

ARTICLE

Two new species of *Microcotyle* (Monogenea: Microcotylidae) on intertidal fish from the south Pacific coast

Dos nuevas especies de *Microcotyle* (Monogenea: Microcotylidae) en peces intermareales de la costa del Pacífico sur

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Resumen.- El género *Microcotyle* es uno de los más diversos y controvertidos dentro de la familia Microcotylidae. A la fecha se han descrito 131 especies; sin embargo, más de la mitad han sido transferidos a otros géneros y varios otros tienen descripciones deficientes, por lo tanto menos de la mitad de las especies descritas podrían considerarse válidas. En Chile, se han reconocido dos especies de *Microcotyle* y existen registros de especímenes no identificados en varios peces costeros, pero que aún no han sido debidamente identificados. En este estudio se describe taxonómicamente, en base a datos morfológicos y moleculares (genes ITS2 y 18S), dos especies de *Microcotyle* encontrados en peces intermareales de la zona central (33°S) y centro-sur (36°S) de Chile: *Microcotyle sprostonae* n. sp. (principalmente de *Scartichthys viridis* en el norte y centro de Chile) y *M. chilensis* n. sp. (principalmente en *Calliclinus geniguttatus* de la zona centro-sur de Chile). Ambas especies de monogéneos se diferenciaron entre sí y de otras especies, principalmente en el número de testículos y clamps. Las dos nuevas especies también difieren entre sí, y con otras especies de *Microcotyle* secuenciadas hasta el momento, desde un par a varios pares de bases en los genes ITS2 y 18S. Los peces intermareales son mayoritariamente endémicos de la costa del Pacífico sudamericano, y tienen una distribución geográfica limitada que no se superpone con los hospedadores tipo de otras especies de *Microcotyle*. Por lo tanto, las especies aquí descritas se distinguen de sus congéneres por su morfología, genética y biología.

Palabras clave: Taxonomía, Monogenea, *Microcotyle*, peces intermareales, Océano Pacífico Sur, Chile

Abstract.- *Microcotyle* is one of the most diverse and controversial genera within the family Microcotylidae. To date, 131 species have been described in *Microcotyle*; however, more than half have been transferred to other genera, and several others have poor descriptions. Therefore, less than half of all *Microcotyle* species may be considered valid. In Chile, two species have been recognized, and unidentified *Microcotyle* have been found on several littoral fish, but there has been no effort to properly identify them. In this study, two new species of *Microcotyle* are taxonomically described from intertidal fish of the central (33°S) and south-central (36°S) regions of Chile. In this study, *Microcotyle sprostonae* n. sp. (collected mainly from *Scartichthys viridis* in central Chile) and *M. chilensis* n. sp. (collected mainly from *Calliclinus geniguttatus* in south-central Chile) were identified based on morphological and molecular analyses (ITS2 and 18S genes). Both species of *Microcotyle* principally differed from one another and from other valid species in the number of testes and clamps. The two new species also differed from one another by one base pair in the ITS2 and 18S genes and differed from other species of *Microcotyle* by several base pairs of both genes. Intertidal fish are mostly endemic to the Pacific coast of South America, and they have a limited geographical distribution that does not overlap with the type hosts of other *Microcotyle* species. Therefore, the two new species described here are distinguished from other congeneric species by morphological, genetic, and biological characteristics.

Key words: Taxonomy, Monogenea, *Microcotyle*, intertidal fish, South Pacific Ocean, Chile

INTRODUCTION

Microcotylidae Taschenberg, 1879 is one of the most controversial families within Monogenoidea Bychowsky, 1937, in which several genera and subgenera have been erected (e.g., Unnithan 1971, Caballero & Bravo-Hollis 1972). Of the 58 genera described within Microcotylidae (Bray 2001), *Microcotyle* van Beneden and Hesse, 1863 is the most diverse genus, comprising 131 species that have been described from 1863 (Van Beneden & Hesse 1863) to

2019 (Bouguerche *et al.* 2019a, b). The genus *Microcotyle* is mainly characterized by a conspicuous genital atrium with well-developed radial muscles, armed with numerous small, conical spines, and by a female reproductive system composed of a long, convoluted ovary, with an unarmed, long, single vaginal duct (see Mamaev 1989 for the emended diagnosis of the genus). Possibly 64 species of all those described correspond to *Microcotyle* (Bray 2001); however, several still require exhaustive revisions.

In recent years, morphological descriptions have been complemented with genetic analysis, with both techniques allowing researchers to clarify species statuses (Verma *et al.* 2018) and verify the proposal of new species. Indeed, in the last decade molecular markers have been used in the identification of *Microcotyle* species (*e.g.*, Hayward *et al.* 2007, Ayadi *et al.* 2017, Bouguerche *et al.* 2019a, b), although there are few species sequenced till now.

In Chile, two species of *Microcotyle* have been recorded in several fish species from different families (Muñoz & Olmos 2007). These include *M. nemadactylus* Dillon and Hargis, 1965, from the fish *Cheilodactylus variegatus* Cuvier and Valenciennes, 1833 (Cheilodactylidae). *Microcotyle moyanoi* (Villalba and Fernández, 1986) (*syn. Paramicrocotyle moyanoi*) had been recorded on the fish *Pinguipes chilensis* (Molina, 1782) (Pinguipidae), *Bovichthys chilensis* Regan, 1913 (Bovichthidae), and *Scartichthys gigas* (Steindachner, 1876) (Blenniidae). Furthermore, unidentified species of *Microcotyle* have been recorded on subtidal fish species, such as *Helicolenus lengerichi* Norman, 1937, *Sebastes oculatus* Valenciennes, 1833 (Balboa & George-Nascimento 1998, Gonzalez & Moreno 2005), and on intertidal fish, such as *Scartichthys viridis* (Valenciennes, 1836), *Auchenionchus crinitus* (Jenyns, 1842), and *A. variolosus* (Valenciennes, 1836) (Díaz & George-Nascimento 2002, Muñoz *et al.* 2002, Flores & George-Nascimento 2009, Muñoz & Delorme 2011, Muñoz & Randhawa 2011, Muñoz & Castro 2012).

Microcotylids found on different intertidal fish species, from the north (23°S) to the south-central (36°S) regions of Chile, have been identified as *Microcotyle* sp. (Muñoz-Muga & Muñoz 2010) or as *M. moyanoi* (Díaz & George-Nascimento 2002, Muñoz *et al.* 2002, Flores & George-Nascimento 2009), which indicates there are doubts as to the taxonomical identification of these parasites. Since the first time *Microcotyle* was recorded as parasitizing an intertidal fish (on the blenny *S. viridis*) (Díaz & George-Nascimento 2002), no researchers have examined the taxonomy of these monogeneans. Therefore, the objective of this study is to describe two species of *Microcotyle* parasitizing intertidal fish based on morphological and molecular analyses.

MATERIALS AND METHODS

COLLECTION OF FISH AND MONOGENEANS

Between 2014 and 2015, eight common fish species were collected from several rocky pools in different localities in Chile. There were 2,473 specimens obtained from central region (Valparaíso, between 33°26'S-71°41'W and 33°30'S-71°37'W) and 1,076 from the central-south region (Biobío, between 36°28'S-72°55'W and 36°41'S-73°08'W) of Chile (Table 1). Of these, it focused on the blenny *Scartichthys viridis* and the labrisomid *Calliclinus geniguttatus*, because they have the highest abundance and prevalence of microcotylid monogeneans.

Table 1. Species, sample sizes, and body lengths (BL) of fish collected from two zones of Chile. Prevalence (PRE, %), abundance (ABU), and intensity range (INT) of microcotylids is shown. / Especies, tamaños muestrales y longitudes corporales (BL) de los peces recolectados desde dos zonas de Chile. Se muestra la prevalencia (PRE, %), abundancia (ABU), y rango de intensidad (INT) de microcotilidos

	<i>M. sprostonae</i> n. sp. Central (33°S)					<i>M. chilensis</i> n. sp. Central-South (36°)				
	N	BL (cm)	PRE	ABU	INT	N	BL (cm)	PRE	ABU	INT
BLENNIIDAE										
<i>H. sordidus</i> *	189	4.4 ± 1.4	0.5	0.01	1	156	7.0 ± 1.5	0.6	0.01	1
<i>S. viridis</i> *	1643	7.7 ± 3.5	19.2	0.68	1 - 30	220	10.5 ± 5.0	0	0	
BOVICHTHYDAE										
<i>B. chilensis</i> *	128	6.9 ± 2.4	0	0		188	7.2 ± 2.5	1.6	0.31	1 - 55
CLINIDAE										
<i>M. cristatus</i>	6	4.6 ± 2.7	0	0		122	9.2 ± 9.8	0.8	0.01	1
LABRISOMIDAE										
<i>A. crinitus</i>	106	7.1 ± 3.7	1.9	0.02	1					
<i>A. microcirrhis</i>	233	8.1 ± 3.9	0.4	<0.01	1	3	13.3 ± 5.0	0	0	
<i>A. variolosus</i> *	59	10.9 ± 4.4	3.4	0.07	1 - 3	6	13.6 ± 5.1	16.7	0.17	1
<i>C. geniguttatus</i> *	109	6.9 ± 2.2	1.8	0.02	1	381	7.6 ± 2.3	16.5	0.64	1 - 3

**Microcotyle* specimens of fish used for molecular analyses

Fish were collected with hand nets during low tide. Of the fish obtained, some were dissected immediately to collect monogeneans for morphological analysis. Each fish was euthanized with an overdose of an anesthetic solution before dissection, according to the bioethics protocols of the Universidad de Valparaíso and Universidad Católica de la SSMA Concepción. The gills were removed from the fish and observed under a light microscope. The monogeneans were collected and fixed in 5% formalin for staining procedure or 96% ethanol for molecular analysis.

Monogeneans were stained with hematoxylin, dehydrated in a graded ethanol series (from 70% to 100%), cleared in methyl salicylate, and mounted in Entellan. The specimens stained and mounted which were sufficiently clear to observe internal morphology were selected for the taxonomical description. The monogeneans were measured with an eyepiece micrometer. For the descriptions, the mean \pm the standard deviation, followed by the range of measurements in parentheses, were recorded and expressed in micrometers

(μm). Drawings were made with a camera lucida attached to a light microscope (Leica® DM LS2). The prevalence and mean intensity of the parasites was calculated according to Bush *et al.* (1997).

MOLECULAR ANALYSES

Total genomic DNA from single specimens of *Microcotyle* from five fish species of the intertidal zone of central and central-south of Chile (Table 2), was extracted using established salt extraction procedures (Aljanabi & Martinez 1997). A few microcotylid specimens of *S. viridis* from northern Chile (23°S) were also considered for molecular analyses. Amplification of the ITS2 nuclear ribosomal DNA region was performed with the 3S forward primer (5'-GGT ACC GGT GGA TCA CGT GGC TAG TG-3') (Bowles *et al.* 1993) and the ITS2.2 reverse primer (5'-CCT GGT TAG TTT CTT TTC CTCCG C-3') (Anderson & Barker 1993). The 18S rDNA fragments were amplified with the 18SF forward primer (5'-AAG GTG TGM CCT ATC AAC

Table 2. Species of the Microcotylidae used in molecular analyses in the present study /
Especies de Microcotylidae usadas en los análisis moleculares en el presente estudio

Parasite species	Fish species	Locality	18S GenBank #	ITS2 GenBank #	Source
<i>M. sprostonae</i> n. sp.	<i>Scartichthys viridis</i>	Valparaíso, Chile	MN429297	MN429299, MN429308, MN429309, MN429310	This study
		Antofagasta, Chile			
	<i>Hypsobleinnius sordidus</i>	Valparaíso, Chile	MN429304, MN429305, MN429306	MN429302	This study
<i>M. chilensis</i> n. sp.	<i>Calliclinus geniguttatus</i>	Bio-Bio, Chile	MN429295, MN429296	MN429303, MN429312, MN429313	This study
	<i>Auchenionchus variolosus</i>	Biobío, Chile	MN429294	MN429311	This study
	<i>Bovichthys chilensis</i>	Biobío, Chile	MN429293	MN429301, MN429314	This study
	<i>Hypsobleinnius sordidus</i>	Biobío, Chile	MN429292	MN429300	This study
<i>Microcotyle</i> sp.	<i>Sebastes oculatus</i>	Valparaíso, Chile	MN429298	MN429307	This study
<i>M. sebastis</i>	<i>Sebastes</i> sp.		AJ287540		Littlewood & Olson (2001)
<i>M. erythrinii</i>	<i>Pagellus erythrinus</i>	France	AM157195		Badets <i>et al.</i> (2011)
Microcotylidae gen sp.	<i>Argyrops spinifer</i>	Oman Sea	JN602094		Yoon <i>et al.</i> 2013
<i>Polylabris</i> sp.	<i>Siganus fuscescens</i>	China	HM545905		Wang <i>et al.</i> (unpublished)*
<i>Cynoscionicola branquialis</i>			AJ287495		Littlewood & Olson (2001)
<i>Bivagina pagrosomi</i>	<i>Chrysophrys auratus</i>		AJ228775		Littlewood & Olson (2001)
<i>Neomicrocotyle pacifica</i>	<i>Caranx hippos</i>		AJ228787		Littlewood & Olson (2001)
<i>Microcotyle bassensis</i>	<i>Platycephalus</i> sp.	Port Lincoln, Australia		EF452636	Hayward <i>et al.</i> (2007)
<i>Microcotyle pomatomi</i>	<i>Pomatomus</i> sp. **	Moreton Bay, Australia		EF452637	Hayward <i>et al.</i> (2007)
<i>Anchoromicotyle gaymensis</i>	<i>Atractoscion nobilis</i>	California, USA		EF452635	Hayward <i>et al.</i> (2007)
<i>Diplostamenides sciaenae</i>	<i>Pemahia argentata</i>	Kagawa Pref, Japan		EF452639	Hayward <i>et al.</i> (2007)
<i>Sciaenacotyle sciaenicola</i>	<i>Argyrosomus japonicus</i>	Port Lincoln, Australia		EF452634	Hayward <i>et al.</i> (2007)
<i>Zeuxapta seriola</i> (Outgroup)	<i>Seriola hippos</i>	Harbor, Australia	AJ228797		Littlewood & Olson (2001)
	<i>Seriola lalandi</i>	Adelaide, Australia		EF452638	Hayward <i>et al.</i> (2007)

* References got from GenBank, Wang JY, Lu YS, Wu ZH & Jian JC. Studies on biodiversity and population ecology of the parasites from *Siganus fuscescens* in Zhanjiang sea area (Unpublished data).

**The fish species was indicated as *Pomatomus pomatomi*, probably a mistake, because this is not recorded in public data base (www.fishbase.org and www.marinespecies.org)

T-3') and the 18SR reversed primer (5'-TTA CTT CCT CTA AAC GCT C-3'). The ITS2 and 18S regions in the sequences were determined using the Internal Transcribed Spacer 2 Ribosomal RNA Database website (from NCBI GenBank)¹.

PCR (Polymerase Chain Reaction) amplification for 18S rDNA was performed using 100- μ l mixtures containing 200 ng of genomic DNA, 0.2 μ M of each of the two primers, 50 μ M of each of the dNTPs, 1 \times PCR buffer (with 2 mM MgCl₂), and 2.5 U of ExTaq DNA polymerase (Takara). Thermocycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min. A final extension was performed at 72 °C for 5 min.

PCR reactions for ITS2 rDNA were performed using the same reaction mixture as described above and the following thermocycling program: initial denaturation at 95 °C for 10 min, followed by a touchdown of 10 cycles of 95 °C for 15 s, 60-50 °C for 30 s, and 72 °C for 45 s. This was followed by the second stage of 35 cycles of 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 45 s. A final extension of 72 °C for 30 min was performed (Peña *et al.* 2014). PCR products were visualized on 0.8% agarose gels using a 1 \times sodium borate (SB) buffer solution.

The final PCR products for the 18S and ITS2 rDNA genes were purified and sequenced using the service of Macrogen, South Korea. Sequencher™ version 4.5 (GeneCodes Corporation) was used to analyze the sequences. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). All positions with less than 95% site coverage were eliminated. More than 5% alignment gaps, missing data, or ambiguous bases were not allowed at any position. For the 18S rDNA gene, the analysis involved 22 nucleotide sequences and a total of 1,493 positions in the final dataset. For the ITS2 gene, the analysis involved 22 nucleotide sequences and a total of 371 positions in the final dataset.

Genetic sequences of *Microcotyle* species of this study were contrasted to other microcotylid species; *M. sebastis* and *M. erythrinii* were used for the 18S gene, and *M. bassensis* and *M. pomatomi* were used for the ITS2 gene, including other species of Microcotylidae listed in Table 2. Evolutionary analyses were conducted using MEGA6 (Tamura *et al.* 2013). The genetic distances were computed with the total number of mutations, and the divergences were calculated among individuals by applying three algorithms: NJ (neighbor-joining), ML (maximum likelihood), and MP (maximum parsimony) (Tateno *et al.* 1994).

RESULTS

SPECIES DESCRIPTIONS

Family Microcotylidae Taschenberg, 1879

Genus *Microcotyle* van Beneden and Hesse, 1863

Microcotyle sprostonae n. sp. (Fig. 1 A-I)

Description (based on 20 mature and stained specimens): Body elongated, fusiform and flattened dorsoventrally, 3917 \pm 522 (3062-5225) long and 883 \pm 156 (687-1250) wide at the ovary level. Thin and smooth tegument. A pair of anterior, oval suckers, 75 \pm 9 (52-87) long and 65 \pm 7 (55-85) wide, with a thin septum located at the middle of each sucker. One row of minute papillae on the anterior portion of each sucker. Oral suckers from 43 \pm 13 (25-69) from the anterior edge. Mouth subterminal, immediately anterior to the pharynx. Pharynx oval to spherical 58 \pm 10 (44-75) long and 51 \pm 6 (37-62) wide. Brain almost circular, at the middle of esophagus level. Two lateral nervous cords. Esophagus 292 \pm 64 (137-387) long and 19 \pm 6 (12-25) wide, with a pair of short lateral diverticula. Gut bifurcates post atrium, at 434 \pm 61 (350-600) from the anterior edge. Caeca laterally ramified, one caecum larger than the other. Last part of caeca without ramification, extend up to the mid-level of the haptor. The gut epithelium, from esophagus up to the end of caeca with pigmented cells.

Genital atrium cavity from oval to as a triangle shape with rounded corners, 101 \pm 43 (60-250) long and 135 \pm 23 (106-181) wide, with anterior wide and rounded border, covered by little spines. Spines also on the peripheral atrium cavity and inside the cavity, which are difficult to count, approximately 200 spines of similar size, 11 \pm 2 (8-15) long. Posterior to the atrial cavity, a concave zone in which the cirrus evert, surround by muscular fibers. Two groups of spines located posterior-laterally to cirrus cavity, with 7 \pm 1 (4-9) spines each group. Atrial cavity surrounded by radial muscles, making a conspicuous atrial zone, 179 \pm 47 (125-262) long, including the cirrus cavity, and 185 \pm 74 (215-475) wide, considering the radial muscles.

Ovary pre-testicular, intercaecal, glomerular and long. The portion with immature cells coiled. The portion of the ovary with mature cells as an inverted U, located at 1,519 \pm 198 (1,200-2,050) from the anterior end of the body. Ovary maximum wide 109 \pm 30 (75-175). Oviduct a long folded tube, connected to an oval seminal receptacle and posteriorly to the vitelline duct. Oviduct turns upwards, followed by the ootype. Ootype almost oval shape, 330 \pm 28 (310-350) long. Mehlis' cells different sizes, short at the beginning and at the end of the ootype (11-18 μ m), and large cells at the middle of the ootype (24-30 μ m). Uterus

¹<<https://www.ncbi.nlm.nih.gov/genbank>>

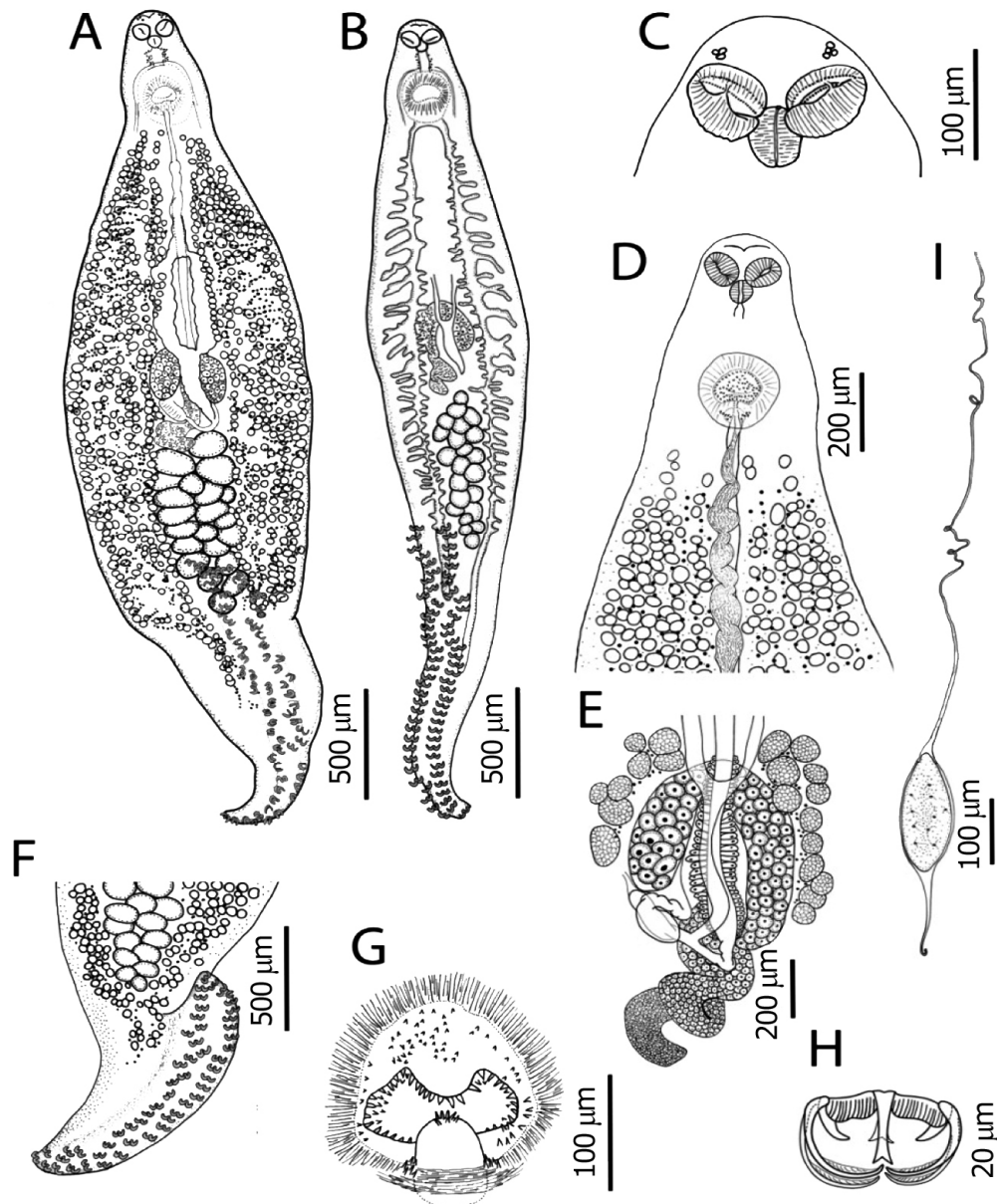


Figure 1. Drawings of *Microcotyle sprostonae* n. sp. A) Ventral view of whole body, B) Ventral view of whole body showing the digestive tract (vitelline glands not drawn), C) oral suckers and pharynx, D) anterior portion of the body, E) female reproductive organs, F) haptor, G) genital atrium, H) clamp, I) egg / Dibujos de *Microcotyle sprostonae* n. sp. A) Vista ventral del cuerpo completo, B) Vista ventral de cuerpo completo que muestra el tracto digestivo (glándulas vitelinas no dibujadas), C) ventosas orales y faringe; D) parte anterior del cuerpo, E) órganos reproductores femeninos, F) haptor, G) atrio genital, H) clamp, I) huevo

tubular, intercaecal, extending up to the atrial cavity. Vitelline glands glomerulated, located laterally in both sides of the body, extending from gut bifurcation up to the haptor mid-level. Two vitelline ducts, intercaecal, at the middle of the body. Vitelline ducts replete with reserves in some specimens. Vitelline ducts united posteriorly and anteriorly; posterior union in short conduct which joined to oviduct; anterior union connect with the vitello-vaginal duct. Female genital pore dorsal, at 717 ± 45 (675-787) from the anterior edge. A short muscular vagina that gradually connects with a short vitelline duct. Female genital pore located at 490 ± 91 (425-595) from the vitelline duct bifurcation, and 308 ± 96 (240-475) from the posterior border of the atrium cavity.

Nine specimens with eggs. One mature egg per monogenean. In two specimens, another egg in development. Egg fusiform, 213 ± 22 (175-237) long and 76 ± 13 (56-95) wide, with polar filaments, one short 79 ± 29 (75-80) directed to posterior side and the other much longer than 10 times the egg length, directed to the genital opening.

Testes intercaecal, post-ovary, from oval to irregular shape, 101 ± 28 (50-150) long and 114 ± 34 (70-200) wide. Number of testes 25 ± 3 (21-31). Seminal vesicle long, located between the ovary and ootype; continuing ventrally along with the uterus. Cirrus oval, 72 ± 25 (54-90) long, 47 ± 7 (41-55), with 7-8 spines on the top, observed in two specimens.

Haptor symmetrical, 1232 ± 204 (975-1750) long, 207 ± 38 (150-312) wide, with two lateral rows, each sidewith 29-40 clamps, microcotyle-type, with a total of 68 ± 8 (58-83) clamps. Clamps oval, the most anterior slightly smaller than others. Clamps size, at the middle of haptor, 76 ± 11 (69-119) long and 45 ± 8 (34-56) wide.

TAXONOMY SUMMARY

Syns: *Paramicrocotyle moyanoi sensu* Díaz & George-Nascimento (2002), Flores & George-Nascimento (2009); *Microcotyle* sp. *sensu* Muñoz-Muga & Muñoz (2010), Díaz & Muñoz (2010), Muñoz & Delorme (2011), Muñoz & Randhawa (2011), Muñoz & Castro (2012).

Type host: *Scartichthys viridis* (Valenciennes, 1836) (Pisces: Blenniidae).

Other hosts: *Hypsoblennius sordidus* (Bennet, 1928) (Pisces: Blenniidae)

Site of infection: Gills.

Type locality: Las Cruces ($33^{\circ} 29' S$, $71^{\circ} 38' W$), Chile.

Other locality: Antofagasta ($23^{\circ} 27' S$, $70^{\circ} 36' W$); Isla Negra ($33^{\circ} 25' S$, $71^{\circ} 41' W$), El Tabo ($33^{\circ} 27' S$, $71^{\circ} 40' W$), Maitencillo ($33^{\circ} 16' S$, $71^{\circ} 40' W$), Tunquén ($33^{\circ} 37' S$, $71^{\circ} 27' W$) and Quintero ($33^{\circ} 45' S$, $71^{\circ} 30' W$), Chile.

Prevalence and intensity: 13.3% prevalence and an intensity range of 1-3 on 30 *S. viridis* at the type locality; 100% prevalence and range intensity of 2-4 on three *S. viridis* at Antofagasta; infections in other hosts are indicated in Table 1.

Deposition of types of specimens: MNHNCL PLAT-15016 (Holotype); MNHNCL PLAT-15017 (Paratype).

Etymology: "*sprostonae*" is dedicated to Nora Georgina Sproston, who made important contributions to the taxonomy of monogeneans.

REMARKS

M. sprostonae n. sp. was compared to all other species based upon two features: the number of testes and number of clamps. Species of the *Microcotyle* that have 20-30 testes and 60-90 clamps were chosen, similar to specimens of *M. sprostonae* n. sp. Thus, the two species examined that resembled *M. sprostonae* n. sp. were *M. ditrematis* Yamaguti, 1940 and *M. emmelichthyops* Yamaguti, 1968.

M. sprostonae n. sp. differs from *M. ditrematis* in pharynx size ($44-75$ long \times $37-62$ wide vs. $30-32$ long \times $33-35$ wide), oral suckers ($52-87$ long \times $55-85$ wide vs. $33-42$ long \times $39-42$ wide), and genital atrium width ($106-181$ vs. 90 maximum width, without surrounding muscles, see Yamaguti 1940).

M. sprostonae n. sp. differs from *M. emmelichthyops* with regard to clamps, displaying a significant difference in the maximum number ($58-83$ vs. $62-115$). Additionally, the genital atrium of *M. emmelichthyops* has "an oval bulb of lamellar muscle fibers," and the clamp structure contains a styliform median piece enclosed in a capsule-like structure at the base, specifically in the median bifid sclerite (Yamaguti 1968), which is not present in *M. sprostonae* n. sp.

M. sprostonae n. sp. differs from the other two *Microcotyle* species previously recorded in Chile, *M. nemadactylus* and *M. moyanoi*. *M. sprostonae* n. sp. shows a smaller number of clamps ($53-83$ vs. $90-104$) and shorter clamp length ($34-56$ vs. $63-77$) than *M. nemadactylus* (Dillon & Hargis 1965), whereas *M. sprostonae* n. sp. shows smaller numbers of clamps and testes than *M. moyanoi* (clamps: $52-83$ vs. $118-150$; testes: $21-31$ vs. $33-44$) (Villalba & Fernández 1986).

The distribution of microcotylids and hosts also supports *M. sprostonae* n. sp. as a new species. This monogenean was recorded in northern and central Chile on six fish species, but with a notorious preference for *S. viridis* (Table 1). The fish hosts of *M. sprostonae* n. sp. are mostly endemic to the Pacific coast of South America, whereas *M. ditrematis* has been recorded on *Ditrema temminki* at the Japanese coast (Yamaguti 1940), and *M. emmelichthyops* has been found on *Emmelichthys* sp. in Hawaii (Yamaguti 1968).

Microcotyle chilensis n. sp. (Fig. 2 A-F)

Description (based on 14 stained specimens): Body elongated, fusiform and flattened dorsoventrally, $3,169 \pm 674$ (2,187-4,062) long, 705 ± 182 (475-1,125) wide at the level of the ovary. Thin and smooth tegument. A pair of oral, rounded suckers, 55 ± 6 (45-65) long, 52 ± 8 (35-63) wide, with a thin septum located at the middle of each sucker. One row of minute papillae on the anterior portion of the sucker. Oral suckers 46 ± 14 (25-62) from the anterior edge. Mouth subterminal, immediately anterior to the pharynx. Pharynx almost spherical, 63 ± 8 (50-75) long, 64 ± 8 (56-81) wide. The brain at the middle of the esophagus, nervous cords directed posteriorly and laterally. Esophagus 265 ± 69 (152-375) long; diverticula not observed. Gut bifurcates post atrium, at a distance of 450 ± 106 (300-625) from the anterior edge. Caeca laterally ramified, from esophagus up to the end of the body reaching some part of the haptor. The gut epithelium with pigmented cells.

Genital atrium shape was similar in structure to *M. sprostonae* n. sp. Atrial cavity 83 ± 18 (50-120) long and 128 ± 22 (75-162) wide. Peripheral spines on the atrium cavity, 12 ± 2 (7-15) long, also spines inside the genital

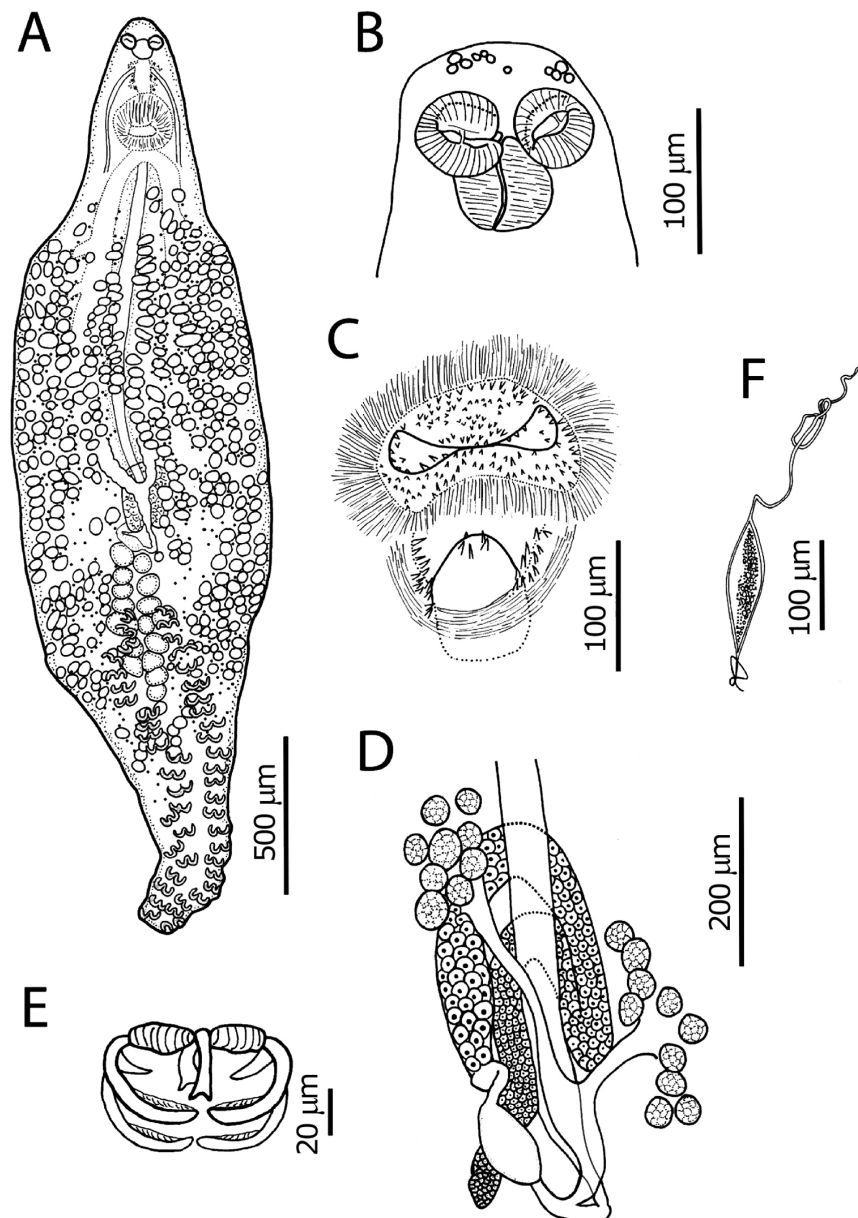


Figure 2. Drawings of *Microcotyle chilensis* n. sp. A) Ventral view of whole body, B) prohaptor, C) genital atrium, D) female reproductive organs, E) clamp, F) egg / Dibujos de *Microcotyle chilensis* n. sp. A) Vista ventral del cuerpo completo, B) prohaptor, C) atrio genital, D) órganos reproductores femeninos, E) clamp, F) huevo

cavity. More than 200 spines of similar size in the atrium cavity. Posteriorly, a cirrus cavity as a concave depression, surround by muscular fibers, armed with two groups of spines located posterior-laterally to this cavity, with 11 ± 2 (7-14) spines each group. Atrial cavity surrounded by radial muscles, making a conspicuous atrial zone, 163 ± 44 (100-225) long and 187 ± 46 (113-250) wide including the cirrus cavity.

Ovary pretesticular, intercaecal glomerular and long twisted extremes; the portion of the ovary with mature cells as an inverted U, located at 1650 ± 440 (1050-2310)

from the anterior end of the body. Ovary maximum wide 94 ± 35 (62-175). Ootype oval, 131 ± 33 (100-165) long, 41 ± 10 (31-60) wide, located posterior to mature part of the ovary. Oviduct a long folded tube, connected to an oval seminal receptacle and posteriorly to the vitelline duct. Oviduct turns upwards, followed by oval, long ootype. Uterus tubular, intercaecal, extending up to atrial cavity. Vitelline glands glomerulated, located laterally in both sides of the body, extending from some distance to gut bifurcation, at 600 ± 137 (350-800) from the anterior end of the body. Vitelline ducts united posteriorly. The anterior union of vitelline ducts was not observed in most

specimens. Anterior union of vitelline conducts connect with the vitello-vaginal duct. Female genital pore dorsal, located at 638 ± 187 (475-900) from the anterior edge. A short muscular vagina that gradually connects with a short vitelline duct. Female genital pore located at 357 ± 201 (215-500) from the vitelline duct bifurcation, and 283 ± 80 (225-375) from the posterior border of the atrium cavity.

Eight specimens with eggs. One or two eggs per monogenean. Egg fusiform, 224 ± 30 (175-256) long, 71 ± 30 (50-110) wide, with polar filaments, one short, approximately 100 μm , directed to the genital opening, the other longer than 10 times the egg length.

Testes intercaecal, post-ovary, from oval to irregular shape, 81 ± 31 (50-125) long and 65 ± 7 (55-72) wide. Number of testes 17 ± 2 (14-18). Seminal vesicle long, ventrally located along with the uterus. Cirrus oval, approximately 100 long, 64 ± 5 (62-70) wide, with two little spines on the top, observed in just one specimen.

Haptor symmetrical, 863 ± 125 (700-1090) long, 285 ± 53 (200-350) wide, with two lateral rows with clamps, 20-28 on the right and 21-27 on the left. Clamp as usual for *Microcotyle*. A total of 48 ± 3 (44-54) clamps. Clamps similar in structure and size, 85 ± 9 (75-100) long and 51 ± 6 (44-62) wide, at the middle of the haptor.

TAXONOMY SUMMARY

Sym: *Paramicrocotyle moyanoi sensu* Muñoz *et al.* (2002).

Type host: *Calliclinus geniguttatus* (Valenciennes, 1836) (Labrisomidae).

Other hosts: *Auchenionchus variolosus* (Valenciennes, 1836) (Labrisomidae), *Bovichthus chilensis* Regan, 1913 (Bovichthidae), and *Hypsoblennius sordidus* (Bennet, 1928) (Blenniidae).

Site of infection: Gills.

Type locality: Burca ($36^{\circ}28'S$, $72^{\circ}55'W$), Chile.

Other locality: Cantera ($36^{\circ}41'S$, $73^{\circ}08'W$) and Merquiche ($36^{\circ}29'S$, $72^{\circ}54'W$), Chile.

Prevalence and intensity: 5.9% of prevalence and intensity range of 1-12 on 268 *C. geniguttatus* at the type locality; infections in other hosts are indicated in Table 1.

Deposition of types of specimens: MNHNCL PLAT-15014 (Holotype); MNHNCL PLAT-15015 (Paratype).

Etymology: "*chilensis*" referred to the country where this monogenean was found.

REMARKS

Microcotyle chilensis n. sp. was compared to other species, which were selected according to two features: 10-20 testes and 40-60 clamps. Four species resembled *M. chilensis* n. sp.: *M. hiatulae* Goto, 1899, *M. furcata* Linton, 1940, *M. pentapodi* Sandars, 1944, and *M. neozealanicus* Dillon and Hargis, 1965.

Microcotyle hiatulae has been poorly described, based only on body length, number of clamps and testes, and spine lengths on the genital atrium (Goto 1899). Thoney & Munroe (1987) redescribed *M. hiatulae* and considered it as a senior synonym of *M. furcata*. We used data from that redescription and found that, compared to the new species, *M. chilensis* n. sp. differs from *M. hiatulae* in a smaller oral sucker length (45-65 vs. 44-112) and in the ratio of haptor length to body length (20-34% vs. 23.4-48.1%). *M. chilensis* n. sp. also has a genital atrium at a greater distance from the anterior edge compared to *M. hiatulae* (8-30% vs. 7.3-12.4%), and the ratio of the oral sucker length to pharynx length was greater in *M. chilensis* n. sp. than in *M. hiatulae* (0.75-1.04 vs. 0.92-1.34)².

M. pentapodi was described using measurements of the holotype and the average of the morphometric measurements, which made it difficult to know the morphometric variability of the species. The average length and width of the body were smaller in *M. pentapodi* (2,060 \times 250) than in *M. chilensis* n. sp. (3,169 \times 705). Oral suckers were longer in *M. pentapodi* than in *M. chilensis* n. sp. (63 vs. 52), and the pharynx was smaller in *M. pentapodi* than in *M. chilensis* n. sp. (length and width: 50 \times 33 vs. 64 \times 64). Moreover, *M. pentapodi* was described as having a particularly shaped genital atrium: a "sucker with a pair of saccular bags without spines" positioned to the right and to the left of the sucker (Sandars 1944). Sandars distinguished this species from others using this distinctive genital atrium shape. However, the general morphology of the genital atrium of *M. pentapodi* seems similar to most *Microcotyle*, although the way to represent this was confusing. Overall, the description of the morphology of this structure in *M. pentapodi* requires some revision.

It is worth noting that *M. pentapodi* has been transferred to the genus *Manterella* Unnithan, 1971, due to the shape of the genital atrium. However, Mamaev (1977) considered this genus to be a synonym of *Cynoscionicola* Yamaguti, 1963. However, the genital atrium of *M. pentapodi* was not well described and drawings conveyed a poor representation of this structure. Therefore, the status of this species is unclear.

²OSL/PhL calculated for *M. hiatulae* considered the averages of oral sucker lengths and pharynx diameters in the data generated by Thoney & Munroe (1987)

M. chilensis n. sp. differs from *M. neozealanicus* with regard to several traits. *M. chilensis* n. sp. has a larger body size (2187-4062 × 475-1125) than *M. neozealanicus* (1460-2920 × 460-770). The pharynx is also bigger in *M. chilensis* n. sp. (54-75 × 56-81) compared to *M. neozealanicus* (44-62 × 47-61), and the distance between the vagina opening and genital atrium is larger in *M. chilensis* n. sp. (225-375) than in *M. neozealanicus* (115-191) (see Dillon & Hargis 1965).

Of the other *Microcotyle* species previously recorded in Chile, *M. nemadactylus* (clamps: 90-104; testes: 16-25) (Dillon & Hargis 1965) and *M. moyanoi* (clamps: 118-150; testes: 33-44) (Villalba & Fernández 1986) are morphologically distinct from *M. chilensis* n. sp. (clamps: 44-54; testes: 14-18). *M. chilensis* n. sp. shares several morphological and morphometrical traits with *M. sprostonae* n. sp., but they differ in the number of testes, and there is only a small overlap in the number of clamps (Table 4). The cirrus cavity length is 37-112 in *M. chilensis* n. sp., which is in contrast to 12-50 in *M. sprostonae* n. sp. There are also differences in the number of spines on the sides of the cirrus cavity (4-9 in *M. sprostonae* n. sp. vs. 7-14 in *M. chilensis* n. sp.). Other differences are in relative measurements, such as larger ratio between oral sucker length and pharynx length and larger distance from posterior edge to last vitellarium gland in *M. sprostonae* n. sp. than *M. chilensis* n. sp. (Table 3).

The distribution of parasites and hosts might support *M. chilensis* as a new species. Similar to the hosts of *M. sprostonae* n. sp., the fish hosts analyzed here are mostly endemic to the Pacific coast of South America, and they are farther away than the type localities of *M. hiatulae*, which are found in the fish *Tautoga onitis* from Newport, Rhode Island, in the United States (Thoney & Munroe 1987). *M. pentapodi* has been found on *Pentapodus milii* fish in Western Australia (Sandars 1944), and *M. neozealanicus* has been found in *Helicolenus percoides* in New Zealand (Dillon & Hargis 1965). *M. chilensis* n. sp. was recorded in south-central Chile on five intertidal fish species, but with a notorious preference for *C. geniguttatus* (Table 1), contrasting with the distribution of *M. sprostonae* n. sp. in northern and central Chile. However, with the information obtained in this study, it is not possible to confirm the existence of geographical overlapping between *M. sprostonae* n. sp. and *M. chilensis* n. sp.

MOLECULAR ANALYSES

Based on the 18S gene, there were two clades of *Microcotyle* in the samples used in this study. One included a specimen *M. sprostonae* n. sp. from *S. viridis* (Fig. 3) from central Chile (33°S), and the other was obtained from different fish species (*C. geniguttatus*, *Auchenionchus variolosus*, *B. chilensis*, and *H. sordidus*) from the south-central region of Chile (36°S) (Fig. 3).

Table 3. Pairwise sequence divergences for the 18S gene of Microcotyloid species obtained by averaging all sequence pairs between groups. The divergence distance was calculated using the maximum composite likelihood model, and it is shown as a percentage (below the diagonal). The mean number of mutations between pairwise comparisons is also shown for each clade (above the diagonal) / Divergencias de secuencia por pares para el gen 18S de las especies de microcotilidos promedio obtenidas de todos los pares de secuencias entre los grupos. La distancia de divergencia se calculó mediante el modelo de probabilidad máxima compuesta, indicada en porcentaje (debajo de la diagonal). El número medio de mutaciones entre comparaciones por pares también se muestra para cada clado (por encima de la diagonal)

	18S	1	2	3	4	5	6	7	8	9	10	11
1	<i>M. chilensis</i> n. sp.		1	2	3	6	14	50	52	38	94	69
2	<i>M. sprostonae</i> n. sp.	0.07		3	4	7	13	49	51	37	94	68
3	<i>Microcotyle</i> sp. So Val	0.13	0.20		3	6	13	50	51	38	96	68
4	<i>M. sebastis</i>	0.20	0.27	0.20		7	11	49	51	37	94	70
5	<i>M. erythrinii</i>	0.40	0.47	0.40	0.47		18	48	54	40	96	71
6	Microcotylidae gen. sp.	0.94	0.88	0.88	0.74	1.22		55	53	39	96	69
7	<i>Polylabris</i> sp.	3.45	3.37	3.44	3.37	3.30	3.80		66	68	102	79
8	<i>C. branquialis</i>	3.58	3.51	3.51	3.51	3.72	3.65	4.58		60	93	59
9	<i>B. pagrosomi</i>	2.60	2.53	2.59	2.53	2.73	2.66	4.72	4.14		96	80
10	<i>N. pacifica</i>	6.61	6.61	6.76	6.61	6.76	6.76	7.21	6.53	6.75		113
11	Outgroup	4.79	4.72	4.72	4.87	4.94	4.79	5.52	4.07	5.59	8.04	

Based upon the ITS2 gene, there were also two clades of *Microcotyle*: one clade composed of monogeneans attached to the fish *S. viridis* and *H. sordidus* from central and from northern Chile (Antofagasta, 23°S) (Fig. 4). These specimens were described as *M. sprostonae* n. sp. Another clade was only composed *Microcotyle* from fish from the south-central region of Chile. These specimens were described as *M. chilensis* n. sp.

Specimens of *M. sprostonae* n. sp. did not differ in the genetic sequences for the ITS2 or 18S rDNA genes (Tables 4 and 5) even when the monogeneans were collected in localities far away from one another (Valparaíso 33°S and Antofagasta 23°S, Table 1). However, *M. sprostonae* n. sp. and *M. chilensis* n. sp. differed by one base pair in both genes (ITS2 and 18S), which was supported by the phylogenetic analyses (NJ, ML, and MP) (Tables 3 and 4). Hence, molecular analyses confirmed the presence of two *Microcotyle* species in intertidal fish on the coast of Chile.

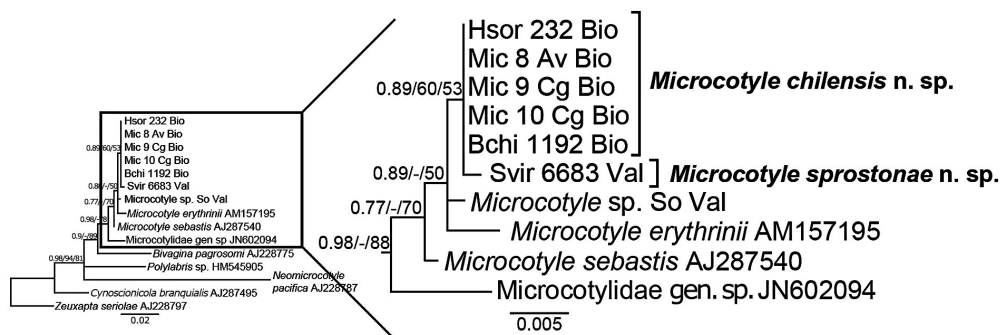


Figure 3. Phylogenetic tree showing the relationships among the microcotylids, including the two new species described in this study. The phylogenetic tree is based on NJ analyses of 18S gene sequences. The numbers along the branches indicate the percentage of supporting values obtained from the different analyses in the following order: NJ, ML, and MP. Low values (<0.5 for NJ and <50% for ML and MP) are indicated with dashes. Abbreviations of regions of Chile: VAL: Valparaíso (central); BIO: Biobío (central-South). Fish names: Hsor: *H. sordidus*, Svir: *S. viridis*, Bchi: *B. chilensis*, Av: *A. variolosus*, Cg: *C. geniguttatus*, So: *S. oculatus* / Árbol filogenético que muestra las relaciones entre las especies de microcotilidos, incluidas las dos nuevas especies descritas en este estudio, basado en los análisis de NJ de las secuencias del gen 18S. Los números a lo largo de las ramas indican el porcentaje de valores de respaldo de los diferentes análisis en el siguiente orden: NJ, ML y MP. Los valores bajos se indican con guiones (<0,5 para NJ y <50% para ML y MP). Abreviaciones de las regiones de Chile: VAL: Valparaíso (central); BIO: Biobío (centro-sur). Nombres de peces: Hsor: *H. sordidus*, Svir: *S. viridis*, Bchi: *B. chilensis*, Av: *A. variolosus*, Cg: *C. geniguttatus*, So: *S. oculatus*

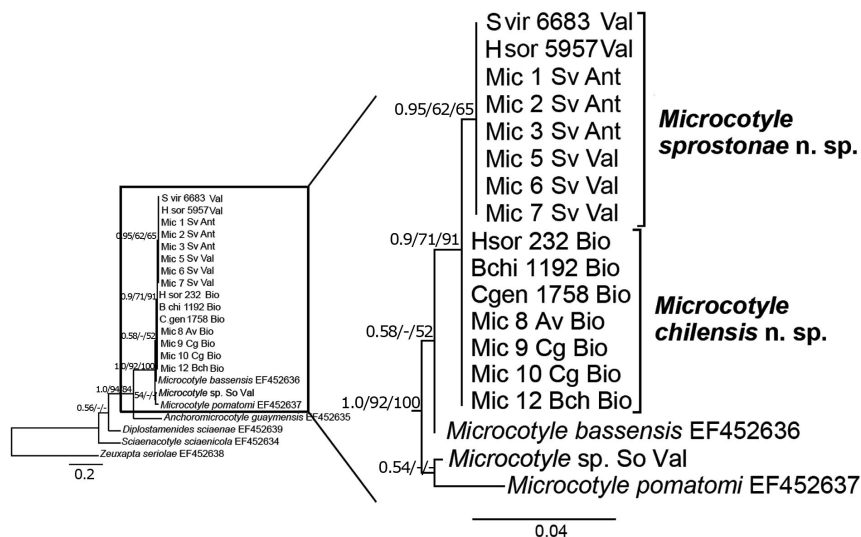


Figure 4.- Phylogenetic tree showing the relationships among the microcotylids, including the two new species described in this study. The phylogenetic tree is based on NJ analyses of ITS2 gene sequences. The numbers along the branches indicate the percentages of supporting values resulting from the different analyses in the following order: NJ, ML, and MP. Low values (<0.5 for NJ, and <50% for ML and MP) are indicated with dashes. Abbreviations as in Figure 3 / Árbol filogenético que muestra las relaciones entre las especies de microcotilidos, incluidas las dos nuevas especies descritas en este estudio, basado en los análisis de NJ de las secuencias del gen ITS2. Los números a lo largo de las ramas indican los porcentajes de los valores de respaldo resultantes de los diferentes análisis en el siguiente orden: NJ, ML y MP. Los valores bajos se indican con guiones (<0,5 para NJ y <50% para ML y MP). Abreviaciones como en la Figura 3

Table 4. Pairwise sequence divergence for the ITS2 gene of Microcotyloid species obtained by averaging all sequence pairs between groups. The divergence distance was calculated using the maximum composite likelihood model, and it is shown as a percentage (below the diagonal). The mean number of mutations between pairwise comparisons is also shown for each clade (above the diagonal) / Divergencia de secuencias por pares para el gen ITS2 de las especies de microcotiloides promedio obtenidas de todos los pares de secuencias entre los grupos. La distancia de divergencia se calculó mediante el modelo de probabilidad máxima compuesta, indicada en porcentaje (debajo de la diagonal). El número medio de mutaciones entre comparaciones por pares también se muestra para cada clado (por encima de la diagonal)

	ITS2	1	2	3	4	5	6	7	8	9
1	<i>M. chilensis</i> n. sp.		1	3	2	6	85	78	97	177
2	<i>M. sprostonae</i> n. sp.	0.27		4	3	7	86	79	98	178
3	<i>Microcotyle</i> sp. So Val	0.81	1.09		1	3	84	77	97	178
4	<i>M. bassensis</i>	0.54	0.81	0.27		4	84	77	97	178
5	<i>M. pomatomi</i>	1.64	1.92	0.82	1.09		86	79	100	179
6	<i>D. sciaenae</i>	27.80	28.22	27.38	27.38	28.37		92	61	174
7	<i>A. guaymensis</i>	25.28	25.68	24.88	24.88	25.77	30.90		100	182
8	<i>S. sciaenicola</i>	32.77	33.23	32.67	32.67	34.17	18.80	34.54		178
9	Outgroup	82.54	83.54	83.15	83.15	84.81	81.99	89.77	85.47	

Table 5. Differences in morphometric measurements of the two new species in this study, *M. sprostonae* n. sp. and *M. chilensis* n. sp. / Diferencias en las mediciones morfométricas de las dos nuevas especies, *M. sprostonae* n. sp. y *M. chilensis* n. sp.

Variables	<i>M. sprostonae</i> n. sp.	<i>M. chilensis</i> n. sp.
Length	3,062-5,225	2,187-4,062
Width	687-1,250	475-1125
Length/Width	3.3-5.8	3.5-5.5
Total number of testes	22-31	14-18
Total number of clamps	52-83	44-54
Distance from anterior end to ovary (DAE-OV)	1,200-2,050	1,050-2,310
% DAE-OV/ length	33.9-46.3%	42.4-61.8%
Distance from anterior edge to first vitellarium gland (DAE-FVG)	112-750	350-800
% DAE-FVG/Length	2.0-19.3%	14.4-28.7%
Distance from posterior edge to last vitellarium gland (DPE-LVG)	500-950	300-550
% DPE-LVG/Length	13.1-24.5	7.5-16.2
Ratio OSL/PhL	1.01-1.75	0.74-1.04

DAE: distance from anterior edge; DPE: distance from posterior edge; FVG: first vitelline follicle; LVG: last vitelline follicle; OV: ovary; OSL: oral sucker length; PhL: pharynx length

DISCUSSION

In this study, two new species of *Microcotyle* were morphologically described, which was also supported by molecular data. Relative measurements, as suggested by Machkewskyi *et al.* (2013) were also useful for making comparisons among species, especially between *M. sprostonae* n. sp. and *M. chilensis* n. sp. The two species described here are parasites of sympatric fish, which are distributed along the Chilean coast. However, these *Microcotyle* species have different distributions. One is in south-central Chile (*M. chilensis* n. sp.), and the other is in northern and central Chile (*M. sprostonae* n. sp.).

Distinctions in morphology between these two species cannot be attributed to the geographic distance for the following four reasons. (1) Molecular data were based on a variable gene (ITS2) and a conserved gene (18S). Both genes differed between species, and that difference was

consistent in all host species and geographical zones. (2) Almost 200 specimens of *S. viridis* were collected in south-central Chile (Table 1), but none of them were parasitized by *M. sprostonae* n. sp., despite *C. geniguttatus* being collected in the same habitat at the same time with a high level of parasitization by *M. chilensis* n. sp. In contrast, in central Chile, most *S. viridis* collected were parasitized by microcotylids (*M. sprostonae* n. sp.). In 109 specimens of *C. geniguttatus*, only two were parasitized by microcotylids (one parasite per fish). One of these monogeneans was fixed in ethanol, but, unfortunately, DNA amplification was not successful. However, its morphology is consistent with that of *M. sprostonae* n. sp. In addition, three specimens of *S. viridis* were collected in northern Chile. All of them were *M. sprostonae* n. sp. (Figs. 3-4). (3) Central (33°S) and south-central (36°) zones of Chile are 600 km away. In both locations, different *Microcotyle* species were found.

While the central (33°S) and northern (23°S) zones of Chile are 1300 km apart, *M. sprostonae* n. sp. was in both localities. Therefore, the geographic distance alone does not explain the presence of different *Microcotyle* species. It is probable that environmental conditions, which change across latitudes, may affect the distribution and host specificity of *Microcotyle*. (4) The presence of *Microcotyle* species in one zone versus another was not due to fish body sizes, because *S. viridis* and *C. geniguttatus* were similar in body length between sampling zones (Table 1). Altogether, there is sufficient morphological, molecular, and ecological evidence to confirm the validity of the two *Microcotyle* species described here.

Microcotyle sprostonae n. sp. and *M. chilensis* n. sp. were present on hosts of different families, although each species showed a preference for a certain host species (based on the high abundance and prevalence of the parasite). Also, a host species, *Hypsoblennius sordidus*, can be parasitized by both monogeneans separately, depending on the geographical distribution of the parasite. This result indicates that it is not possible to assume fish of the same species from different localities have the same *Microcotyle* species or that *Microcotyle* from different host species are different (Martínez & Barrantes 1977).

The genus *Microcotyle* requires the reassessment of many species, which is a very difficult task to carry out because several of the species considered valid need revision. Moreover, over time, more morphological details have been incorporated, and thus descriptions have become more complex. Consequently, the characteristics and distinctions of the recent new species are well understood, but the simplicity of the original descriptions for many of the species described long ago generates more doubts about the validity of those species. Also, it is worth noting that some characteristics may change with age, such as body width, number of clamps, and egg size (Sproston 1946). Thus, future studies need to considerate several specimens in order to establish the variability of any morphological trait. The descriptions of a species should consider as many morphological features as possible, which should be documented in measurements and figures. Molecular techniques are good tools for species descriptions; however, the molecular approach has been applied only recently, considering so far just a few species of *Microcotyle*, and therefore the advantages of genetic analyses for identification purposes are still limited for this genus. Therefore, future studies may include molecular methods to complement the species descriptions (new and already known), in order to clarify the status of numerous species in *Microcotyle* and in any other genera within Microcotylidae.

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