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Research paper

Novel diversity and distributions of myxozoans in amphibians from Ecuador with the description of a new species of *Cystodiscus*

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ABSTRACT

Among parasites with vertebrate hosts, myxozoans (Cnidaria) remain some of the least studied both taxonomically and geographically. We conducted the first reported surveys for myxozoans from amphibian hosts in Ecuador at two localities: a mid-elevation cloud forest on the Chocó region (western slopes of the Andes) and a lowland Amazonian tropical forest, east of the Andes. We sampled 177 gall bladders and 17 kidneys across the surveys. We found no evidence of myxozoans in the cloud forest site. Myxozoans of the genus *Cystodiscus* were encountered in the gallbladders of multiple amphibian species from the Amazonian rainforest site, including new host records. Our molecular phylogenies show that, while many of these myxozoans were referable to a clade of *C.* cf. *immersus*, we also discovered a divergent lineage of *Cystodiscus* in the gall bladder of a host that, unlike other known amphibian hosts, has arboreal oviposition. We describe this lineage as a new species, *Cystodiscus insperatus* n. sp., and infer transmission scenarios consistent with the unique ecology of its frog host. We also report for the first time molecular evidence of a possible new lineage of *Sphaerospora* living in the kidneys of *Osteocephalus taurinus* (Anura). Collectively, our study highlights the potential for (i) large biogeographic barriers (like the Andes) to influence the distribution of myxozoans and (ii) intermediate host ecology to drive the evolution of novel lineages of these parasites.

1. Introduction

Myxozoans are simplified multicellular microscopic cnidarians that are obligatory parasites found in marine and freshwater environments. There are two classes – Malacosporea Canning, Curry, Feist, Longshaw & Okamura, 2000 with five species, and Myxosporea Bütschli, 1881 which contains the vast majority of 2596+ described species [1]. Myxozoans have complex life cycles involving an intermediate vertebrate host and a definitive invertebrate host [2,3]. Among vertebrates, myxozoans are mainly known from fish [2], but they are also found in reptiles [4,5], amphibians [6,7], terrestrial mammals [8,9] and waterfowl [10]. The only completely known life cycles are from myxozoans that infect fish as intermediate hosts [11]. Transmission to new hosts in the life cycle is achieved by multicellular infectious spores produced within plasmodia and pseudoplasmodia. Studies to date indicate that short-lived spores are released by invertebrate hosts (annelids or freshwater bryozoans) in water to infect vertebrate hosts. Spores produced by myxosporeans ('myxospores') during infection of vertebrate hosts are long-lived with transmission eventually achieved by ingestion of spores by invertebrate hosts.

In amphibians, myxozoan infections are mostly found in the gall bladder and urogenital system (see [8] and references therein), and rarely in skin [12]. Infections can also occur in liver and brain [13]. There are currently 29 species of myxosporean myxozoans reported from amphibians (Supplementary Table S1), including all three orders of Amphibia (Anura, Caudata and Gymnophiona). These myxosporean species belong to seven genera (number of species in parenthesis): *Caudomyxum* (1), *Chloromyxum* (4), *Cystodiscus* (11), *Hoferellus* (1), *Myxobolus* (6), *Sphaerospora* (4), *Zschokkella* (2). No malacosporean infections have been reported in amphibians. However, myxozoans have been largely overlooked in amphibians. Thus, there are major gaps in our understanding of the diversity, taxonomy, geographical

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distributions, and transmission of myxozoans that infect amphibians. For instance, it remains unknown whether infection occurs during the amphibian host's larval phase and/or post-metamorphosis. Amphibians have the largest diversity of breeding modes among tetrapod vertebrates, varying from fully aquatic to fully terrestrial reproduction [14]. Whether this diversity of amphibian reproductive modes is linked with

Table 1
Anurans from Ecuador and prevalence of *Cystodiscus* in gall bladder and *Sphaerospora* in kidney. Microscopy: numbers indicate infected specimens/examined specimens. PCR: numbers indicate positive result/specimens analysed. Names in bold denotes positive infection. (*) New host record; (†) PCR shows faint band but Sanger sequencing did not work. Abbreviations: gall bladder, Gb; kidney, K; life cycle, LC; biphasic (i.e., part of the cycle involves water), B; terrestrial, T; Amazon - eastern side of the Andes, E; cloud forest - western side of the Andes, W.

Taxa	Microscopy Gb	PCR Gb	PCR K	LC	Site
ANURA					
Aromobatidae					
Allobates femoralis* (Boulenger, 1884)	1/2	1/2	–	B	E
Allobates insperatus (Morales, 2002)	0/1	0/1	–	B	E
Bufonidae					
Rhaebo guttatus* (Schneider, 1799)	2/2	2/2	–	B	E
Rhaebo haematiticus (Cope, 1862)	0/3	0/2	–	B	W
Rhinella bella Menéndez-Guerrero, Santos, SalazarNicholls, Green, and Ron, 2024	0/2	0/2	–	B	W
Rhinella acutirostris* (Spix, 1824)	4/5	5/5	0/5	B	E
Rhinella* cf. *dapsilis* (Myers and Carvalho, 1945)	1/1	1/1	0/1	B	E
***Rhinella marina* (Linnaeus, 1758)**	3/5	3/4	0/1	B	E
Centrolenidae					
Espadarana prosoblepon (Boettger, 1892)	0/8	0/8	–	B	W
Sachatamia ilex (Savage, 1967)	0/1	0/1	–	B	W
Craugastoridae					
Craugastor longirostris (Boulenger, 1898)	0/6	–	0/1	T	W
Dendrobatidae					
Ameerega bilinguis (Jungfer, 1989)	0/1	0/1	–	B	E
Ameerega hahneli (Boulenger, 1884)	0/1	0/1	–	B	E
Ranitomeya ventrimaculata (Shreve, 1935)	0/1	0/1	–	B	E
Hylidae					
Boana calcarata (Troschel, 1848)	0/4	–	0/1	B	E
Boana cinerascens* (Spix, 1824)	1/2	1/2	–	B	E
Boana lanciformis (Cope, 1871)	0/4	–	0/2	B	E
Boana pellucens (Werner, 1901)	0/1	–	–	B	W
Boana picturata (Boulenger, 1899)	0/1	–	–	B	W
Callimedusa tomopterna (Cope, 1868)	0/3	0/1	–	B	E
Dendropsophus bifurcus (Andersson, 1945)	0/6	–	–	B	W
Dendropsophus cannatellai Aguirre, Apunte, and Ron 2025	0/5	–	–	B	W
Dendropsophus rhodopeplus (Günther, 1858)	0/3	–	–	B	W
Hyloscirtus mashpi Guayasamin, et al., 2015	0/2	–	–	B	W
Osteocephalus planiceps Cope, 1874	0/1	–	–	B	E
Osteocephalus taurinus* Steindachner, 1862	0/5	0/4	3/4	B	E
Osteocephalus yasuni* Ron and Pramuk, 1999	1/12	1/5	0/1	B	E
Phyllomedusa tarsius (Cope, 1868)	0/2	0/1	–	B	E
Phyllomedusa vaillantii* Boulenger, 1882	1/1	1/1	–	B	E
Scinax cruentomma (Duellman, 1972)	0/1	–	–	B	W
***Scinax ruber* (Laurenti, 1768)**	1/3	1/3	0/1	B	E
Scinax sugillatus (Duellman, 1973)	0/4	–	–	B	W
Scinax tsachila Ron, Duellman, Caminer, and Pazmiño, 2018	0/7	–	–	B	W
Leptodactylidae					
Adenomera andreae (Müller, 1923)	0/1	–	–	B	E
Engystomops petersi* Jiménez de la Espada, 1872	1/1	1/1	–	B	E
Leptodactylus pentadactylus (Laurenti, 1768)	0/1	–	–	B	E
Leptodactylus ventrimaculatus Boulenger, 1902	0/9	0/4	–	B	W
***Leptodactylus wagneri* (Peters, 1862)**	1/4	1/2	–	B	E
Lithodytes lineatus (Schneider, 1799)	0/2	–	–	B	E
Microhylidae					
Chiasmocleis bassleri* Dunn, 1949	1/5	1/2	–	B	E
Strabomantidae					
Oreobates quixensis Jiménez de la Espada, 1872	0/5	0/4	–	T	E
Pristimantis achatinus (Boulenger, 1898)	0/11	0/5	–	T	W
Pristimantis conspicillatus (Günther, 1858)	0/8	0/4	–	T	E
Pristimantis kichwarum Elmer and Cannatella, 2008	0/1	0/1	–	T	E
Pristimantis diadematus (Jiménez de la Espada, 1875)	0/1	–	–	T	E
Pristimantis labiosus (Lynch, Ruiz-Carranza, and Ardila-Robayo, 1994)	0/11	–	–	T	W
Pristimantis lanthanites (Lynch, 1975)	0/1	–	–	T	E
Pristimantis malkini (Lynch, 1980)	0/4	0/2	–	T	E
Pristimantis pteridophilus (Lynch and Duellman, 1997)	0/2	–	–	T	W
Pristimantis variabilis (Lynch, 1968)	0/1	–	–	T	E
Ranidae					
Lithobates palmipes* (Spix, 1824)	1/2	1/2	1/1†	B	E
CAUDATA					
Plethodontidae					
Bolitoglossa sp.	0/1	–	–	T	E
Total: 11 new host species	**19/177**	**19/67**	**4/17**	**B = 125; *T* = 52**	**E = 104; W = 73**

myxozoan infection or evolution is unknown.

Here we report on the first surveys for myxozoans in amphibians with diverse reproductive modes from two disparate sites in Ecuador. Our data include many new host records. We also describe a new myxozoan species discovered in a host characterised by exclusively terrestrial adults. This is the first myxozoan known to exploit amphibians with a life history involving adults that do not lay eggs in water.

2. Material and methods

We examined a total of 177 gall bladders of adult amphibians from Ecuador. Species sampled belong to two orders (Anura and Caudata), 10 families and 23 genera. Their breeding modes vary from fully aquatic (i.e., eggs and larvae developing in water) to fully terrestrial (i.e., direct development). Table 1 indicates species with biphasic (at least part of the life cycle involves water) and terrestrial life cycles. Specimens were collected from two sites: Milpe Bird Sanctuary, a montane evergreen forest site in the western slopes of the Andes (0°01′51.2"S, 78°52′01.9"W, ca. 1100 m above sea level), and the Yasuní Field Station, a humid tropical rainforest site within the Ecuadorian Amazon (0°40′26.0"S, 76°23′54.6"W, ca. 200 m above sea level). Although both sites are geographically close (airline distance ~280 km) they are separated by Andean highlands above 4000 m. Variation in the amphibians sampled may thus reflect the barrier effect of the Andes in driving distinct amphibian communities. The complete list of amphibian specimens analysed in this study is shown in Table S2.

Amphibians were collected by hand during day and night surveys, placed in plastic bags and taken to the laboratory where they were humanely euthanised using an overdose of Tricaine Methanesulfonate (MS222). Gall bladders were removed, using tools that were sterilised with bleach between samples, and preserved in 100 % ethanol. Kidneys were opportunistically collected from 17 individuals and preserved in 100 % ethanol (see Table S2). The gall bladder material and kidney samples were shipped to the Natural History Museum, London for further study. Voucher specimens of the hosts were deposited in the Museo de Zoologia of the Pontificia Universidad Católica del Ecuador (Quito, Ecuador) and the Natural History Museum, London (London, United Kingdom). Fieldwork and sample exportation were conducted with permits from the Ministerio del Ambiente, Agua y Transición Ecológica of Ecuador (No 009–2024-EXP-IC-DBI/MAATE).

A chi-squared test (two tailed) of the null hypothesis that infection is independent of provenance (east and west of the Andes) was implemented in RStudio [15]. Given the low number of infected specimens from each species, we pooled all hosts within provenances for this analysis. Although there were no species in common between the East and West sites, we did sample closely related, ecologically similar species (e.g., *Rhinella bella* [West] / *Rhinella marina* [East]; *Rhaebo haematiticus* [West] / *Rhaebo guttatus* [East]; *Leptodactylus wagneri* [West] / *Leptodactylus ventrimaculatus* [East]).

2.1. Morphological analyses

For wet mount examination of gall bladders (previously preserved in 100 % ethanol) and their contents, an incision was made along the length of the organ. Bile was then drained onto a slide and immediately covered with a cover slip. Slides were examined at 10× and 40× magnification using a Nikon Eclipse 50i. Pipette tips were used to scrape the gall bladder to release potential myxozoan plasmodia or earlier stages associated with the wall of the organ. Photographs were taken of positive samples using a 100× oil immersion objective (Leica CTR500). Slides that contained myxospores were washed with 100 % ethanol into a sterile watch glass, and pipetted into a 1.5 ml microtube, and retained for morphological investigation. Additional digital photographs of myxospores and plasmodia were taken at 100× magnification using Olympus BX46 with a BP21 Camera. Myxospore measurements followed recommendations of [16] but using the term polar tubule instead of polar filament [17]. Measurements were taken from digital images using ImageJ 1.47v [18] and are given in μm as the mean ± standard deviation with the range in parentheses. Myxospores were air dried directly onto glass slides, stained with Epredia™ Shandon™ Kwik-Diff™ Stains (Thermo Fisher Scientific; Waltham, Massachusetts) and mounted with Neo-mount™ non-aqueous mounting medium (Merck; Darmstadt, Germany).

2.2. Molecular analyses

Total DNA was extracted from half the gall bladder of 54 samples, including all confirmed by microscopy to be positive for myxozoans, using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol. Total DNA was also extracted from small fragments of the kidney ($n = 17$). For gall bladders, two adjustments were made to the extraction protocol: Proteinase K input was 5 μl, and a third wash stage using 200 μl ethanol was added to further reduce potential contaminants.

In order to compare our samples to other amphibian myxozoans for which molecular sequence data are available, we amplified a fragment of the small ribosomal subunit (18S rRNA) using myxosporean specific primers Myxospec F (5'-TTC TGC CCT ATC AAC TWG TTG-3′) and Myxospec R (5′-GGT TTC NCD GRG GGM CCA AC-3′) [19]. Additionally, the ITS*1*–*5.8S*–ITS*2* region (referred to as ITS) was amplified using G-ITS-For (5′-GGG ATC CGT TTC CGT AGG TGA ACC TGC-3′) and ITS-Zschok-Rev (5′-GAT TCT CAT AGT AAC TGC GAG TG-3′) [20]. The relative positions of the DNA regions amplified here are illustrated in Fig. 1. The expected sizes of the amplified fragments for the 18S and ITS regions for gall bladder myxozoans are 850 bp and 900 bp, respectively. Polymerase Chain Reactions (PCRs) were conducted in 20 μl reactions using the Promega GoTaq G2 Master Mix. Each reaction contained 2 μl of the extracted genomic DNA, 1 μl of each primer, 5 μl of 5× Buffer, 4 μl of 25 mM MgCl2, 0.2 μl dNTPs 10 mM and 0.125 μl TaqG2Flexi and was completed with DNA-grade water. PCR reactions were run for both 18S rRNA and ITS on a Veriti 96 Well Thermal Cycler (Applied Biosystems) under the following conditions: 95C for 5 min; 35 cycles of 95C for 1 min, 55C for 1 min, 72C for 1 min; elongation at 72C for 7 min. All reactions were run with negative controls containing PCR water. PCR products were visualised in 1 % agarose gel in Tris-acetate-EDTA buffer stained with GelRed (Biotium) and visualised via a UV-transilluminator. Uncleaned PCR products were purified using Appleton Woods AppMag magnetic PCR purification beads. Samples were then quantified and normalised before being Sanger sequenced, using BigDye Terminator V3.1. Reactions were then loaded onto a 3730xl DNA capillary analyser (Applied Biosystems) at the NHM sequencing facility. Chromatogram inspection and sequence assemblies were performed in Geneious Prime 2023.1.2 (https://www.geneious.com). Sequences generated in this study are deposited at GenBank (Accession numbers PX603274-PX603293 and PX606605-PX606621).

2.3. Phylogenetic analyses

Sequences produced in this study were compared to the online nucleotide database from NCBI using the Basic Local Alignment Search Tool (BLAST®). The closest hits for both genes belonged to species from the genus *Cystodiscus* Lutz, 1889, and these sequences were downloaded for phylogenetic analyses. There are currently 11 species of *Cystodiscus* formally described, the nine included in Hartigan et al. (2012b), plus *C. boulengeri* Li, 1993 and *C. elachsitocleis* Vieira et al., 2021, but DNA sequences are only available for six of them. Sequences of 18S rRNA of *Sphaerospora* sp. (EU371498) and *Zschokkella auratis* (ON054249) were downloaded as outgroups for the gall bladder samples (no outgroups were available for the ITS region), and of *Sphaerospora truttae* (AJ581915) as an outgroup for the kidney samples.

Multiple sequence alignments were made using MAFFT v7.490 [22,23] applying 200PAM (k = 2) scoring matrix, a gap open penalty

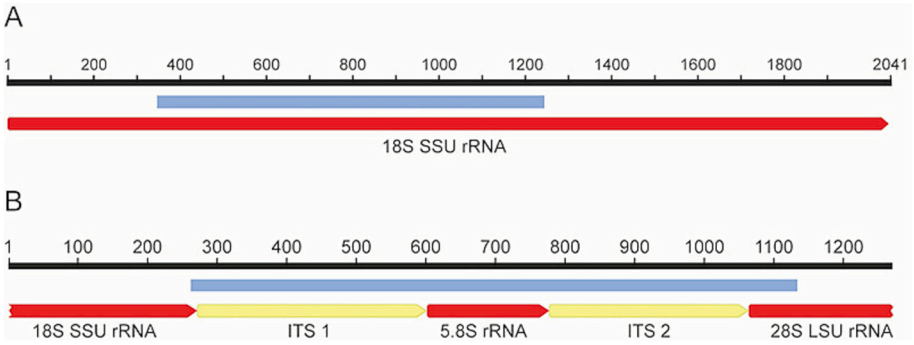

Fig. 1. Molecular regions sequenced for this study. A) 18S SSU rRNA region; B) Partial 18S SSU rRNA, ITS 1, 5.8S, ITS 2 and partial 28S LSU rRNA. A blue bar indicates the PCR-amplified regions while red and yellow arrows indicate the annotated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1.53, offset value 0.123, and automatic selection of best algorithm according to data size. Poorly aligned positions were masked using Gblocks v0.91b [24] with 'Minimum Length of a Block' set to 5 (following the developers' recommendation for DNA alignments), and 'Allowed Gap Positions' set to 'All' (gall bladder sequences) and 'Half' (kidney sequences). Default settings were used for all other parameters. ModelTest [25] was implemented in RAxML-NG GUI 2.0 [26] to determine the best nucleotide substitution model using the corrected Akaike information criterion (AICc). A phylogeny was inferred using maximum likelihood (ML) in RAxML-NG applying the ML + thorough bootstrap + consensus analysis, with 1000 bootstrap replicates. The consensus trees were visualised in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Uncorrected pairwise distances were calculated in PAUP* v.4.0a [27] using the alignments from the ML analyses. Number of nucleotide differences per site was calculated in Geneious using the same alignments with all sequences trimmed to the same length as the shortest sequence.

3. Results

Myxozoans were only detected in samples from the Yasuní Field Station, in the eastern side of the Andes (Table 1). Samples from this site represent 59 % (104/177) of all frogs examined, and 19/104 (18 %) were confirmed to be infected either by microscopy or by PCR. Only frogs with biphasic life cycles were infected. A chi-square test of independence was performed to test the null hypothesis that the probability of infection is independent of sample location, and thus an equal chance of finding myxozoans in specimens from the east and west sides of the Andes. Our significant result indicates that the probability of infection likely depends on site (x^2 (df = 3, N = 176) = 15.498, p = 0.0014).

3.1. Morphological analyses

Microscopic analyses revealed the presence of myxosporean plasmodia and/or myxospores in 19 individuals from 13 species of anurans (Table 1). The infected species belong to five families (Aromobatidae, Bufonidae, Hylidae, Leptodactylidae, Microhylidae). Plasmodia were found in the bile or attached to the internal wall of the gall bladder, and myxospores were observed floating in the bile. Kidney samples of three individuals of *Osteocephalus taurinus* were confirmed to be infected (PCR analyses) but no microscopy was conducted. Eleven of the 14 species of anurans infected represent novel host records for myxozoans (*Allobates femoralis*, *Boana cinerascens*, *Chiasmocleis bassleri*, *Engystomops petersi*, *Lithobates palmipes*, *Osteocephalus taurinus, O. yasuni*, *Phyllomedusa vaillantii*, *Rhaebo guttatus*, *Rhinella acutirostris*, and *Rhi.* cf. *dapsilis*), three of them include new records for the host genus (*Osteocephalus*, *Phyllomedusa* and *Rhaebo*). The morphology of myxospores found in the gall bladders was consistent with myxosporean species in the genus *Cystodiscus* Lutz, 1889 (Figs. 2 and 3). Herein, we describe a new species (Fig. 2 A-D, Table 2) and provide morphometric data of myxospores obtained in this study (Fig. 3 A-E and Table 2).

3.2. Description of new species

Phylum Cnidaria Hatschek, 1888
Unranked subphylum Myxozoa Grassé, 1970
Class Myxosporea Bütschli, 1881
Order Bivalvulida Schulman, 1959
Suborder Variisporina Lom et Noble, 1984
Family Myxidiidae Thélohan, 1892
Genus *Cystodiscus* Lutz, 1889
***Cystodiscus insperatus* n. sp.**

3.2.1. Description of myxospores

Based on 18 myxospores fixed in 100 % ethanol from the bile of one host by light microscopy. Myxospores ellipsoid in valvular view, fusiform in sutural view (Fig. 2 A-D) measuring 11.8 ± 0.7 μm (11.0–13.2 μm; n = 18) in length, 7.7 ± 0.9 μm (6.3–9.9 μm; n = 15) in width, and 6.9 ± 0.6 μm (6.4–7.5 μm; n = 3) in thickness. Two valves with 4–5 surface ridges, transverse to sutural plane. S-shaped suture bisects the spore. Two equal subspherical polar capsules, 3.6 ± 0.5 μm (2.7–5.0 μm; n = 35) long and 3.3 ± 0.4 μm (2.7–4.3 μm; n = 35) wide (Fig. 2 A-D). Polar capsules at each end of the spores, opening in opposite directions at sutural plane. Polar tubule allocated in 3 coils. Sporoplasm binucleate, in middle of spore.

3.2.2. Taxonomic summary

Type-host: *Phyllomedusa vaillantii* Boulenger, 1882.

Type-locality: Yasuní Biological Station, Orellana, Ecuador (0°40′26.1"S 76°23′54.5"W; 223 m).

Site of infection: Gall bladder.

Prevalence: Detected in one male host (prevalence 1/1).

Type material: DNA extraction (deposited at QCAZ) along with photos of spores QCAZA78186.

Representative DNA sequences: GenBank PX603274 and PX606605 ex *Phyllomedusa vaillantii* (846 bp; 845 bp).

ZooBank registration: The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub: 0D3BF1A5-74CC-4C68-B42C-AE916CBD649D. The LSID for the new name *Cystodiscus insperatus* n. sp. is urn:lsid:zoobank.org:act: 55AE36A9-0955-46AD-AE4B-BAB60E64704E.

Etymology: The specific epithet *insperatus* is a Latin adjective meaning unexpected/unforeseen and is used in reference to the unexpected discovery of this species that occurs in sympatry with other *Cystodiscus*.

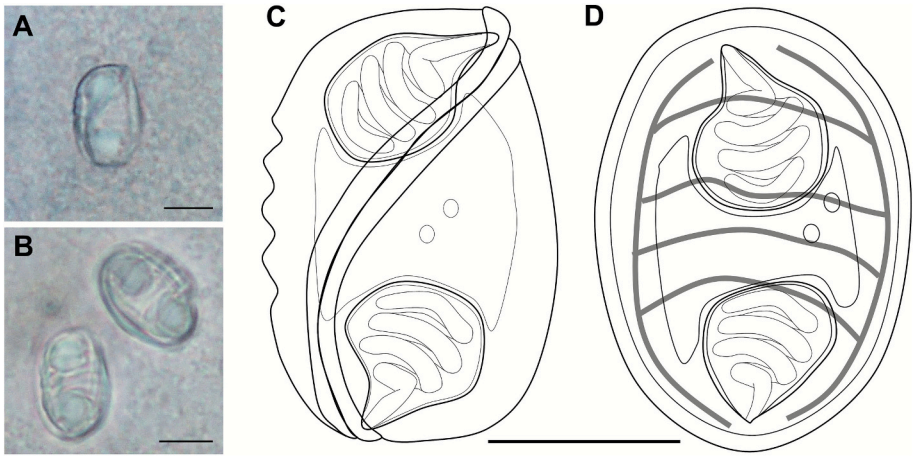

Fig. 2. . Myxospores morphology of *Cystodiscus insperatus* sp. n. A) light microscopy images of a spore, sutural view, showing S-shaped suture and surface ridges on the side; B) valvular view, notice surface ridges on both spores; C—D) Schematic drawing of the myxospore, C) sutural view and D) valvular view. Scales: 5 μm.

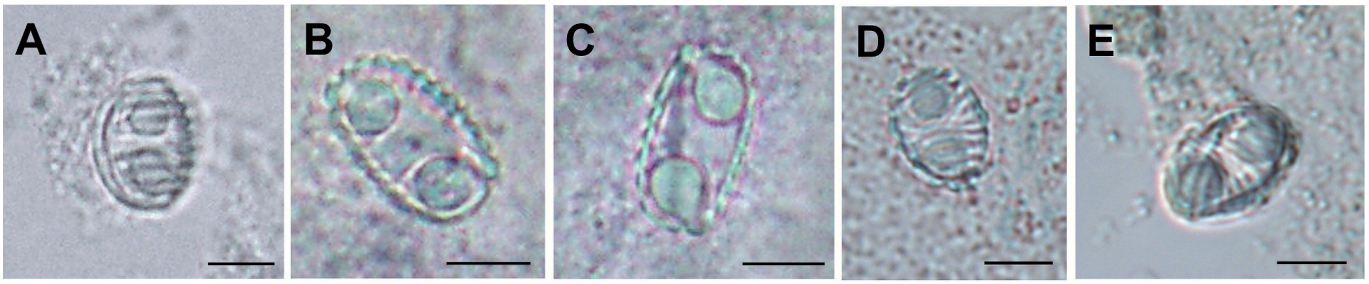

Fig. 3. Myxospores morphology of *Cystodiscus* spp. A) *Cystodiscus* sp. (Ecuador 2) ex *Leptodactylus wagneri* (GB00214), valvular view; B) *Cystodiscus* sp. (Ecuador 2) ex *Rhinella marina* (GB00216), valvular view and C) sutural view; D) *Cystodiscus* sp. (Ecuador 2) ex *Osteocephalus yasuni* (GB00256), valvular view; E) *Cystodiscus* sp. (Ecuador 3) ex *Scinax ruber* (GB00196) (partial) sutural view. Scales: 5 μm.

Table 2
Morphological data of myxospores detected from gall bladders of frog hosts collected in this study. Myxospores were fixed in 100 % ethanol. Data for Cystodiscus species refer to clades in Table 3 and the new species. Abbreviations: SL.- spore length; SW.- spore width; ST.- spore thickness; PCL.- Polar capsule length; PCW.- Polar capsule width. Measurements are given in μm as the mean ± standard deviation with the range in parentheses. Spore morphology shown in Fig. 2 (*C. insperatus* n. sp.) and Fig. 3 A-E for other undescribed species.

Cystodiscus spp.	Host	Number of spores	SL	SW	ST	PCL	PCW
Ecuador 2	*Leptodactylus wagneri* (GB00214)	6	10.6 ± 0.5 (10.2–11.5)	7.5 ± 0.4 (6.9–7.9)	NA	3.3 ± 0.3 (2.8–3.8)	2.8 ± 0.3 (2.2–3.3)
Ecuador 2	*Rhinella marina* (GB00216)	46	11.6 ± 0.6 (10.2–12.7)	7.1 ± 0.7 (5.9–8.5)	6.8 ± 0.3 (6.4–7.4)	3.6 ± 0.4 (2.9–4.5)	3.1 ± 0.3 (2.3–3.8)
Ecuador 2	*Osteocephalus yasuni* (GB00256)	9	10.3 ± 0.5 (9.5–11.0)	7.7 ± 0.6 (6.9–8.7)	NA	3.2 ± 0.2 (2.8–3.6)	3.0 ± 0.2 (2.6–3.4)
Ecuador 3	*Scinax ruber* (GB00196)	13	10.3 ± 0.6 (9.1–11.0)	7.0 ± 0.6 (5.5–7.6)	NA	3.1 ± 0.3 (2.4–3.5)	2.8 ± 0.3 (2.1–3.6)
C. insperatus n. sp.	*Phyllomedusa vaillantii* (GB00314)	3–35	11.8 ± 0.7 (11.0–13.2; n = 18)	7.7 ± 0.9 (6.3–9.9; n = 15)	6.9 ± 0.6 (6.4–7.5; n = 3)	3.6 ± 0.5 (2.7–5.0; n = 35)	3.3 ± 0.4 (2.7–4.3; n = 35)

3.2.3. Remarks

Cystodiscus insperatus n. sp. has overall myxospore morphology characteristic of the genus *Cystodiscus*. For differential diagnosis we selected all species of *Cystodiscus* described to date (Table S3). The new species differs from its congeners in sizes of spores and polar capsules (Table S3), in addition to the frog host species. Further notable differences include the number of polar tubule coils and variation in transverse ridges on valves. *Cystodiscus australis* Hartigan, Fiala, Dyková, Rose, Phalen & Šlapeta, 2012, *C. axonis* Hartigan, Fiala, Dyková, Rose, Phalen & Šlapeta, 2012, *C. boulengeri* (Li, 1993), *C. melleni* (Jirků, Bolek, Whipps, Janovy, Kent and Modrý 2006) and *C. typhonius* (Gray, 1993) possess ≥4 polar tubule coils while the new species has 3 coils. *Cystodiscus immersus* has 7–9 transverse ridges, *C. typhonius* has 9–11, *C. serotinus* (Kudo and Sprague, 1940) has 10–13, *C. lesminteri* (Delvinquier, Markus and Passmore, 1992) has none, and the new species has 4–5.

The new species shows the greatest morphometric similarity to *C. immersus* and *C. lindoyense* (Carini, 1932), with overlapping ranges in several measurements. However, *C. insperatus* n. sp. has narrower spores, smaller polar capsules and differing number of transverse ridges (4–5 vs 7–9) than these two species and differs in frog host (*Phyllomedusa vaillantii* vs *R. marina*) and geographic location (Ecuador vs Brazil).

3.3. Molecular and phylogenetic analyses

Table 1 summarises the results from myxozoan DNA amplification of

gall bladder and kidney samples from the sampled Ecuadorian amphibians. A total of 17 novel sequences of the 18S rRNA and 17 of the ITS region were generated from the myxozoans found in gall bladder samples. Two additional samples (*Lithobates palmipes* GB00234 and *R. marina* GB00278) had positive PCR results using the 18S rRNA primers (Myxospec F + R) but the sequences were of poor quality possibly due to mixed infection. BLAST searches resulted in closest hits to the genus *Cystodiscus*.

For the gall bladder samples, the 18S rRNA alignment consisted of 53 sequences and 862 positions, of which 3.54 % are gaps and 71.93 % invariant sites (data based on model of nucleotide substitution TPM2uf + FO + I + G4m). The ITS alignment consisted of 63 sequences and 887 positions, of which 6.33 % are gaps and 63.36 % invariant sites (data based on model of nucleotide substitution K81uf + FO + G4m). For the kidney sequences, the 18S rRNA alignment consisted of seven sequences and 1147 positions, of which 7.56 % are gaps and 59.02 % invariant sites (data based on nucleotide substitution model TN93 + FO + G4m).

With the exception of a single sample isolated from the host *Scinax ruber* and the new species, samples from Ecuador form two clades referred to here as Ecuador 1 and Ecuador 2 (see Table 3 for lists of hosts associated with each clade). The 18S rRNA and ITS trees (Fig. 4) have different sets of taxa complicating comparison. However, in terms of shared taxa, in both trees the samples from Ecuador are all more closely related to *C. australis* and *C.* cf. *immersus* Brazil 1 and 2 than they are to *C. axonis* in both trees. The ITS tree has strong bootstrap support suggesting that *C.* cf. *immersus* is not monophyletic.

There is incongruence in the position of *C.* cf. *immersus* Brazil 1 and 2, which is flipped in the two trees and in each showing a closer relationship with the samples from Ecuador. There is also incongruence regarding the position of clades Ecuador 1 and 2, and the *Cystodiscus* found in *S. ruber*. However, while there is no support in the ITS tree, the relationship among these groups is otherwise well supported in the 18S tree.

Cystodiscus insperatus n. sp. from *P. vaillantii*, a tree frog that lays its eggs on leaves above water, does not group within *C.* cf. *immersus* (in both trees) and the other samples from Ecuador.

Average uncorrected pairwise distances (upd) and number of nucleotide differences per site for both genes are shown in Tables S4 and S5. Within-clade upd is <1 % for 18S, and ≤ 1 % for ITS, except *C. axonis* (2.1 %). Regarding the new species, *C.* cf. *immersus* Brazil 1 show the lower upd for 18S (3 %) and *C.* cf. *immersus* Brazil 2 (9.6 %) for ITS. The highest are *C.* sp. C WC2020 from China (9.7 %) for 18S and *C. axonis* (20.6 %) for ITS. Average pairwise distance between the two main clades from Ecuador (Ecuador 1 and 2) is 2 % for 18S and zero for ITS. Maximum likelihood trees characterising all samples included in the analyses is shown in Fig. S1.

The 18S region of *C. insperatus* n. sp. has a minimum of 27 (*C.* cf. *immersus* Brazil 1) and a maximum of 93 (*C.* sp. c WC-2020) nucleotide differences over 862 nucleotides. The ITS region of *C. insperatus* n. sp. has a minimum of 47 (*C.* cf. *immersus* Brazil 2) and a maximum of 113 (*C. axonis*) nucleotide differences over 887 nucleotides.

The PCRs of kidney samples revealed, for the first time, a myxozoan infection in *Osteocephalus taurinus* (prevalence = 3/4). No evidence of myxozoan infection was found in the gall bladders of these specimens. Sanger sequencing data revealed the average length of our 18S sequence is 1075 base pairs (bp), ca. 250 bp longer than the sequences of the gall bladder myxozoans (see comments in Discussion). The closest hits of the BLAST search are *Sphaerospora ranae* (EF211975; ex *Rana dalmatina*), *S.* sp. (JX286622; ex *Ptychadena anchietae*), and *S. ohlmacheri* (JX286619; ex *Aquarana catesbeiana*) – all known to infect renal system of anurans (e.g., [28,29]). Table 4 shows query cover, e-values and percentage identity of our samples in relation to their closest matches from GenBank. Phylogenetic analysis suggests closer relationship with *S. ohlmacheri* (Fig. 4 C).

4. Discussion

Our morphological and molecular data have identified 10 new amphibian hosts for myxozoans and have revealed high genetic diversity within the genus *Cystodiscus* in Ecuador (potentially four new species). We have described a new species of myxozoan based on morphology and molecular data that infects an amphibian host with non-aquatic oviposition, which suggests that infection may occur during larval development (in water) or directly to adult (out of water). In addition, we present the first record of *Sphaerospora* from a Neotropical amphibian and which may also be a new species.

Our study indicates that the taxonomy of even the better-studied groups of myxozoans that infect amphibians, like *Cystodiscus*, remains poorly resolved (e.g., [7,21,30]). For example, *C.* cf. *immersus* sensu Hartigan et al. ([21]) is not monophyletic (this work and [7]). Our results thus support the pervasive view that myxozoan taxonomy is challenged by a focus on unreliable characters that lead to extensive poly- and paraphyly [31–33]. Furthermore, because sequences from *C.* cf. *immersus* Brazil 1 and 2 belong to pools of samples of *R. marina* (from Manaus, Brazil), the phylogenetic position of these samples should be interpreted with caution. The type locality of *C. immersus* is not specified in the original description so it is reported as 'Brazil' [21]. Addition of further samples and new molecular markers may help to improve the resolution of *Cystodiscus* taxonomy.

The molecular phylogenetic placement of the *C. insperatus* n. sp. from

Table 3
***Cystodiscus* clades inferred by maximum likelihood analyses, amphibian hosts, and collection site.** (*) Specimens found around the same pond; (†) Sample only included in the 18S trees (‡) Sample only included in the ITS tree.

Cystodiscus clade	Field ID	Amphibian host	Latitude	Longitude
Ecuador 1	GB00201*	*Rhinella acutirostris*	0° 40′ 26.112" S	76° 23′ 54.492" W
	GB00236	*Rhinella acutirostris*	0° 40′ 18.3" S	76° 23′ 58.956" W
	GB00237	*Rhinella* cf. *dapsilis*	0° 40′ 19.992" S	76° 23′ 56.724" W
	GB00242	*Engystomops petersi*	0° 40′ 9.156" S	76° 24′ 5.148" W
	GB00268	*Chiasmocleis bassleri*	0° 40′ 25.608" S	76° 24′ 2.232" W
	GB00312	*Rhaebo guttatus*	0° 40′ 26.112" S	76° 23′ 54.492" W
Ecuador 2	GB00200*	*Rhinella acutirostris*	0° 40′ 26.112" S	76° 23′ 54.492" W
	GB00214	*Leptodactylus wagneri*	0° 40′ 26.112" S	76° 23′ 54.492" W
	GB00216	*Rhinella marina*	0° 40′ 26.112" S	76° 23′ 54.492" W
	GB00217	*Rhinella marina*†	0° 40′ 26.112" S	76° 23′ 54.492" W
	GB00234	*Lithobates palmipes*‡	0° 40′ 8.94" S	76° 24′ 3.492" W
	GB00256	*Osteocephalus yasuni*	0° 40′ 31.512" S	76° 23′ 55.536" W
	GB00258	*Allobates femoralis*	0° 40′ 30.036" S	76° 23′ 54.6" W
	GB00260	*Rhinella acutirostris*	0° 40′ 25.896" S	76° 24′ 2.376" W
	GB00295	*Boana cinerascens*	0° 40′ 31.404" S	76° 23′ 36.312" W
	GB00311	*Rhaebo guttatus*	0° 40′ 26.112" S	76° 23′ 54.492" W
Ecuador 3	GB00196	*Scinax ruber*	0° 40′ 26.112" S	−76.39847
C. insperatus **n. sp.**	GB00314*	*Phyllomedusa vaillantii*	0° 40′ 26.112" S	−76.39847

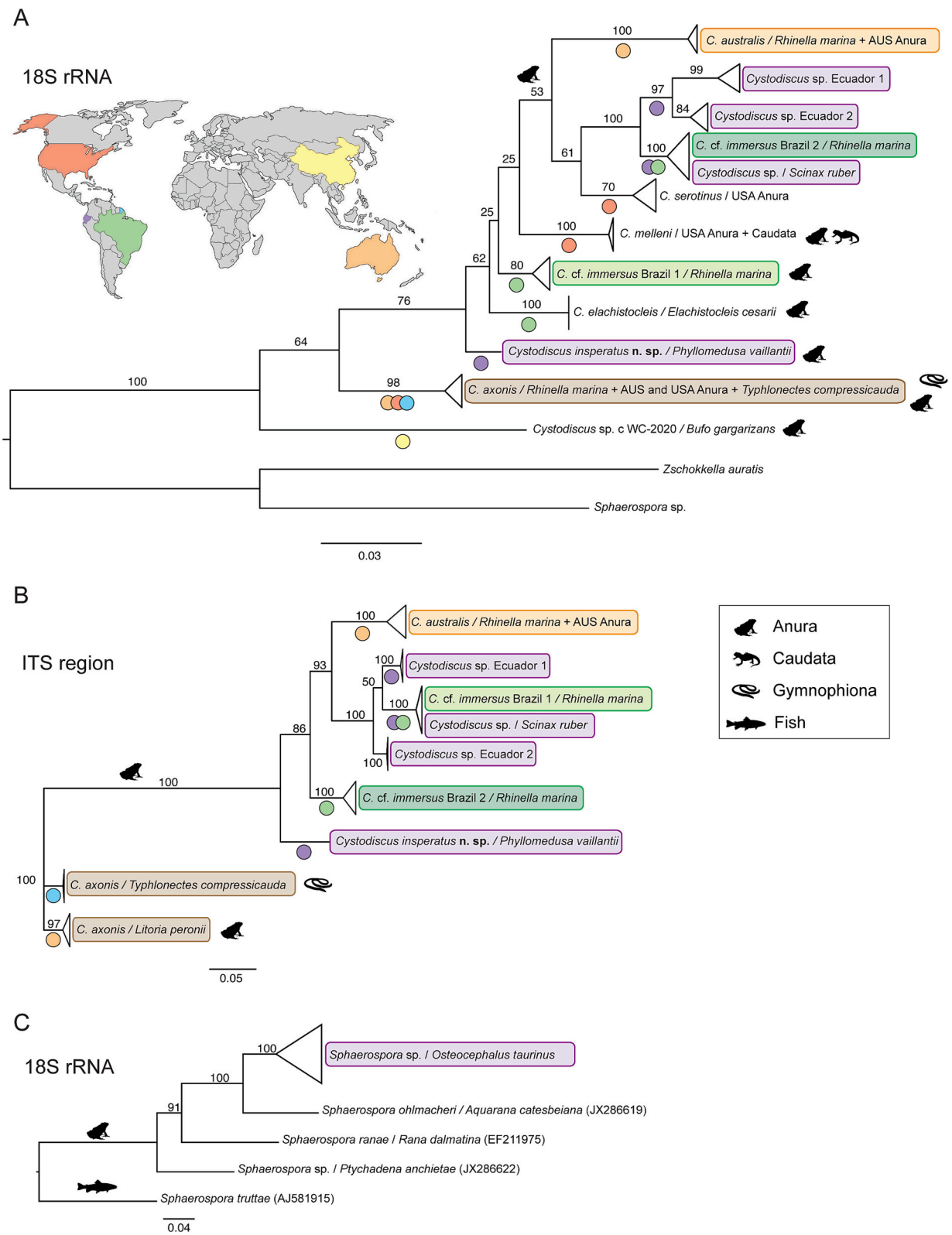

Fig. 4. Maximum likelihood phylogenies for the focal myxozoans inferred from 18S rRNA (A) and ITS (B) regions of gall bladder samples, respectively, and (C) 18S region of kidney samples. Note ITS region includes the *5.8S* rRNA sequence and a partial sequence of *28S* rRNA. Support values shown above branches correspond to support obtained from 1000 bootstrap replicates. Intermediate amphibian hosts are partially indicated using silhouetted images for anurans (frogs and toads), caudatans (newts and salamanders) and gymnophionans (caecilians). Scale bars values correspond to estimated substitutions per site. Coloured circles below branches and background colour of terminal names indicate the geographic distribution. Blue circles indicate occurrence in amphibians from the pet trade, so the point of infection is unclear. AUS = Australia, USA = United States of America. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Results from BLAST search of 18S sequence of myxozoan from *Osteocephalus taurinus* against GenBank nucleotide database. Abbreviations: Perc. Id = percentage identity.

Host ID	Length (bp)	Query cover	e-value	Perc. Id	Species	Accession #
		83 %	5e-155	**96 %**	*S. ranae*	EF211975
GB00210	1192	66 %	8e-143	94 %	*S.* sp.	JX286622
		85 %	0	88 %	*S. ohlmacheri*	JX286619
		83 %	3e-142	**95 %**	*S. ranae*	EF211975
GB00211	1175	65 %	3e-132	94 %	*S.* sp.	JX286622
		85 %	0	88 %	*S. ohlmacheri*	JX286619
		44 %	2e-78	**91 %**	*S.* sp.	JX286622
GB00248	829	43 %	8e-72	90 %	*S. ranae*	EF211975
		60 %	4e-104	86 %	*S. ohlmacheri*	JX286619

P. vaillantii suggests the evolutionary distinctiveness of the new species may be related to the ecology of its intermediate host. To the best of our knowledge, this is the first record of a myxozoan infecting an anuran that does not lay eggs directly in water. *Phyllomedusa vaillantii* is a tree frog that lays its eggs on leaves above water. The tadpoles drop into the water after hatching from the eggs to complete their metamorphosis [34]. At present, it is unclear whether infection of anuran hosts by myxozoans occurs during the host's larval phase or after metamorphosis. We speculate that infection of anurans may be achieved at the larval stage because adults of *P. vaillantii* rarely, if ever, enter the water. However, an alternate explanation is that transmission to adults is achieved on land – for example by ingestion of prey that are infected with *C. insperatus* n. sp., by direct adult frog-to-adult frog passage, or by consumption of or contact with infectious material (e.g., spores from moist leaf litter or within faeces) [35]. Despite its arboreal habits, *P. vaillantii* is often found in leaf litter [36]. Either speculative explanation provides novel insights on the ecology and biology of myxozoans infecting amphibians. At present terrestrial oligochaetes have not been identified as myxozoan hosts, although this is suggested in view of myxozoan infections in European moles (*Talpa europaea*) and several species of shrews (*Sorex araeus, Sorex minutus*, and *Crocidura suaveolens*) [35]. The divergence of the novel *P. vaillantii* lineage could thus reflect the adoption of a new mode of transmission in terrestrial environments.

All *Sphaerospora* reported from amphibians form a subclade within the freshwater *Sphaerospora* sensu stricto from fish [31]. The 18S rRNA sequences we obtained from Ecuadorean *Sphaerospora* are markedly longer than sequences of the same region from *Cystodiscus*. The notably longer size of the *Sphaerospora* SSU rRNA sequences is probably explained by extensive nucleotide insertions in the V4 and V7 regions [37,38]. The *Sphaerospora* found in the kidney of *Osteocephalus taurinus* from Ecuador is likely a new species – and represents the first case of finding *Sphaerospora* in a Neotropical amphibian. Further investigation is needed to compare the morphology of this lineage with *Sphaerospora* from other amphibians.

It is noteworthy that none of the amphibians we examined from the western side of the Andes were infected with myxozoans. Similarly, to the best of our knowledge, the only two records of freshwater myxozoan infections to the west of the Andes are from Chinese-native goldfishes *Carassius auratus* in Peru [39]. Considering that this is an ornamental fish, these records should be interpreted as suspicious. While this pattern allows us to speculate about the Andes being a geographical barrier to freshwater myxozoans, further research will be necessary to confirm this putative distributional pattern.

5. Conclusions

This study reveals a hidden diversity of myxozoans in anuran amphibians from Ecuador. Part of this reflects that many myxozoan infections are asymptomatic and thus go unnoticed [40,41]. However there has also been a long tradition of focusing on myxozoans in fish hosts [3]. Over 100 years have passed since the first amphibian myxozoan was discovered by Adolph Lutz [42] and still no life-cycle has been resolved. It is clear that there is much to be learned about interactions between amphibians and myxozoans, including when, where and how transmission is achieved in vertebrate hosts that use both aquatic and terrestrial environments. Myxozoan diversity is poorly known [43] and we predict that many amphibians are overlooked hosts. It is to be hoped that further work may begin to clarify patterns of diversity, host specificity, geographical distributions, host-parasite interactions, and co-infections with other parasites for myxozoans affiliated with amphibian hosts.

CRediT authorship contribution statement

Gabriela B. Bittencourt-Silva: Writing – original draft, Supervision, Project administration, Investigation, Conceptualization. **Gema Alama-Bermejo:** Writing – review & editing, Investigation. **Rebecca Higham:** Writing – review & editing, Investigation. **Santiago R. Ron:** Writing – review & editing. **Beth Okamura:** Writing – review & editing, Project administration, Funding acquisition. **Jeffrey W. Streicher:** Writing – original draft, Supervision, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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