

An integrative taxonomy study reveals a rare new species of the genus *Creptotrema* (Trematoda: Allocreadiidae) in an endangered frog in South America

Research Paper

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Abstract

During an ecological study with a near-endangered anuran in Brazil, the Schmidt's Spinythumb frog, *Crossodactylus schmidti* Gallardo, 1961, we were given a chance to analyze the gastrointestinal tract of a few individuals for parasites. In this paper, we describe a new species of an allocreadiid trematode of the genus *Creptotrema* Travassos, Artigas & Pereira, 1928, which possesses a unique trait among allocreadiids (i.e., a bivalve shell-like muscular structure at the opening of the ventral sucker); the new species represents the fourth species of allocreadiid trematode parasitizing amphibians. Besides, the new species is distinguished from other congeners by the combination of characters such as the body size, ventral sucker size, cirrus-sac size, and by having small eggs. DNA sequences through the 28S rDNA and COI mtDNA further corroborated the distinction of the new species. Phylogenetic analyses placed the newly generated sequences in a monophyletic clade together with all other sequenced species of *Creptotrema*. Genetic divergences between the new species and other *Creptotrema* spp. varied from 2.0 to 4.2% for 28S rDNA, and 15.1 to 16.8% for COI mtDNA, providing robust validation for the recognition of the new species. Even though allocreadiids are mainly parasites of freshwater fishes, our results confirm anurans as hosts of trematodes of this family. Additionally, we propose the reallocation of *Auriculostoma oclaya* Liquin, Gilardoni, Cremonte, Saravia, Cristóbal & Davies, 2022 to the genus *Creptotrema*. This study increases the known diversity of allocreadiids and contributes to our understanding of their evolutionary relationships, host-parasite relationships, and biogeographic history.

Introduction

The diversity of amphibians in Brazil is extraordinarily high (1,222 species) (Segalla *et al.* 2021; Frost 2023). Most of this diversity comprises anurans with 1,178 species, followed by caecilians (39 species) and salamanders (five species) (Segalla *et al.* 2021; Frost 2023). Despite this high biodiversity, the diversity of amphibian parasites is underestimated (Camião *et al.* 2014; Alcantara *et al.* 2021, 2022). The genus *Crossodactylus* Duméril and Bibron, 1841 presently includes 13 species of frogs distributed across South America (Frost 2023). One of these species, Schmidt's Spinythumb frog, *Crossodactylus schmidti* Gallardo, 1961 (Anura: Hylodidae Günther, 1858), was first described in Argentina (Misiones) by Gallardo (1961) and later reported in Paraguay (southeastern Itapúa province) and Brazil (western Paraná, western Santa Catarina, and northern and western Rio Grande do Sul) (Frost 2023). *Crossodactylus schmidti* is currently considered a federally threatened species, catalogued as Near Threatened (NT) in the IUCN Red List (IUCN 2023), as their populations are declining since the anuran occurs in severely fragmented environments. Data on parasites associated with this anuran species include only one study carried out by Forti *et al.* (2020), in which the presence of helminths was reported as a part of an ecological study aimed at reporting the infection by chytrid fungus in relation to forest cover, although macroparasites were not taxonomically identified to species level. One of them was morphologically identified as belonging to the trematode genus *Creptotrema* Travassos, Artigas & Pereira, 1928.

The genus *Creptotrema* includes parasites of freshwater teleosts and anurans distributed across the Neotropical region (Franceschini *et al.* 2021; Liquin *et al.* 2022). To date, the genus includes 20 valid species (Franceschini *et al.* 2021; Liquin *et al.* 2022), with 16 of those occurring

in South America – that is, the type-species *Creptotrema creptotrema* Travassos, Artigas & Pereira, 1928, *Creptotrema conconae* Franceschini, Aguiar, Zago, Yamada, Ebert & Silva, 2021, *Creptotrema diagonale* (Curran, Tkach & Overstreet, 2011), *Creptotrema foliaceum* (Curran, Tkach & Overstreet, 2011), *Creptotrema guacurarii* Montes, Barneche, Croci, Balcazar, Almirón, Martorelli & Pérez-Ponce de León, 2021, *Creptotrema lamothei* Curran, 2008, *Creptotrema lynchi* Brooks, 1976, *Creptotrema macrorchis* (Szidat, 1954), *Creptotrema megacetabulare* Franceschini, Aguiar, Zago, Yamada, Ebert & Silva, 2021, *Creptotrema oclroye* (Liquin, Gilar-doni, Cremonte, Saravia, Cristóbal & Davies, 2022) n. comb., *Creptotrema pati* Lunaschi, 1985, *Creptotrema paraense* Vicente, Santos & Souza, 1978, *Creptotrema platense* (Szidat, 1954), *Creptotrema schubarti* Franceschini, Aguiar, Zago, Yamada, Ebert & Silva, 2021, *Creptotrema stenopteri* (Mañé-Garzón & Gascón, 1973), and *Creptotrema sucumbiosa* Curran, 2008. *Creptotrema lynchi* is the only species reported from an anuran host, *Rhinella marina* (Linnaeus, 1758) (= *Bufo marinus*), in Colombia (Brooks, 1976), although the definitive hosts of *Creptotrema* spp. are preferentially freshwater fishes belonging to multiple orders (Characiformes, Gymnotiformes, Perciformes, and Siluriformes). A new species of *Auriculostoma* Scholz, Aguirre-Macedo & Choudhury, 2004 was described from Argentina in 2022 as a parasite of siluriforms, named *A. oclroya* (Liquin *et al.* 2022). Apparently, the authors were unaware of the publication by Franceschini *et al.* (2021) in which the genus *Auriculostoma* was synonymized with *Creptotrema*. Our study offers a possibility to test for the phylogenetic position of that species within Alloecreadiidae Looss, 1902.

Although molecular tools and bioinformatics have revolutionized biodiversity research and can be used to address some of the Linnaean shortfalls (Rubio-Godoy and Pérez-Ponce de León 2023; Vergara-Asenjo *et al.* 2023), parasite fauna is still considered largely unknown, especially in tropical regions (Dobson *et al.* 2008; Carlson *et al.* 2020a). This taxonomic gap holds back the progress of ecological knowledge on parasites and impairs conservation actions (Carlson *et al.* 2020b). Even though parasite taxonomy is in crisis with only small groups of prolific authors describing new species (Poulin and Presswell 2022), the recent discovery of new species of *Creptotrema* and other parasite groups is due to the utilization of integrative approaches and the use of distinct tools for morphological analyses, such as light and scanning electron microscopy, and molecular investigations, with the employment of different markers, and phylogenetic analyses.

As a part of a survey on the helminth parasite fauna of Brazilian anurans, we had an opportunity to study the internal organs of *C. schmidti* in Paraná, Rio Grande do Sul and Santa Catarina states; among them, some individuals were identified as belonging to the genus *Creptotrema*. A further detailed morphological assessment of the specimens, accompanied by molecular analyses (28S rDNA and COI mtDNA genes) confirmed they represented an undescribed species of trematode parasitizing the near-threatened frog *C. schmidti*. The new species is described herein, and its phylogenetic position within the genus *Creptotrema* is tested. Our results expand the knowledge of the interaction of *Creptotrema* species with amphibian hosts in South America.

Materials and methods

Host sampling and parasitological procedures

Twenty-seven specimens of *C. schmidti* were sampled in three national parks of Brazil: the Parque Nacional do Iguaçu,

municipality of Céu Azul (25°9'4.036"S, 53°50'28.777"W); Parque Estadual Rio Guarani, municipality of Três Barras do Paraná, Paraná state (25°26'42.871"S, 53°9'37.879"W); and Parque Estadual do Turvo, municipalities of Derrubadas (27°8'31.68"S, 53°52'39.35"W) and Frederico Westphalen (27°21'43.36"S, 53°24'38.32"O), Rio Grande do Sul state, and municipality of São Miguel do Oeste (26°45'36.10"S, 53°31'30.47"O), Santa Catarina state. The frogs were euthanized with 2% lidocaine hydrochloride. Frogs were dissected, and all internal organs were screened for parasites under a stereomicroscope.

Trematodes were collected from the small intestine of the anurans, and two fresh specimens were transferred directly to 99.8% ethanol for molecular study while the other specimens were fixed in alcohol-formalin-acetic acid solution under light pressure of a coverslip for 10 min and transferred to 70% alcohol for further processing. At the laboratory, trematodes were stained with alcoholic chloride carmine solution, cleared with eugenol, and analyzed in a computerized system for image analysis (V3 Leica Application Suite, Leica Microsystems, Wetzlar, Germany) in a microscope with differential interference contrast. Morphological descriptions followed the recommendations of Travassos *et al.* (1928) and Fernandes and Kohn (2014), and the observations provided by Scholz *et al.* (2004) and Razo-Mendivil *et al.* (2014b). Measurements of the specimens are presented as the values of the holotype followed by the range in parentheses (reported in micrometers). Illustrations of the structures were produced with the aid of a camera lucida mounted on a Leica DMLS microscope with phase-contrast optics.

Holotype and paratypes of the new species of *Creptotrema* were deposited in the Helminthological Collection of the Oswaldo Cruz Institute (CHIOC – Holotype: number 40421a; Paratypes: numbers 40421b, 40422, 40423, 40424, 40425), Rio de Janeiro State, Brazil. The host specimens (*C. schmidti*) were deposited at the Museu de Zoologia 'Prof. Adão José Cardoso' of the Universidade Estadual de Campinas (Unicamp), Campinas, São Paulo, Brazil (ZUEC24284 to 24295).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from two *Creptotrema* specimens using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, United States), following the manufacturer's protocol. Fragments of the 28S rDNA gene and the COI mtDNA gene were amplified using the primers and cycling conditions described in Franceschini *et al.* (2021). Conventional polymerase chain reaction (PCR) amplifications were performed on a final volume of 25 µl containing 12.5 µl of 2× MyFi™ Mix (Bioline, Taunton, MA, USA), 3.0 µl of extracted DNA, 7.5 µl of pure water, and 1.0 µl of each PCR primer. PCR products (2.0 µl) were run on an agarose gel (1%) using GelRed™ fluorescent nucleic acid dye and loading buffer to confirm amplicon size and yield. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's instructions. Automated sequencing was performed directly on purified PCR products using a BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction kit on an ABI 3500 DNA genetic sequencer (Applied Biosystems). Forward and reverse sequences were assembled and edited using Sequencher v. 5.2.4 (Gene Codes, Ann Arbor, MI, USA).

Phylogenetic analyses

To perform the phylogenetic analyses, two independent datasets were created: the first contained the newly generated 28S rDNA

sequences, published sequences of Allocreadiidae retrieved from GenBank, and sequences of *Prosthenhystera* Travassos, 1922 (Callodistomidae Odhner, 1910), *Dicrocoelium* Dujardin, 1845 (Dicrocoeliidae Looss, 1899), *Degeneria* Campbell, 1977 (Gorgoderidae Looss, 1899), and *Phyllodistomum* Braun, 1899 (Gorgoderidae), which were used as outgroups (Table S1). The second dataset contained the newly generated COI mtDNA sequence, published sequences of Allocreadiidae retrieved from GenBank, and sequences of *Phyllodistomum parasiluri* Yamaguti, 1934 (Gorgoderidae), *Dicrocoelium dendriticum* (Rudolphi, 1819), and *Dicrocoelium chinensis* (Sudarikov & Ryjikov, 1951) Tang & Tang, 1978 (Dicrocoeliidae) as outgroups (Table S1).

The alignments of the two datasets were performed separately using the MUSCLE algorithm implemented on Geneious 7.1.3 (Kearse *et al.* 2012) with default settings. The presence of stop codons and indels for the COI mtDNA alignment was verified by amino acid translation using the trematode mitochondrial code table on Geneious 7.1.3 (Kearse *et al.* 2012). Prior to the phylogenetic analyses, the best-fitting model of nucleotide substitution for the aligned datasets was selected in the JModelTest software (Posada 2008) using the Akaike information criterion, as GTR + G + I for the 28S rDNA dataset and HKY + G for the COI mtDNA dataset.

Phylogenetic trees were obtained using Bayesian Inference (BI) and Maximum Likelihood (ML). BI was performed using MrBayes 3.2 (Ronquist *et al.* 2012) on the online platform CIPRES. The Markov chain Monte Carlo (MCMC) was run with 10^6 generations saving one tree every 100 generations, with a burn-in set to the first 25% of the trees. Only nodes with posterior probabilities (pp) greater than 95% were considered well supported. The ML analyses were run in RAxML (Guindon and Gascuel 2003) at the online platform CIPRES with 1000 bootstrap replicates. Only nodes with bootstrap values greater than 70% were considered well supported. The BI and ML trees were visualized in FigTree v. 1.3.1 software (Rambaut 2009) and edited in CorelDraw X6.

Pairwise genetic distances among sequences were calculated using the Kimura-2-parameter (K2P) model and a bootstrap procedure with 1,000 replicates in the software MEGA7 version 7.0 (Kimura 1980; Kumar *et al.* 2016).

Results

Morphological description

Allocreadiidae Looss, 1902

Creptotrema Travassos, Artigas & Pereira, 1928

Creptotrema cruste n. sp. Alcantara, Ebert, Ferreira-Silva, Pérez-Ponce de León & Silva (Figures 1–3)

(Based on seven adult specimens). Body elongated, 2,240 (1,950–2,860) long, 559 (436–688) wide. Oral sucker subterminal, subspherical, 209 (204–241) long, 214 (182–215) wide, with a single muscular lobe on either side of oral sucker ('auricles'), stretching from ventral side to lateral area. Oral sucker with 4 inner and 6 outer papillae. Two other papillae anteriorly to auricles. Six pairs of papilla on anterior border, close to oral sucker. Inconspicuous pre-pharynx. Pharynx muscular, subspherical, 89 (89–118) long, 99 (99–128) wide. Oesophagus, 88 (88–162) long. Intestinal caeca extending to posterior body end. Ventral sucker pre-equatorial, 358 (355–406) long, 348 (275–394) wide, narrow and elongated opening, with two bivalve shell-like muscular structures. Genital pore close to caecal bifurcation, anterior to ventral sucker. Ratio oral sucker length to ventral sucker length 1:0.6 (0.5–0.7); ratio oral

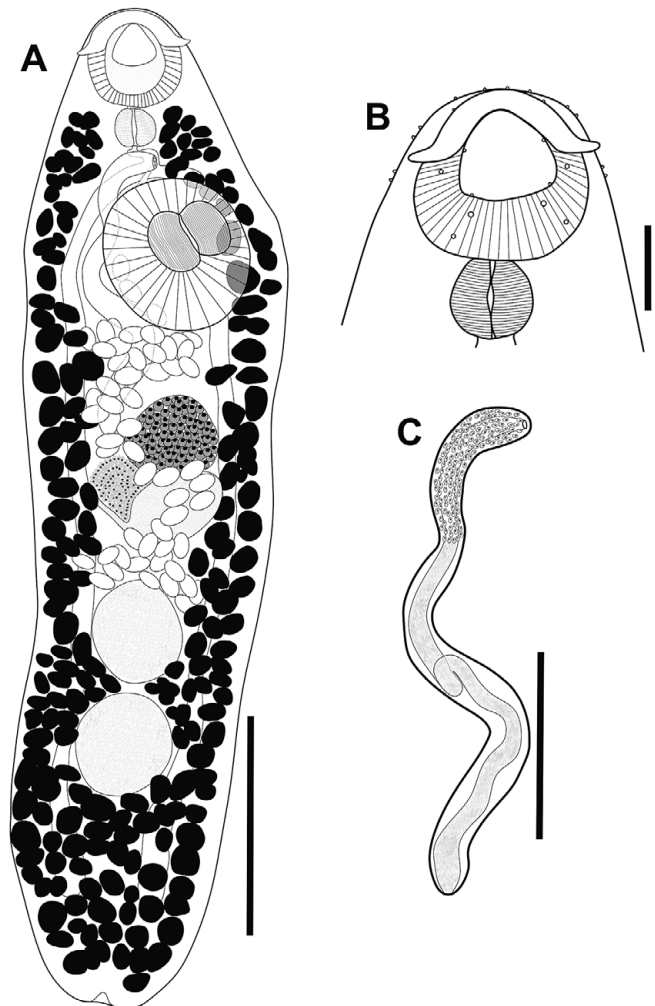


Figure 1. *Creptotrema cruste* n. sp. (Holotype) from the anuran-host *Crossodactylus schmidti* Gallardo, 1961. A. Total view. B. Ventral view, with detail of a discrete single ventrolateral muscular lobe on either side of the oral sucker, with distribution of papillae over the oral sucker. C. Detail of the cirrus-sac in ventral view. Scale bar: 500 µm (A), 100 µm (B), and 200 µm (C).

sucker width to ventral sucker width 1:0.6 (0.5–0.7). Testes two, rounded, in tandem, anterior testis 231 (151–291) long, 204 (172–248) wide; posterior testis 222 (210–276) long, 211 (185–301) wide, juxtaposed next to each other, intercecal. Cirrus-sac well developed, 768 (577–768) long, 59 (55–69) wide, sinuous, passing posterior to ventral sucker, reaching ovary region posteriorly, enclosing seminal vesicle, and unarmed and eversible cirrus. Prostate glands about 25% of cirrus sac length. Pars prostatica not observed. Ovary posterior to ventral sucker, pretesticular, slightly sinistral, obliquely oval to irregular in shape, with entire margin, 180 (143–234) long, 213 (142–213) wide. Vitelline follicles marginal, large, extra- and intra-cecal, not overlapping gonads, extending from pharynx level to posterior end of body, completely separated into two lateral fields but confluent in post-testicular region. Mehlis' gland close to ovary. Laurer's canal not observed. Uterus pretesticular, intra-cecal. Eggs operculate, (53–63) long, (35–40) wide. Excretory pore terminal; excretory vesicle I-shaped, reaching anterior testis.

Taxonomic summary

Type host: *Crossodactylus schmidti* Gallardo, 1961.

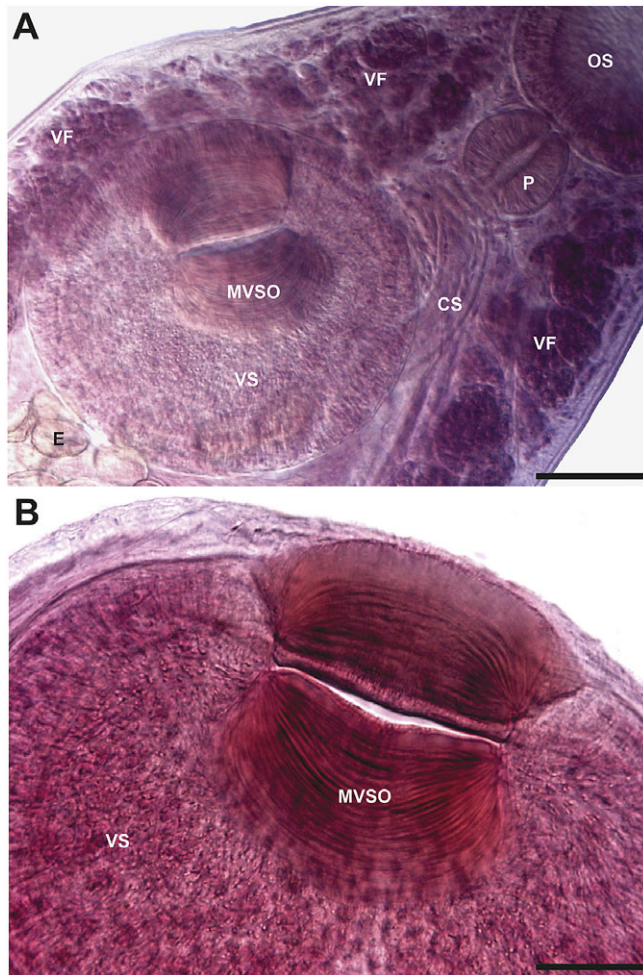


Figure 2. Anterior region of *Creptotrema cruste* n. sp., highlighting the bivalve shell-shaped musculature associated with the ventral sucker opening. A) General view. B) Detail of the musculature of the ventral sucker opening. VS, ventral sucker; CS, cirrus sac; MVSO, musculature associated with the ventral sucker opening; OS, oral sucker; P, pharynx; VF, vitelline follicles. Scale bar: 100 µm (A) and 50 µm (B).

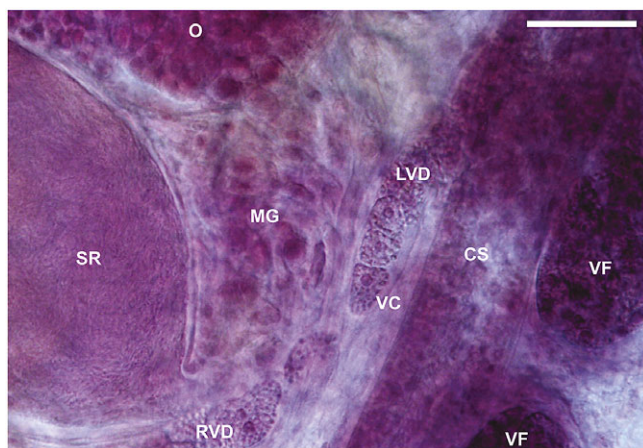


Figure 3. Detail of the Mehli's gland region of *Creptotrema cruste* n. sp. CS, cirrus sac; LVD, left vitelline duct; MG, Mehli's gland; O, ovary; RVD, right vitelline duct; SR, seminal receptacle; VC, vitelline cells. Note the nucleus and the cluster of shell protein globules; VF, vitelline follicles. Scale bar: 50 µm.

Type locality: Parque Nacional do Iguaçu, municipality of Céu Azul, Paraná state, Brazil (25°9'4.036"S, 53°50'28.777"W).

Other localities: Parque Estadual Rio Guarani, municipality of Três Barras do Paraná, Paraná state (25°26'42.871"S, 53°9'37.879"W) and the municipality of São Miguel do Oeste, Santa Catarina state, Brazil (26°45'36.10"S, 53°31'30.47"W).

Site of infection: small intestine.

Infection level: Nine specimens were found in the small intestines of seven host specimens.

Etymology: The specific epithet is derived from the Latin *crusta* (= shell) and refers to the presence of a remarkable bivalve shell-shaped musculature at the opening of the ventral sucker.

Remarks

Creptotrema cruste n. sp. is morphologically distinguished from its congeners mainly by the presence of a bivalve shell-shaped musculature at the opening of the ventral sucker. This structure was never reported before in *Creptotrema* spp., making it a specific differential character.

Morphologically, *Creptotrema cruste* n. sp. differs further from *C. creptotrema*, *C. foliaceum*, *C. guacurarii*, *C. lamothei*, *C. lynchi*, *C. macrorchis*, *C. megacetabulare*, *C. pati*, *C. platense*, *C. schubarti*, *C. stenopteri*, and *C. totonacapanense* (Razo-Mendivil, Mendoza-Garfias, Pérez-Ponce de León & Rubio-Godoy, 2014a) by having two lateral vitelline fields that extend along the body and are confluent at the posterior end, filling the entire posterior end, while these species have two lateral vitelline fields that extend to the posterior end of the body but are not confluent. *Creptotrema cruste* n. sp. also differs from *C. diagonale*, *C. ocluye* n. comb., *C. sucumbiosa*, and *C. tica* (Hernández Mena, Pinacho-Pinacho, García-Varela, Mendoza-Garfias & Pérez-Ponce de León, 2019) by having testes in tandem, while these species have oblique testes. The new species differs from *C. lobatum* (Hernández-Mena, Lynggaard, Mendoza-Garfias & Pérez-Ponce de León, 2016) by presenting testes with a smooth surface, while *C. lobatum* possesses lobated testes. *Creptotrema cruste* n. sp. differs from *C. astyanace* (Scholz, Aguirre-Macedo & Choudhury, 2004), *C. conconae*, *C. foliaceum*, and *C. paraense* by presenting a ventral sucker much larger than the oral sucker, while in *C. astyanace* and *C. conconae*, the oral and ventral suckers are of similar sizes, and in *C. foliaceum* and *C. paraense*, the oral sucker is much larger than the ventral sucker. Furthermore, the new species exhibits morphometric differences with respect to those observed in other *Creptotrema* species (Table 1).

Interestingly, a similar bivalve shell-shaped musculature at the opening of the ventral sucker was reported for *Australotrema brisbanense* Khalil, 1981, a trematode parasite of the intestine of the pinkete mullet, *Trachystoma petardi* (Castlenau, 1875) from Brisbane River, Australia (Khalil 1981). In the description, the author pointed out that the ventral sucker of their specimens presented a 'transverse opening guarded by a strong muscular sphincter'. Other than the presence of this structure, *Creptotrema cruste* n. sp. and *A. brisbanense* are very different, as they belong to Allocreadiidae and Cladorchiidae Fischoeder, 1901, respectively.

Phylogenetic analyses

Two partial sequences of the 28S rDNA gene (GenBank accession numbers OR557501 and OR557502) and one partial sequence of the COI mtDNA gene (GenBank accession number OR552537) of *Creptotrema cruste* n. sp. were obtained. The two newly generated 28S rDNA sequences of *Creptotrema cruste* n. sp. were each 1,254

Table 1. Summary of morphometrics for the most useful characteristics distinguishing species of *Creptotrema*. The morphological measurements were reported in micrometers. Measurements are sourced from original descriptions plus selected redescrptions

| Species | Host type | Country | Body length | Body width | Oral sucker | Pharynx | Esophagus | Cirrus-sac | Ventral sucker | Ovary | Testis | References |
|---|---------------------------------------|---------------|--------------------|----------------|--------------------------|------------------------|---------------|----------------|--------------------------|--------------------------|--------------------------|-------------------------------------|
| <i>Creptotrema astyanace</i> | <i>Astyanax fasciatus</i> | Nicaragua | 1,900–2,900 | 400–488 | 202–234 x 256–304 | 93–115 x 90–118 | 70–141 | | 250–285 x 250–330 | 160–230 x 144–202 | 166–349 x 157–218 | Scholz <i>et al.</i> (2004) |
| <i>Creptotrema conconae</i> | <i>Imparfinis mirini</i> | Brazil | 990–1,860 | 126–421 | 126–188 x 127–209 | 31–50 x 57–61 | 128–156 | 288–371 | 116–169 x 150–179 | 100–175 x 87–163 | 93–179 X 100–176 | Franceschini <i>et al.</i> (2021) |
| <i>Creptotrema creptotrema</i> | <i>Megaleporinus obtusidens</i> | Brazil | 1,120–1,410 | 513–663 | 141–206 x 175–194 | 67–82 x 68–93 | 87–216 | 258–420 | 232–278 x 223–305 | 151–181 x 119–182 | 125–184 x 89–143 | Franceschini <i>et al.</i> (2021) |
| <i>Creptotrema cruste n. sp.</i> | <i>Crossodactylus schmidti</i> | Brazil | 1,950–2,860 | 436–688 | 204–241 x 182–215 | 89–118 x 99–128 | 88–162 | 577–768 | 355–406 x 275–394 | 143–234 x 142–213 | 151–290 x 171–301 | Present study |
| <i>Creptotrema diagonale</i> | <i>Stethapron cf. erythropros</i> | Peru | 1,210–1,600 | 352–474 | 70–190 x 170–208 | 45–57 x 63–68 | | | 213–221 x 213–227 | 156–161 x 102–145 | 193–304 x 110–159 | Curran <i>et al.</i> (2011) |
| <i>Creptotrema foliaceum</i> | <i>Bryconops cf. caudomaculatus</i> | Peru | 1,990 | 450 | 279 x 374 | 95 x 100 | 111 | | 207 x 202 | 145 x 117 | 106–167 x 134–145 | Curran <i>et al.</i> (2011) |
| <i>Creptotrema guacurarii</i> | <i>Characidium heirmostigmata</i> | Argentina | 1,080–1,440 | 259–456 | 93–137 x 106–160 | 41–60 x 50–69 | 41–76 | | 122–157 x 120–148 | 101–145 x 77–104 | 126–173 x 68–83 | Montes <i>et al.</i> (2021) |
| <i>Creptotrema lamothei</i> | <i>Ageneiosus brevifilis</i> | Paraguay | 1,150–1,410 | 359–457 | 223–258 x 173–228 | 62–100 x 57–78 | | | 223–258 x 173–228 | 72–99 x 79–102 | 91–153 x 85–159 | Curran (2008) |
| <i>Creptotrema lobatum</i> | <i>Brycon guatemalensis</i> | Mexico | 2,540–3,010 | 502–639 | 153–234 x 243–245 | 114–139 x 82–112 | 71–141 | | 238–319 x 233–324 | 164–217 x 92–165 | 267–396 x 167–274 | Hernández-Mena <i>et al.</i> (2016) |
| <i>Creptotrema lynchi</i> | <i>Rhinella marina</i> | Colombia | 850–1,490 | 390–670 | 156–276 x 192–336 | 60–84 x 120 | | | 264–396 x 276–372 | 204–264 x 216–288 | 340–540 x 180–265 | Brooks (1976) |
| <i>Creptotrema macrorchis</i> | <i>Pachyurus bonariensis</i> | Argentina | 1,500 | 500 | 180 x 200 | 70 x 50 | | | 150 | | | Szidat (1954) |
| <i>Creptotrema megacetabulare</i> | <i>Auchenipterus osteomystax</i> | Brazil | 930–1,550 | 332–625 | 101–151 x 115–190 | 37–56 x 45–68 | 52–54 | 393–484 | 190–270 x 160–247 | 116–185 | 105–213 x 78–134 | Franceschini <i>et al.</i> (2021) |
| <i>Creptotrema oclroye n. comb.</i> | <i>Heptapterus qenqo</i> | Argentina | 850–1,520 | 216–393 | 117–176 x 113–193 | 49–74 x 56–86 | 49–73 | | 122–193 x 113–208 | 95–154 x 95–184 | 110–174 x 98–149 | Liquin <i>et al.</i> (2022) |
| <i>Creptotrema paraense</i> | <i>Pimelodus sp.</i> | Brazil | 1,840 | 510 | 220 x 180 | 80 x 90 | | | 160 x 150 | 140 x 180 | 210 x 250 | Vicente <i>et al.</i> (1978) |
| <i>Creptotrema pati</i> | <i>Luciopimelodus pati</i> | Argentina | 943–1,415 | 687–856 | 174–207 x 185–207 | 50–81 x 59–67 | | | 205–251 x 208–262 | 174–218 x 87–119 | 272–398 x 82–174 | Lunaschi (1985) |
| <i>Creptotrema platense</i> | <i>Pimelodus maculatus</i> | Argentina | 800 | 200 | 120 | 30 x 30 | | | 120 | | | Szidat (1954) |
| <i>Creptotrema schubarti</i> | <i>Characidium schubarti</i> | Brazil | 670–910 | 324–394 | 91–122 x 122–138 | 60 x 54 | 62–89 | 250–420 | 71–149 x 75–138 | 140 x 122 | 104–179 x 82–122 | Franceschini <i>et al.</i> (2021) |
| <i>Creptotrema stenopteri</i> | <i>Charax stenopterus</i> | Uruguay | 750–1,320 | 216–308 | | 60–96 x 60–88 | 40–80 | | 148–190 x 140–240 | 52–120 x 32–52 | 60–120 x 72–150 | Mañé-Garzón and Gascón (1973) |
| <i>Creptotrema succumbiosa</i> | <i>Tetragonopterus argenteus</i> | Ecuador | 1,750–1,880 | 472–584 | 212–296 x 234–279 | 89–100 x 67–83 | 89–100 | | 250–340 x 284–307 | 156–170 x 122–140 | 307–390 x 139–195 | Curran (2008) |
| <i>Creptotrema tica</i> | <i>Gymnotus maculosus</i> | Costa Rica | 550–2,120 | 424–568 | 240–27 x 216–260 | 74–98 x 81–115 | | | 143–179 x 161–192 | 90–112 x 75–129 | 135–182 x 83–144 | Hernández-Mena <i>et al.</i> (2019) |
| <i>Creptotrema totonacapanense</i> | <i>Astyanax mexicanus</i> | Mexico | 1,030–2,000 | 287–568 | 117–212 x 128–223 | 39–92 x 44–101 | 71–96 | | 153–240 x 154–259 | 93–210 x 68–167 | 103–259 x 62–239 | Razo-Mendivil <i>et al.</i> (2014a) |

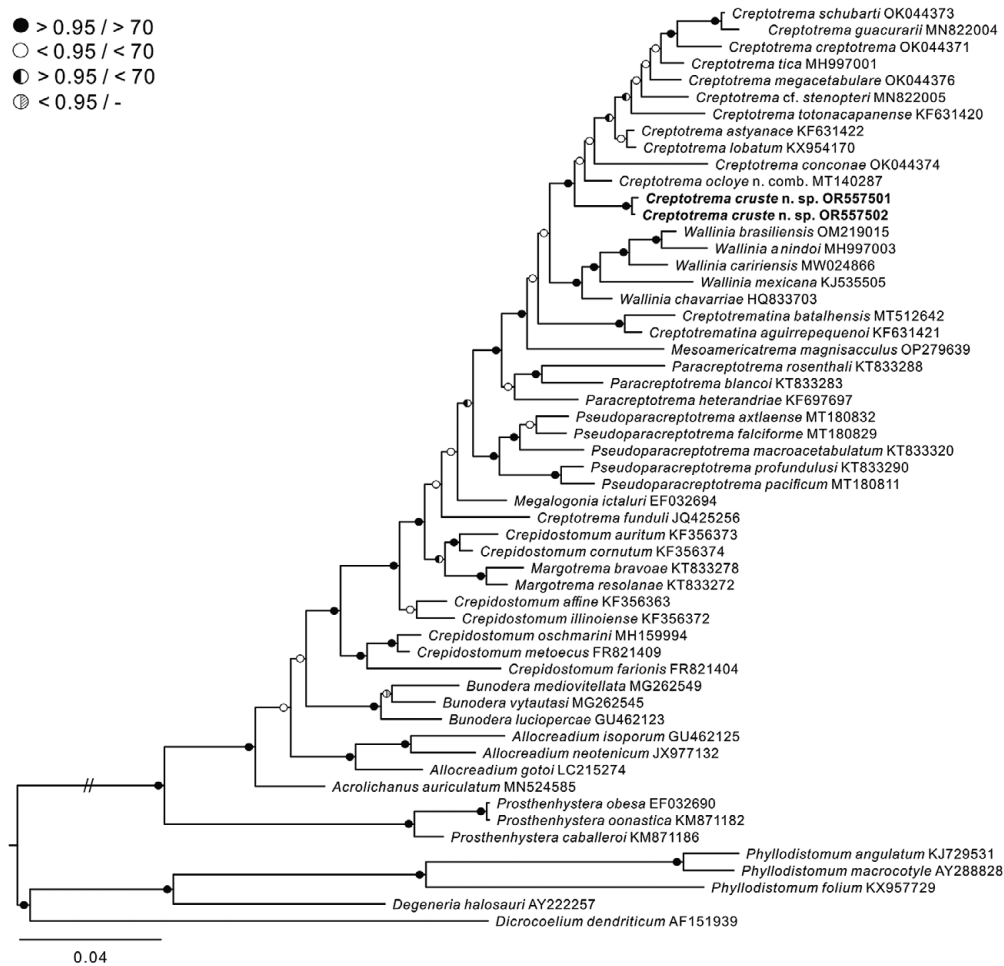


Figure 4. Bayesian inference phylogram based on 28S rDNA sequences of Allocreadiidae, showing the phylogenetic position of the new species, *Creptotrema cruste* n. sp. from the Schmidt's Spinythumb frog *Crossodactylus schmidti* Gallardo, 1961. Branch length scale bar indicates the number of substitutions per site. The new sequences are highlighted in bold.

and 1,253 bp in length and, after trimming the ends to the shortest sequence, the final alignment was 1,037 bp long. The ML and BI analyses of the partial 28S rDNA alignment produced phylograms with consistent topologies, most nodes were highly supported (Figure 4). Both analyses recovered Allocreadiidae as a monophyletic group. The newly generated sequences of *Creptotrema cruste* n. sp. grouped together with all the other sequences of *Creptotrema* in a monophyletic clade (except for *Creptotrema funduli* Mueller, 1934) with *Wallinia* spp. recovered as its sister group. For the partial 28S rDNA, the interspecific genetic divergences found among the sequences of *Creptotrema cruste* n. sp and *Creptotrema* spp. varied from 2.0% (*C. astyanace*) to 4.2% (*C. guacurarii*).

The newly generated COI mtDNA sequence of *Creptotrema cruste* n. sp. was 446 bp in length, and the final alignment was 351 bp long after trimmed to the shortest sequence. The ML and BI analyses of the partial COI mtDNA alignment recovered identical phylograms with most clades well supported (Figure 5). Both analyses also recovered the sequences of Allocreadiidae as a monophyletic group (i.e., *Creptotrema* spp., *Margotrema* spp., *Wallinia chavarriae* Choudhury, Daverdin & Brooks, 2002, and *Allocreadium lobatum* Wallin, 1909). The newly generated sequences of *Creptotrema cruste* n. sp. grouped together with all the other sequences of *Creptotrema* for which sequences are available in a well-supported monophyletic clade, with *Wallinia* spp. also

recovered as its sister group. For the COI mtDNA gene, the interspecific genetic divergences among the new species and *Creptotrema* spp. were very high and varied from 15.1% (*C. megacetabulare*) to 16.8% (*C. conconae*). Furthermore, in both phylograms, *C. oclroye* n. comb. was recovered as a member of *Creptotrema* with high nodal support values.

Discussion

The integrative taxonomic approach employed in this study provided robust validation for the recognition of a new species of *Creptotrema* parasitizing the near-threatened anuran *C. schmidti* in Brazil. *Creptotrema cruste* n. sp. is the 17th species known from South America, the second species of the genus found parasitizing an anuran, and the fourth species of allocreadiid parasitizing amphibians. *Creptotrema lynchi* was first described from the toad *Rhinella marina* in Colombia (Brooks 1976). After that, some reports on *C. lynchi* parasitizing fishes were published (Kohn *et al.* 1985; Lunaschi and Sutton 1995; Curran *et al.* 2008). However, Franceschini *et al.* (2021) demonstrated that the individuals identified as *C. lynchi* in fishes from South America were, in fact, juvenile forms of *C. creptotrema*, and after studying voucher specimens, these authors proposed the synonymy of these records infecting

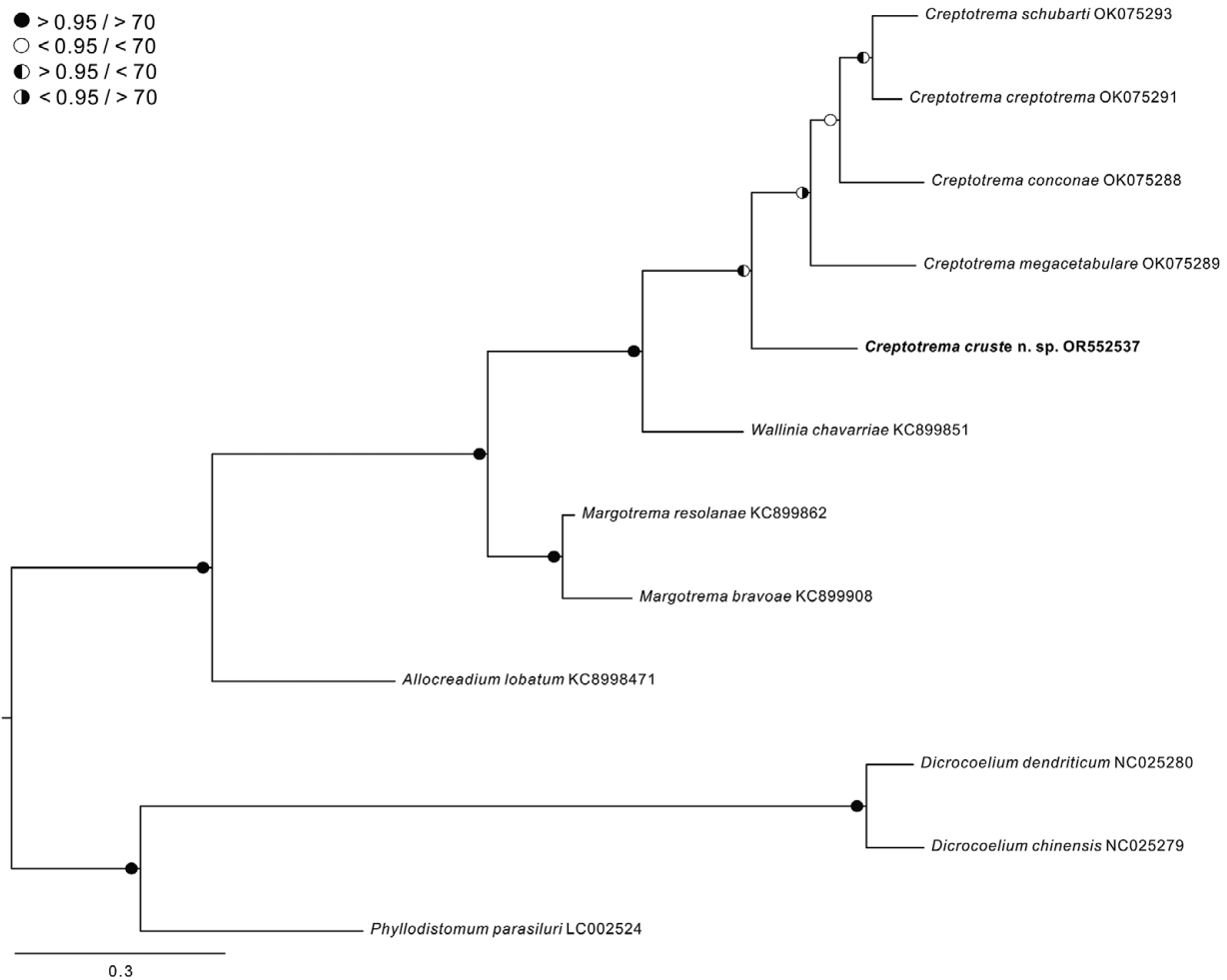


Figure 5. Bayesian inference phylogram based on COI mtDNA sequences of Allocreadiidae, showing the phylogenetic position of the new species, *Creptotrema cruste* n. sp. from the Schmidt's Spinythumb frog *Crossodactylus schmidti* Gallardo, 1961. Branch length scale bar indicates the number of substitutions per site. The new sequence is highlighted in bold.

fishes with *C. creptotrema*. Despite that, *C. lynchi* remained as the only valid species of the genus *Creptotrema* reported from an amphibian in South America. Still, the absence of subsequent reports of *C. lynchi* from anurans led Curran (2008) to suggest that the toad *R. marina* may have been an accidental host for the species. Therefore, our results not only formally describe a new *Creptotrema* species but extend the knowledge on host species for the genus and confirm anurans and fishes as hosts of *Creptotrema* spp. We also provide the first molecular data of a *Creptotrema* species parasitizing an anuran host, since *C. lynchi* still lacks molecular information. As most of the definitive hosts of *Creptotrema* spp. are preferentially freshwater fishes, it would be necessary to generate new molecular data on *C. lynchi* to compare with *Creptotrema cruste* n. sp. and test hypotheses of host specificity of these two species.

Additionally, Freitas (1960) erected a new genus, *Maicuru* Freitas, 1960, to accommodate *Maicuru solitariu* Freitas, 1960, an allocreadiid parasite of *Rhinella granulosa* (Spix, 1824) in Brazil. However, Caira and Boguea (2005) considered that, although the species present muscular lobes associated with the lateral margins of the oral sucker, the only species in the genus lacks an oesophagus and has bilobed instead of smooth testes, concluding that this taxon should be considered as *incertae sedis*. However, Curran *et al.* (2011) examined photomicrographs of the holotype of *M. solitariu* deposited at the Helminthological Collection of the

Oswaldo Cruz Institute, Rio de Janeiro, Brazil (CHIOC No. 26646) and concluded that the holotype exhibits a suite of features that strongly confirm this digenean as a member of the family Allocreadiidae. Despite that, molecular studies of *M. solitariu* are necessary to prove its identity as an allocreadiid.

Furthermore, another allocreadiid trematode, *Caudouterina rhyacotritoni* Martin, 1966, was described as a parasite of the Olympic salamander (*Rhyacotriton olympicus* [Gaije, 1917]) in western Oregon, USA (Martin 1966). The species has not been reported ever since. While describing a new genus and species of trematode, *Parabrachycoelium longicaecum* Pérez-Ponce de León, Mendoza-Garfias, Razo-Mendivil, and Parra-Olea, 2011, parasitizing a salamander from cloud forests of Veracruz, Mexico, the authors observed a remarkable resemblance with *C. rhyacotritoni* with the exception of the presence of a spined tegument in *P. longicaecum* (Pérez-Ponce de León *et al.* 2011). The species was assigned to the family Brachycoeliidae Looss, 1899. After examining the type-material, the authors confirmed that *C. rhyacotritoni* does not possess spines in the tegument. It is also necessary to obtain DNA sequences of specimens from the type locality to test the validity of the species parasitizing the Olympic salamander.

Our phylogenetic results are in agreement with previous topologies, which resolved the genus *Creptotrema* as monophyletic (Francheschini *et al.* 2021), with the new species placed as the early

divergent species of all other *Creptotrema* spp. Even though *C. funduli*, a Nearctic species, was described as belonging to *Creptotrema*, molecular evidence unequivocally has shown that the species does not belong in the new concept of the genus (Franceschini *et al.* 2021), in the same way *Creptotrema agonostomi* Salgado-Maldonado, Cabañas-Carranza & Caspeta-Mandujano, 1998 from the Mountain Mullet in Mexico was shown not to belong in *Creptotrema* (Pérez-Ponce de León *et al.* 2020). Actually, these authors suggested that a new genus was necessary to accommodate the species occurring in the Nearctic, while Franceschini *et al.* (2021) proposed the species as *species inquirenda*.

The lowest 28S rDNA interspecific genetic divergence found between *Creptotrema cruste* n. sp. and its congeners was 2.0% (*C. astyanace*). Franceschini *et al.* (2021) found lower 28S rDNA interspecific genetic divergence between sequences of *C. schubarti* and *C. guacurarii* (0.4%), although the divergence value was accepted by those authors as evidence for differentiating between these species, along with meticulous morphological examination. Although it is known that the 28S rRNA gene is a conserved gene and is not an ideal molecular marker for the delimitation of species, we consider our values of interspecific divergence as strong evidence to support the erection of *Creptotrema cruste* n. sp. The 28S rRNA gene remains the basis of the species-based molecular classification scheme of trematodes thus far (Pérez-Ponce de León *et al.* 2019). This output is also concordant with the phylogeny based on the COI mtDNA, in which the lowest interspecific genetic divergences found between *Creptotrema cruste* n. sp. and its congeners was 15.1% (*C. megacetabulare*). Franceschini *et al.* (2021) found COI mtDNA genetic divergences between *Creptotrema* spp. varying from 6.6 to 16.4%, which is also in agreement with our results. Previous studies have suggested that trematodes show maximum intraspecific divergence up to 2% for mtDNA to be considered a single species (Vilas *et al.* 2005). This is considerably below the observed values for the *Creptotrema* species analyzed here. Therefore, the COI mtDNA interspecific values yielded in this study strongly validate *Creptotrema cruste* n. sp. as a new species.

Recently, a revised diagnosis of *Creptotrema* was proposed by Franceschini *et al.* (2021). By using molecular evidence, those authors identified the genus *Auriculostoma* as a synonym of *Creptotrema*, reallocating all described species of the former *Auriculostoma* as members of *Creptotrema*. Therefore, considering the new diagnosis of *Creptotrema* proposed by Franceschini *et al.* (2021), *A. oclroya* (Liquin *et al.* 2022), which was described after the new revision of the genus, should now be considered as *Creptotrema oclroya* n. comb.

The current knowledge of parasites associated with amphibians is still limited, and further parasitological surveys are needed to increase our understanding of the helminth diversity and their relationships with this host group. Reducing taxonomic gaps is a challenge that implies the parasitological exploration of more host species and the use of standard methods for comparative morphological and molecular analyses. Our results contribute substantially to the progress of the global parasite taxonomy, improve the understanding of the phylogenetic relationships of allocreadiid trematodes, and expand the knowledge on the interaction network of parasite species with amphibian hosts in South America.

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Competing interest. The authors declare no competing interests with anyone.

Ethical standard. All applicable institutional, national, and international guidelines for the ethical handling of animals and collection of zoological material were followed (SISBio #61940), including recommendations from the Ethics Committee for Animal Experimentation (CEUA-UNESP #1061). According to Brazilian laws, species registration for scientific research purposes was carried out at SisGen (#A5DB3BE).

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