharax anisitsi*: 0>2.

Char. 3 (4 steps): Root: 2; *Gymnocorymbus ternetzi*: 2>3; Node 10: 2>12; Node 9: 12>1; *Cheirodon interruptus*: 12>2; *Aphyocharax anisitsi*: 12>1; *Bryconamericus thomasi*: 1>0.

Char. 4 (3 steps):Root: [0,1]; Node 13: 0,1>1; *Characidium borellii*: [0,1]>0, *Aphyocharax anisitsi*: 1>0, *Bryconamericus thomasi*: 1>0.

Char. 5 (3 steps): Root: 1;*Astyanaxendy*: 1>0, *Cheirodon interruptus*: 1>0; *Bryconamericus thomasi*: 1>0.

Char. 6 (3 steps): Root: 1, *Astyanax endy*: 1>0; *Cheirodon interruptus*: 1>0; *Bryconamericus thomasi*: 1>0.

Char. 7 (4 steps) Root: [0,1]; *Characidium borellii*: [0,1]>0; *Gymnocorymbus ternetzi*: [0,1]>1; *Astyanax endy*: [0,1]>0; *Astyanax rutilus*: [0,1]>1; *Cheirodon interruptus*: [0,1]>0; *Aphyocharax anisitsi*: [0,1]>1; *Bryconamericus thomasi*: [0,1]>0; *Markiana nigripinnis*: [0,1]>1.

Char. 8 (3 steps): Root: [0,1]; *Characidium borellii*: [0,1]>1; *Gymnocorymbus ternetzi*: [0,1]>0; *Astyanax endy*: [0,1]>0; *Astyanax rutilus*: [0,1]>1; Node 14: [0,1]>1; Node 9: [0,1]>0.

Char. 9 (3 steps): Root: 1; Node 12: 1>[0,1]; *Astyanax endy*: [0,1]>0; *Astyanax rutilus*: [0,1]>1; Node 14: [0,1]>0; *Bryconamericus thomasi*: [0,1]>1; *Markiana nigripinnis*: [0,1]>0.

Char. 10 (3 steps): Root: 1; *Gymnocorymbus ternetzi*: 1>0; *Astyanax rutilus*: 1>0; *Markiana nigripinnis*: 1>0.

Char. 11 (1 steps): Root: 0; *Cheirodon interruptus*: 0>1.

Autapomorphies:

Characidium borellii: No autapomorphies
Markiana nigripinnis: No autapomorphies
Astyanax rutilus: Char. 2: 0>1, Char. 8: 0>1.
Astyanax endy: Char. 5: 1>0; Char. 6: 1>0.
Gymnocorymbus ternetzi: Char. 1: 1>0; Char. 3: 2>3; Char. 7:0>1; Char. 10: 1>0 *Bryconamericus thomasi*: Char. 3: 12>0; Char. 4: 1>0; Char. 5: 1>0; Char. 6: 1>0.
Aphyocharax anisitsi: Char. 2: 0>2.
Cheirodon interruptus: Char. 11: 0>1.

Synapomorphies:

Node [*A. rutilus*, *M. nigripinnis*]: Char. 7: 0>1, Char. 10: 1>0.
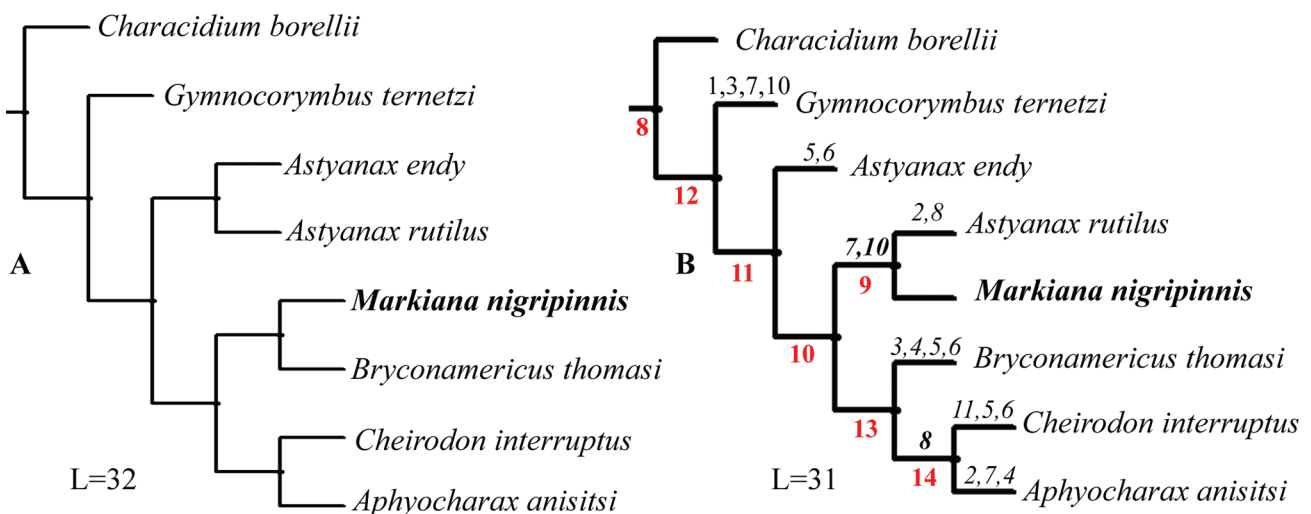Node [*C. interruptus*, *A. anisitsi*]: Char. 8: 0>1.

Fig. 7. Phylogenetic relationship of the selected species after Mirande (2009, 2010) final hypothesis of phylogenetic relationships. A) Considering *Markiana nigripinnis* as a member of Stevardiinae *sensu* Oliveira *et al*. (2011) and Baicere-Silva *et al*. (2011) and B) with *M. nigripinnis* as a member of *Astyanax* clade *sensu* Mirande. Synapomorphies and autapomorphies obtained from optimization of the characters proposed herein are presented in italics above branches as character number. Node numbers are presented in red below branches.

Discussion

This paper represents the first comparative full description of the anatomy of the alimentary tract of fishes of the Characidae discussed in a phylogenetic context and provides a new set of morphological characters that can be used in future phylogenetic analysis. Previous related works had focused on studying some anatomical aspects on particular species of the family, (*i.e.*: Leknes, 2005, 2011; Medina Perozo *et al.*, 2009; Gómez-Ramírez *et al.,* 2011). Herein we analyze general anatomical patterns with a taxon sampling that takes into account previous phylogenetic hypotheses for the family (Mirande, 2009, 2010; Oliveira *et al.*, 2011). Also, species with no evident specializations regarding feeding, for example, which could mask variations strictly correlated with phylogeny were selected. Then, taken together this provides a general framework for the study of this system in the characids and also allows us to qualitatively estimate the level of variability expected among groups and to evaluate its potential phylogenetic information by comparing it with previous phylogenetic hypotheses.

The alimentary tract of the selected species presented a relatively conserved general pattern, especially at the histological level, nevertheless considerable variations on the development of liver lobes, intestine coiling and number and disposition of intestinal *caeca* were observed.

At the histological level, intraspecific variation was only observed in the relative length of mucosa foldings of the alimentary tract that was also variable among the species analyzed herein, which seems to be more related to specimens size rather than to species identity, therefore not used as a character here. The only interspecific difference observed at the histological level was: taste buds in the esophagus were only present in *Cheirodon interruptus* among the selected species. According to Cazorla *et al.* (2003) and Escalante (1987), this species has predatory habits, feeding mainly on Chironomidae and Trichoptera larvae. This feature together with the presence of striated muscle in the esophagus and first stomach portion may allow this species to eliminate preys that are being swallowed and are not palatable, what could happen in case of predation on a "wrong" prey, for example in case of prey confusion. Predation is supposed to be guided by several stimuli and a "specific searching image" the predator has (Tinbergen, 1960; also reviewed in Bond, 2007). Interestingly striated muscle in this portion is also present in the other species herein examined which is probably related to elimination of large size ingested items that cannot pass through the alimentary tract. Additionally, *C. interruptus* would present a second control related to taste buds for avoiding ingestion of unpalatable preys.

Pyloric *caeca* of the studied species presented foldings mainly in the longitudinal direction, in contrast with those in the rest of the intestine, that are mainly transverse. The disposition of the pyloric foldings probably facilitates its filling and emptying in a bidirectional way. Its main function is supposed to be absorptive, in contrast of those of mammals for example, which are supposed to have a fermentative function instead. These are filled and emptied together with the first portion of the intestine, increasing the whole absorptive surface of it. In trout and cod they account for more uptake capacity than all remaining regions of the gut combined (Buddington & Diamond, 1986). This absorptive function was corroborated here histologically by the presence of a striated border present in its epithelium related to the presence of microvilli in these cells. The number of intestinal caeca and intestine length is expected to be increased in herbivorous species, intermediate in omnivorous ones, and minimized in carnivorous fish but this is not always the rule. For example, Pogoreutz & Ahnelt (2014) studied four sympatric species of Gobiidae from Indonesia and found that one herbivorous species presented a short gut, there attributed to a phylogenetic inertia due to a carnivorous plesiomorphic state for that species. In the present work, *A. anisitsi* and *C. borellii* presented a reduced intestine, qualitatively observed (Figs.1-2), and low number of intestinal caeca. Concordantly, *A. anisitsi* in the Pantanal feeds mainly on aquatic insects during the rainy season and terrestrial insects in the dry season (Corrêa *et al.*, 2009) while in the upper Paraná River floodplain in Brazil, this species fed mainly on microcrustaceans (Cladocera and Copepoda), in addition to Decapoda larvae, insect larvae and detritus (Russo & Hahn, 2006). Although there is no information on *C. borellii* diet, other *Characidium* species show a carnivorous diet: *C. rachovii* feed on Diptera (aquatic stage) and Amphipoda (Bastos *et al.*, 2013), and *C. lanei* and *C. pterostictum* feed mainly on insects larvae and arthropods (Aranha *et al.*, 2000). *Cheirodon interruptus* also had a carnivorous diet (Cazorla *et al.*, 2003; Escalante, 1987) but a "medium" sized intestine and number of caeca (Figs. 1-2; Table 1). In the other hand, two omnivorous Anostomidae species of the genus *Leporinus*, with omnivorous diet, presented 10 and 12 intestinal caeca respectively and a relatively developed intestine (Albrecht *et al.*, 2001). *Paracheirodon axelrodi*, a carnivorous miniature species presented no intestinal caeca (Gómez-Ramírez *et al.,* 2011) what may be related both to its diet and miniature reductive characteristics. *Markiana nigripinnis* basically ingested food from vegetable origin, consisting on roots, fruit, seeds, and fibers (Resende *et al.*, 1998) and presents a moderate development of intestinal length and caeca. *Astyanax rutilus* has an omnivorous diet also, although the main items ingested by this species were terrestrial insects and micro-crustaceans and the development of intestinal caeca and intestine was also moderate. This seems to be also the case of *B. thomasi* and *A. endy*, both species with no data on their feeding habits but examination of the guts during dissections and observation of histological sections of these species indicate that they may be omnivorous,

eating insects and also algae (observed in histological preparations of *B. thomasi*). Also it is relevant to note that those species with omnivorous diets herein mentioned seems to have a tendency to a greater development of their liver and pancreas. This means that, in general terms, herbivorous species have a greater portion of its body devoted to feeding and in consequence it may be expected from this, that miniature species are more likely to evolve in "carnivorous clades", or less likely to occur in herbivorous ones, although herbivorous miniatures do occur as in the case of *Otothyris* (Loricariidae) (Rosa *et al.*, 2014), for example.

The alimentary tract of the selected species presented a distinctively general pattern. Many of the characters of gross anatomy proposed herein appear as autapomorphies of some of the selected species. In fact, a search using implicit enumeration with the selected characters resulted in 4 most parsimonious trees with L=22 (results not shown). This means an increase on tree length in about 40% when optimized in Mirande (2010) topology (L=31), reflecting the great level of homoplasy of most of these characters and the relatively scarce phylogenetic information they present at this level. Nevertheless, these types of characters could have more relevant phylogenetic information for resolving relationships of closely related species, and therefore should not be discarded. In the other hand, histology of these species seems to be greatly conserved and therefore its use as a source of characters for this family is not recommended, especially considering its laborious acquisition. In contrast, histology may be a very interesting source of characters for resolving relationships between distant phylogenetic groups.

The tree length calculated for the optimization of the present characters in the topology proposed by Mirande (2009, 2010), in which *Markiana nigripinnis* is part of the "*Astyanax* clade", was L=31 (Fig. 7). The optimization when this species is presented as a basal member of the Stevardiinae presented a tree length decreased on one step (L=32, under equal weights). This is because a synapomorphy of the clade composed by *M. nigripinnis* and *A. rutilus* is lost: distal margin of right dorsal lobe of liver not surpassing vertical through anterior margin of gas bladder posterior chamber (Char. 10.1). Also, an ambiguous reconstruction of Char. 7 it is observed (left ventral lobe submedially located (state 1) present in *M. nigripinnis* and paralleled in *A. anisitsi*, *A. rutilus* and *G. ternetzi*). The information presented here therefore is supporting a closer relationship of *M. nigripinnis* with "*Astyanax* clade" than with Stevardiinae, although only supported by one synapomorphy. Considering also the very few species analyzed herein and the need of the evaluation of the general congruence of this information altogether with information of other sources (osteological, molecular, etc.) in a phylogenetic framework, we consider that the present results are not conclusive with respect to the phylogenetic position of *Markiana*.

The node containing *Cheirodon interruptus* and *Aphyocharax anisitsi* presented as a synapomorphy character 8 (0>1), left ventral lobe less developed and distal portion not reaching curve 2 of the intestine, paralleled in *A. rutilus*. This group is congruent with the node 195 of Mirande (2009, 2010) including the subfamilies Aphyocharacinae, Aphyoditeinae, and Cheirodontinae.

The state 2 of character 2 (Curve 4 of alimentary tract reaching the posterior most region of stomach or not reaching the stomach), present in *A. anisitsi* and *C. borelli* may be a restriction due to the body shape of these species with an elongated cylindrical body and carnivorous feeding habits. The great differences in *Gymnocorymbus ternetzi* gastrointestinal tract morphology with respect to the other studied species is probably related to restrictions due to its particular body shape, laterally compressed and with a great relative body height. Then, these cases may be indicating that general body shape is probably restricting the alimentary tract development at a certain level at least. However, it can be also said that both the anatomy of alimentary tract as the general form of body are, at least at some point, influenced by phylogeny and those characters should not be discarded from phylogenetic analyses.

Characters 4, 5 and 6, present autapomorphies for 2, 3 and 3 species respectively, which may represent synapomorphies in future analyses including closest species of these taxa. Also character 9 presented and ambiguous reconstruction of the characters states in the internal nodes which may be clarified in future phylogenetic analyses including more taxa.

Finally, we highlight the needing of studies integrating data from ecology, compared anatomy, behavior, and physiology in phylogenetic contexts for a better understanding of these features and their interactions in an evolutionary frame. Therefore, we consider that this work could be the basis for further investigations on the relationships between the anatomy of the alimentary system, its physiology, the diet and ecology of these species and their feeding behaviors, in a phylogenetic framework.

Material Examined. *Aphyocharax anisitsi*: **Argentina:** MACN-ict 10912, 2 males and 2 females, 31-51 mm SL; La Leonesa, Chaco, kept in aquarium for one year; Aug 2011; I. García, P. Calviño & F. Alonso. *Astyanax endy*: **Argentina:** MACN-ict 10913: 3 males and 2 females, 43-52 mm SL; arroyo "el Oculto", Oran, Salta, río Bermejo drainage; 20 Aug 2012; J. M. Mirande, G. Terán & F. Alonso. *Astyanax rutilus*: **Argentina:** MACN-ict 10914, 3 males and 3 females, 38-54 mm SL; río Gualeguay, Entre Ríos, río Paraná drainage; 28 Mar 2012; P. Calviño, R. Petracini & F. Alonso. *Bryconamericus thomasi*: **Argentina:** MACN-ict 10915, 2 males and 2 females, 49-65 mm SL; arroyo Gallinato, Salta, río Bermejo drainage; 21 Aug, 2012; J. M. Mirande, G. Terán & F. Alonso. *Characidium borellii*: **Argentina:** MACN-ict 10916, 1 male, 54 mm SL; río Mandolo, río Salí-Dulce drainage, Tucumán,

Nov 2003; J. M. Mirande & G. Terán. *Cheirodon interruptus*: **Argentina:** MACN-ict 10917, 2 males and 2 females, 37-45 mm SL, arroyo San Marcos, Ceibas, Entre Ríos, río Paraná drainage; Nov 2012; I. García, P. Calviño & F. Alonso. *Gymnocorymbus ternetzi*: **Argentina:** MACN-ict 10918, 2 males and 2 females, 41-41 mm SL, La Leonesa, Chaco, kept in aquarium for one year; Aug 2011; I. García, P. Calviño & F. Alonso. *Markiana nigripinnis*: **Argentina:** MACN-ict 10919, 1 male, 88 mm SL, La Leonesa, Chaco, kept in aquarium for one year; Aug 2011; I. García, P. Calviño & F. Alonso.

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