

Broad but restricted detection of malacosporeans in a Neotropical cradle of diversification

Research Article

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Abstract

This study undertook the first investigation of malacosporean infections in Neotropical fish. We used polymerase chain reaction detection with a primer set generally targeting known malacosporeans to assay for infection in the kidney of 146 fish in 21 species belonging to 12 families collected from two areas in the Amazon Basin. Infections were found in 13 fish variously belonging to seven species in six families and included the first identification of a malacosporean infection in cartilaginous fish (a freshwater stingray). Based on ssrDNA, all infections represented a single *Buddenbrockia* species (*Buddenbrockia* sp. E) that demonstrates an exceptionally broad range of fish species infected, and countered our expectations of high Neotropical malacosporean diversity. Infections were characterized at varying and often high prevalences in fish species but sample sizes were small. Ascertaining whether highly divergent malacosporeans have not been detected by current primers, and more comprehensive sampling may reveal whether malacosporeans are truly as species poor in the Amazon Basin as present data suggest. Our results prompt speculations about evolutionary scenarios including introduction *via* marine incursions and patterns of host use over time.

Introduction

Parasites are diverse, ubiquitous and functionally important components of ecosystems (Hudson *et al.*, 2006; Kuris *et al.*, 2008; Carlson *et al.*, 2020). It is thus increasingly realized that parasites can pose major challenges for conservation biology, disease management and zoonoses in our changing world (Stephens *et al.*, 2016; Schmeller *et al.*, 2020). However, many parasites themselves may be more prone to extinction than their free-living hosts (Carlson *et al.*, 2020). Conservation of non-charismatic taxa such as parasites is poorly appreciated but nevertheless important in view of their ecological and evolutionary roles (Dougherty *et al.*, 2016; Okamura *et al.*, 2018). In addition, poor sampling compromises knowledge of patterns of host use (e.g. host specificity) and of parasite diversity (Poulin, 2014; Okamura *et al.*, 2018), thus greatly diminishing our understanding of organismal interactions and the tree of life. Parasite diversity has been shown to correlate with the diversity of potential hosts (Kamiya *et al.*, 2014) and would therefore be expected to peak in biodiversity hot spots. However, a recent investigation of spatial congruence between vertebrate host and helminth parasite diversity using global datasets highlights that poor knowledge of parasite diversity in regions of highest host species richness can obscure this relationship (Jorge and Poulin, 2018). Detecting and mapping global parasite diversity is therefore fundamental for identifying geographical hot spots of diversity, potential disease emergence, predicting and mitigating the impacts of climate change on the distributions of parasites and hosts, and improved understanding of biodiversity (Carlson *et al.*, 2020).

The Amazon River system has served as a cradle of diversification with lineages originating and accumulating over geological time in a biogeographic core of Neotropical diversity, as particularly documented for fishes (Reis *et al.*, 2016). The high diversity of this freshwater fauna reflects both ancient association with continental freshwaters since the formation of South America and enrichment by a number of marine-derived lineages *via* marine incursions from the Eocene to the Miocene (Bloom and Lovejoy, 2017). The presence of varied invertebrate and vertebrate hosts in this Neotropical cradle of diversification suggests that affiliated parasitic taxa may have diversified in concert with their hosts over time. The aim of this study was to begin to address this issue by examining a range of freshwater fish from the Amazon Basin for infections of an early diverging and poorly sampled clade of myxozoan parasites – the Malacosporea.

Myxozoans are microscopic obligate cnidarian endoparasites with complex life cycles, exploiting invertebrate (annelids and bryozoans) and vertebrate (primarily fish) hosts in freshwater and marine environments, comprising some 2600 species worldwide (Okamura *et al.*,

Table 1. Fish sampled from the Amazon Basin infected with *Buddenbrockia* sp. E

Region	Fish species (Family – Order)	Prevalence	ssrDNA sequence length and GenBank accession numbers	Closest malacosporean species in GenBank (% similarity)
Near Santarém	Pescada, <i>Plagioscion squamosissimus</i> (Sciaenidae – Perciformes)	5.7% (2/35)	1732 bp MT993755	<i>Buddenbrockia</i> sp. A (F) or <i>Buddenbrockia</i> sp. 3 (BS) KF731708 (97.96%)
	Tucunaré, <i>Cichla pinima</i> (Cichlidae – Cichliformes)	40% (2/5)	814 bp MT993756	<i>Buddenbrockia</i> sp. A (F) or <i>Buddenbrockia</i> sp. 3 (BS) KF731708 (98.16%)
	Branquinha, <i>Curimata inornata</i> (Curimatidae – Characiformes)	100% (4/4)	755 bp MT993762	<i>Buddenbrockia</i> sp. A (F) or <i>Buddenbrockia</i> sp. 3 (BS) KF731708 (97.88%)
	Piracatinga, <i>Calophysus macropterus</i> (Pimelodidae – Siluriformes)	25% (1/4)	783 bp MT993760	<i>Buddenbrockia</i> sp. A (F) or <i>Buddenbrockia</i> sp. 3 (BS) KF731708 (97.45%)
Near Manaus	Pacu, <i>Mylossoma aureum</i> (Serrasalmidae – Characiformes)	25% (1/4)	815 bp MT993757	<i>Buddenbrockia</i> sp. A (F) or <i>Buddenbrockia</i> sp. 3 (BS) KF731708 (98.16%)
	Mandi moela, <i>Pimelodina flavipinnis</i> (Pimelodidae – Siluriformes)	13.3% (2/15)	827 bp MT993758	<i>Buddenbrockia</i> sp. A (F) or <i>Buddenbrockia</i> sp. 3 (BS) KF731708 (98.07%)
	Stingray, <i>Potamotrygon motoro</i> (Potamotrygonidae – Myobatiformes)	50% (1/2)	759 bp MT993761	<i>Buddenbrockia</i> sp. A (F) or <i>Buddenbrockia</i> sp. 3 (BS) KF731708 (97.50%)

bp, base pairs.

Details are provided on the region of collection of fish (near Santarém or Manaus), fish according to common name and species (and family and order), prevalence of infection (and number of infected individuals/number of fish examined), small subunit ribosomal DNA (ssrDNA) sequence lengths and GenBank accession numbers, and closest malacosporean species in GenBank (and % similarity according to BLASTn search). Notations (F) and (BS) refer to species identified by Fiala et al. (2015) and Bartošová-Sojková et al. (2014), respectively.

2018). Together with the monotypic *Polypodium hydriforme*, myxozoans form the Subphylum Endocnidozoa in the Phylum Cnidaria (Giribet and Edgecombe, 2020). There are two major myxozoan clades: the relatively speciose Myxosporea and the species-poor Malacosporea (Fiala et al., 2015). Malacosporeans have only been observed in freshwater environments and evidence to date indicates they exclusively use freshwater bryozoans (Class Phylactolaemata) as definitive hosts and fish as intermediate hosts.

The five described malacosporean species are variously assigned to the genera *Buddenbrockia* and *Tetracapsuloides* (Patra et al., 2018). *Tetracapsuloides bryosalmonae* is the causative agent of proliferative kidney disease, an emerging disease exacerbated by warming waters and changing climates (Okamura et al., 2011; Borgwardt et al., 2020). *Tetracapsuloides bryosalmonae* infects a range of wild and farmed salmon and trout (Family Salmonidae) in Europe and North America (Hedrick et al., 1993; Skovgaard and Buchmann, 2012). Molecular data have identified 12 undescribed malacosporeans (Bartošová-Sojková et al., 2014; Hartikainen et al., 2014; Naldoni et al., 2019) most of which have been detected in fish kidney. These include kidney infections in 17 fish species in the family Cyprinidae (Bartošová-Sojková et al., 2014; Hartikainen et al., 2014; Naldoni et al., 2019) and single species in the families Percidae (Bartošová-Sojková et al., 2014) and Nemacheilidae (Naldoni et al., 2019). Nearly all malacosporeans have been detected in Europe and North America, exceptions being the detection of a *Buddenbrockia* species in bryozoan hosts in Borneo (Hartikainen et al., 2014) and an early observation of *Buddenbrockia* in a Brazilian bryozoan host (Marcus, 1941). Our knowledge of host species and of malacosporean diversities and distributions is thus highly biased and a hidden malacosporean diversity may await discovery. In view of the poor sampling of malacosporeans in tropical environments, we used molecular tools to survey for malacosporean infections in the kidney of a diversity of fishes in the Amazon Basin. Here we report the results of our survey, explore their implications and provide the first insights on malacosporean infections in fishes in the Southern Hemisphere.

Materials and methods

Sample collection

The kidney of 146 fish specimens belonging to 21 Amazonian fish species of 12 families was screened for the presence of malacosporean DNA (Tables 1 and 2). The fish were caught near the Brazilian municipalities of Santarém from the Tapajós River and from Manaus on the Amazon River (Fig. 1) in May (high water season) of 2018. The areas sampled near Manaus are some ~600 km upstream from the areas sampled near Santarém. The fish were caught by local fishermen using fishing nets and fishhooks and transported live to a nearby field laboratory, where they were euthanized by severance of the spinal cord. The methodology of the present study was approved by the Ethics Research Committee of the Federal University of São Paulo (CEUA N 92090802140), in accordance with Brazilian law (Federal Law No. 11794, dated 8 October 2008). Approximately 27 mm³ of tissue was immediately dissected from the posterior portion of the kidney and fixed in 99% ethanol for the molecular analysis. The fish sampling and access to the genetic data were authorized by the Brazilian Ministry of the Environment (SISBIO n° 44268-9 and SISGEN No. A656D8E, respectively).

DNA extraction, polymerase chain reaction amplification, sequencing and species identification

The DNA was extracted using a DNeasy® Blood & Tissue kit (QIAGEN Inc., California, USA), following the manufacturer's instructions. Malacosporean-specific mala-f and mala-r primers (Grabner and El-Matbouli, 2010) were used in polymerase chain reactions (PCRs) of all samples, amplifying approximately 680 pair of bases (bp) of the small subunit ribosomal DNA (ssrDNA) to confirm infection. The positive samples were then selected to conduct further PCRs (Fig. 2). Malacosporean-specific budd-f and budd-r primers (Grabner and El-Matbouli, 2010) were used in the first-round PCR giving a product of

Table 2. Fish sampled from the Amazon Basin in which infections were not detected by PCR

Region	Fish species and family	Number sampled
Near Santarém	Mapará, <i>Hypophthalmus marginatus</i> (Pimelodidae)	2
	Cachorra, <i>Rhaphiodon vulpinus</i> (Cynodontidae)	4
	Piau, <i>Leporinus</i> sp. (Anostomidae)	3
	Stingray, <i>Potamotrygon motoro</i> (Potamotrygonidae)	22
	Jaraqui, <i>Semaprochilodus insignis</i> (Prochilodontidae)	12
	Aracu, <i>Leporinus</i> sp. (Anostomidae)	4
	Aramaçá, <i>Apionichthys nattereri</i> (Achiridae)	2
	Surubim, <i>Pseudoplatystoma trigrinum</i> (Pimelodidae)	3
	Mandi, <i>Pimelodus blochii</i> (Pimelodidae)	1
Near Manaus	Pescada, <i>Plagioscion squamosissimus</i> (Sciaenidae)	13
	Sardinha, <i>Triportheus</i> sp. (Triportheidae)	1
	Tambaqui, <i>Colossoma macropomun</i> (Serrasalmididae)	5
	Jaú, <i>Zungaro zungaro</i> (Pimelodidae)	2
	Uaru, <i>Uaru amphiacanthoides</i> (Cichlidae)	1
	Cascudo, <i>Pterygoplichthys</i> sp. (Loricariidae)	1
	Cara bicuda, <i>Satanoperca jurupari</i> (Cichlidae)	1

Details are provided on the region of collection of fish (near Santarém or Manaus), common and species (and family) names of fish, and number sampled.

approximately 1800 bp. The product was then used as a template in the second round of PCRs. In two hemi-nested reactions, the primers budd-f and mala-r amplified a fragment of approximately 1500 bp, and mala-f and budd-r amplified approximately 1050 bp. Sequencing of these two partially overlapping fragments provided a contig of approximately 1800 bp. When the hemi-nested PCR failed, the PCR product from the first-round PCR (budd-f /r) was reamplified in a nested PCR using the primers mala-f and Malac18S_R3 (Hartikainen *et al.*, 2014), amplifying a fragment of approximately 900 bp (Fig. 2). The primers used here are conserved and amplify both of the major lineages of malacosporeans – *Buddenbrockia* and *Tetracapsuloides* (Supplementary Fig. S1).

The PCR was carried out with a final volume of 25 µL, composed of 10–50 ng of extracted DNA, 0.2 pmol of each primer, 12.5 µL of DreamTaqGreen PCR Mater Mix (all reagents from Invitrogen, Thermo Fisher Scientific, USA) in an Eppendorf AG 22331 Hamburg Thermocycler (Eppendorf, Hamburg, Germany).

The original cycling conditions were used for mala-f and mala-r primers as described by Grabner and El-Matbouli (2010). For runs using all other primer combinations, an initial denaturation stage at 95°C for 5 min was followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 61°C for 45 s, extension at 72°C for 145 s, finishing with an extended elongation stage at 72°C for 8 min.

PCR products were electrophoresed in 2.0% agarose gel in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8.0), stained with

SYBRsafe® (Invitrogen) and analysed by a MiniBis Pro Transilluminator. The size of the amplicons was estimated by comparison with the 1 kb Plus DNA Ladder (Invitrogen). After purification of the PCR products using the QIAquick PCR Purification Kit (QIAGEN Inc., California, USA), sequencing was carried out using the same primer pairs used in the amplification stage, and with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) in a 3500 DNA sequencing analyser (Applied Biosystems, USA) at the Helixxa Company (Paulinia City, São Paulo State, Brazil). Basic Local Alignment Search Tool (BLAST) searches were performed to verify the similarity of the sequences obtained in this study with other sequences available in the GenBank database (Altschul *et al.*, 1997). Phylogenetic analysis was performed using the malacosporean sequences from Fiala *et al.* (2015) and Patra *et al.* (2018), all available in GenBank. *Aurelia aurita* (AY039208) and *Hydra magnipapillata* (HQ392522) sequences were used as the outgroup. Nucleotide sequences were aligned using ClustalW within BioEdit version 7.0.9.0 (Hall, 1999) and alignments improved manually. Phylogenetic analysis was conducted using maximum likelihood in the PhyML 3.0 implemented via the web server (<http://www.atgc-montpellier.fr/phyml/>) (Guindon *et al.*, 2010), with topology assessed by bootstrapping with 1000 replicates, using the GTR + I + G model of evolution. The resulting tree was visualized with FigTree v1.3.1 (Rambaut, 2008). Only bootstrap values above 50 were considered well supported. The data for families of host fish were obtained from Fiala *et al.* (2015), Patra *et al.* (2018), Naldoni *et al.* (2019) and Fishbase (Froese and Pauly, 2013). A second alignment, including only the malacosporean sequences generated in this study, obtained from different fish species, was used to produce a pairwise dissimilarity matrix using MEGA 7.0 (Kumar *et al.*, 2016). All new sequences were deposited in GenBank (accession numbers are provided in Table 1). We employed the accepted range of variation for identifying malacosporean species as <1% sequence dissimilarity of ssrDNA (Bartošová-Sojková *et al.*, 2014; Hartikainen *et al.*, 2014; Atkinson *et al.*, 2015).

Results

Of the total of 146 fish from 12 different families examined from the Amazon Basin in Brazil (Tables 1 and 2), 13 specimens belonging to six families were positive by PCR for malacosporean infection and were thus subject to ssrDNA sequencing (Table 1 and Fig. 3). The sequences generated ranged from 755 to 1732 bases pair (bp) and the genetic divergence among these sequences ranged from 0 to 0.3% (Table 3). Blastn searches of the sequences generated here against the NCBI GenBank database returned *Buddenbrockia* species with similarities ranging from 97.45 to 98.16% (Table 1). This surprising result implies that we detected only one malacosporean species infecting fish from various families. We therefore refer to this Amazonian *Buddenbrockia* as *Buddenbrockia* sp. E, following on with the sequential alphabetical lettering used to designate malacosporean species yet to be described (Fiala *et al.*, 2015). *Buddenbrockia* sp. E clustered in the *Buddenbrockia* clade as a sister species of *Buddenbrockia* sp. A in our molecular phylogenetic analysis (Fig. 4).

Infection prevalences ranged from some 6 to 100% for fish species with infected individuals (Table 1) but most sample sizes were small. Among fish species sampled in both regions, two (*Potamotrygon motoro* and *Plagioscion squamosissimus*) showed infections, but not in both regions (Santarém and Manaus). For the interesting case of the stingray, *P. motoro*, infection was detected in one of two individuals sampled in the Manaus region, but not in 22 individuals sampled downstream in the Santarém region.

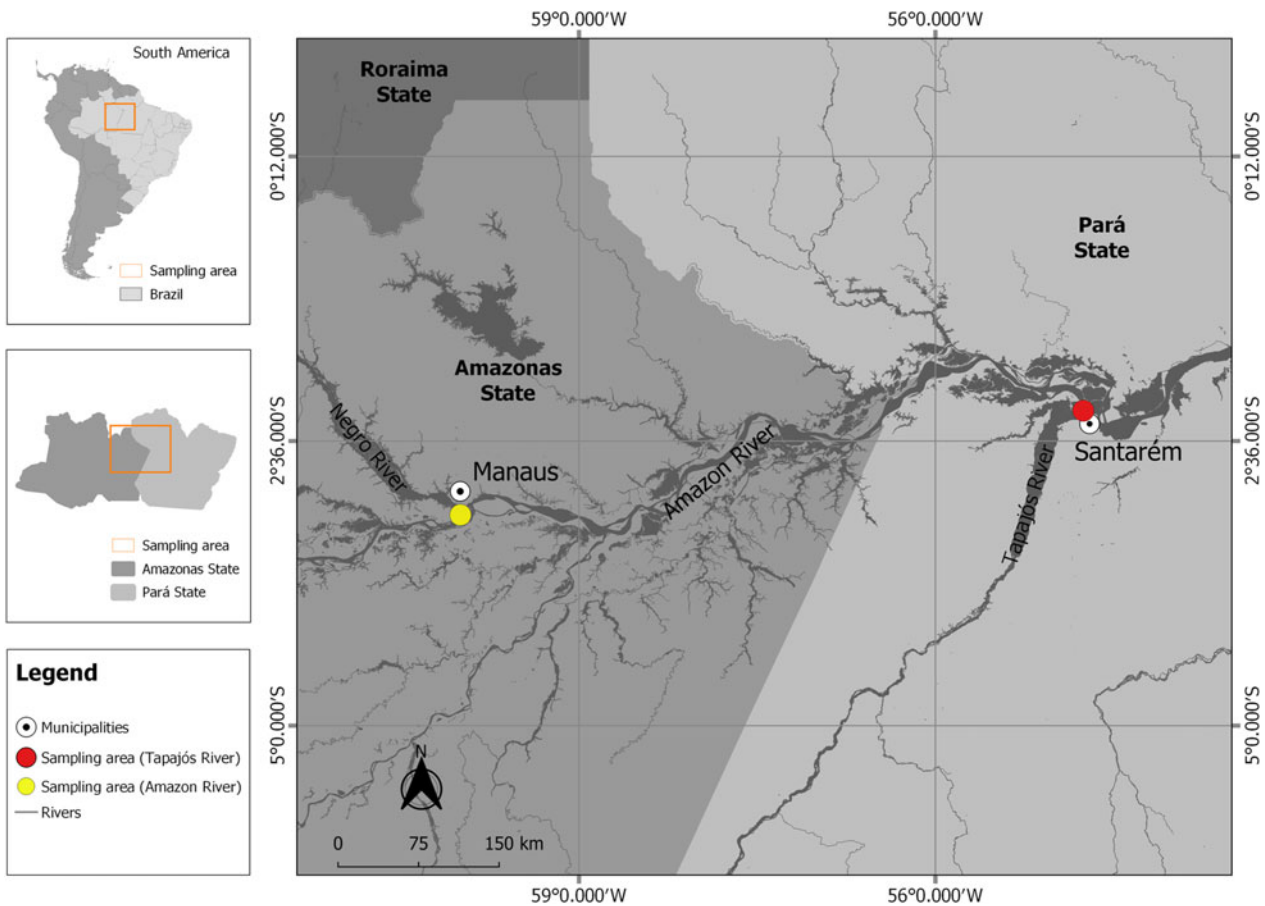


Fig. 1. Sampling localities in the Amazon Basin.

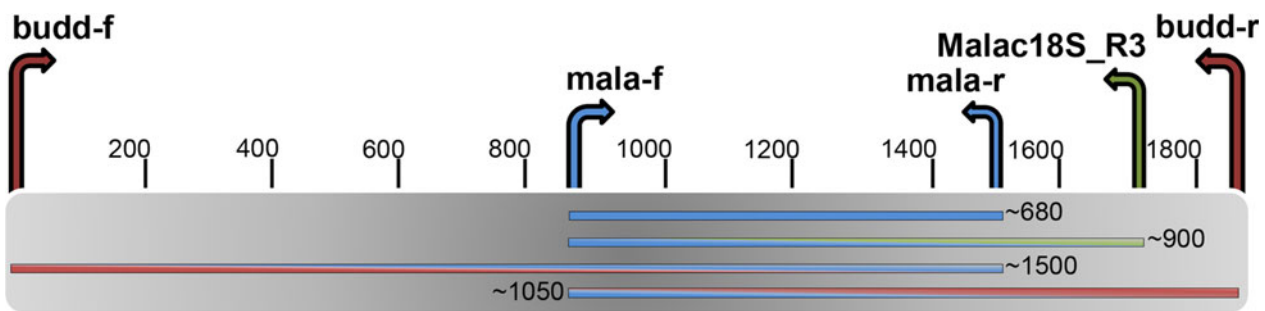


Fig. 2. Schematic representation of part of the partial small subunit ribosomal DNA (ssrDNA) of *Buddenbrockia*. sp. E, which shows locations of PCR primers and the approximated (~) bases pair (bp) amplified using the various combinations of primers.

Discussion

Incidence of infection of Amazonian fishes

Here we report the first case of a malacosporan infecting fish in the Southern Hemisphere. This is not unexpected given the observation of a *Buddenbrockia* species in freshwater bryozoans in São Paulo State (Marcus, 1941). Unexpectedly, however, our molecular phylogenetic analysis demonstrated that only a single species of *Buddenbrockia*, referred to here as *Buddenbrockia* sp. E, infected a wide range of Amazonian fish hosts. Infections were often detected even when sampling <5 fish per species (in four of seven fish species, Table 1). Infections were not detected in nine fish species from Santarém and in seven fish species from Manaus, although sample sizes were generally very low (Table 2). The seven fish species identified to harbour infections of *Buddenbrockia* sp. E belong to six families and five orders

(Table 1). All six families (Sciaenidae, Cichlidae, Curimatidae, Pimelodidae, Serrasalminidae and Potamotrygonidae) and four of the five orders (Cichliformes, Siluriformes, Characiformes and Myliobatiformes) are new records for malacosporan infections. We note that fish hosts include species with varying ecologies – e.g. *P. squamosissimus* is a benthic carnivore in deep waters feeding on fish and shrimp; *Calophysus macropterus* inhabits channels and floodplain lakes and consumes fish and invertebrates; the toothless characin *Curimata inornata* feeds on detritus and surface films (Santos et al., 2006; Pérez and Fabrè, 2009). Our results therefore are inconsistent with the infection of fish with common ecologies.

Knowledge of malacosporan host specificity is still incipient, in keeping with the generally poor sampling and biased reporting of parasite occurrence in general (Dallas et al., 2017). Nevertheless, the current picture suggests that *Buddenbrockia* sp. E is characterized by a remarkable ability to infect a broad

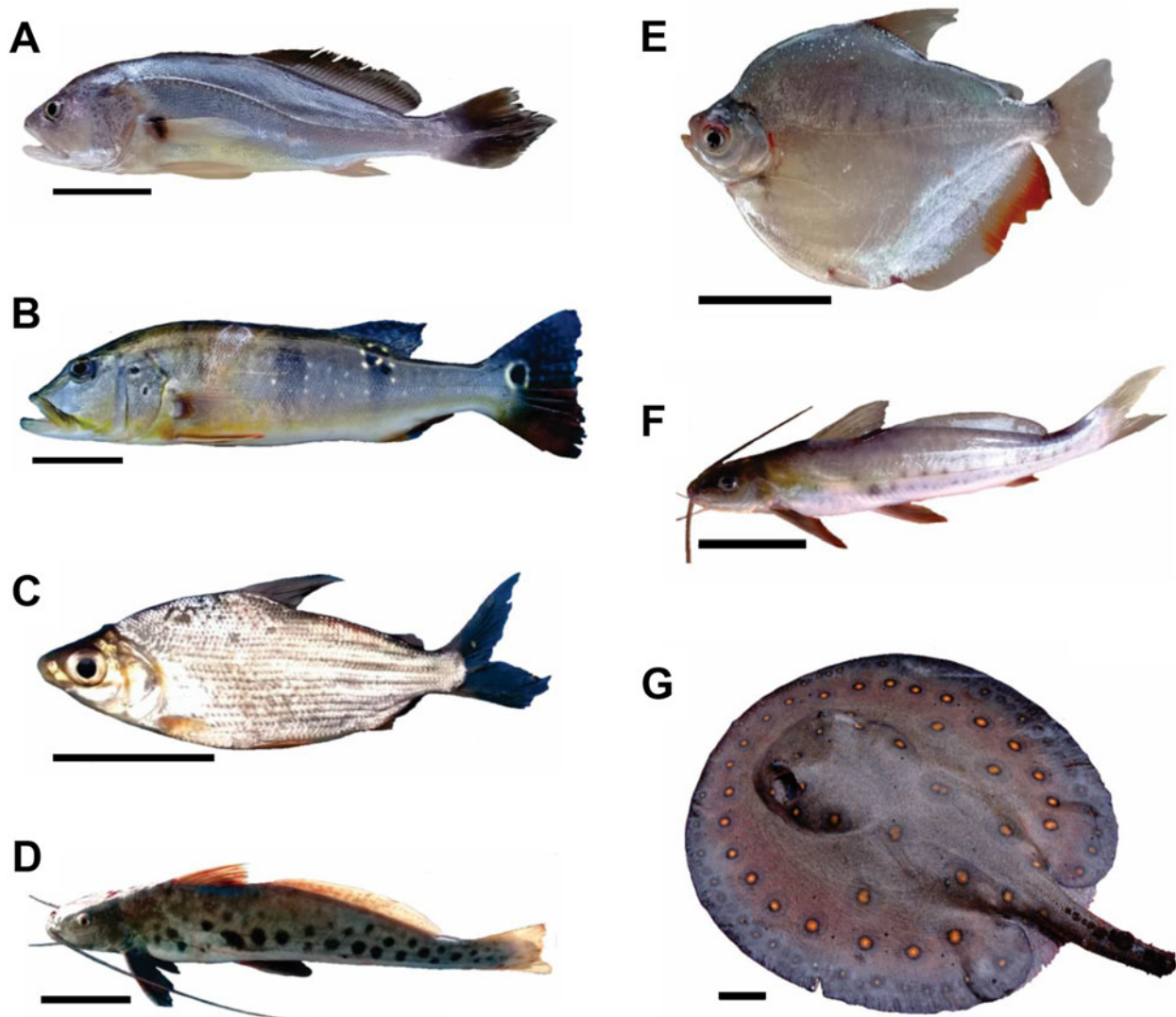


Fig. 3. Fish species infected by *Buddenbrockia* sp. E. (A) *Plagioscion squamosissimus*. (B) *Cichla pinima*. (C) *Curimata inornata*. (D) *Calophysus macropterus*. (E) *Mylossoma aureum*. (F) *Pimelodina flavipinnis* (G) *Potamotrygon motoro*. (A–D) Fish collected near Santarém. (E–G) Fish collected near Manaus. Scale bar: 5 cm.

range of fish. *Buddenbrockia* sp. E infections have been detected in five families of teleosts and one family of chondrichthyans (Potamotrygonidae). In particular, *Buddenbrockia* sp. E was detected in the kidney of four of 13 fish species from the Santarém region and from three of 10 fish species from the Manaus region (total number of fish sampled = 146). Only two fish species examined were common to both regions: the freshwater stingray (*P. motoro*) and pescada (*P. squamosissimus*).

Our study only found one malacosporan species despite the use of general malacosporan primers for detection. Several previous studies using the same primers in temperate regions show higher malacosporan diversity, although direct comparisons are difficult due to different evolutionary histories of fish host fauna and relative sampling effort. Five malacosporan species were detected in six of nine fish species sampled (total number of fish sampled = 117) from the River Stour (UK) (Naldoni *et al.*, 2019) and five malacosporans were detected in nine of 10 species of fish sampled (total examined = 48) from the River Dyje in the Czech Republic (Bartošová-Sojtková *et al.*, 2014). In the current study, seven of 21 species of fish sampled from sites some 600 km apart in the Amazon Basin yielded only the one malacosporan species with comparative detection methods.

The detection of a single species is somewhat surprising, as the collective data do not support the general expectation that parasite

diversity will mirror host diversity in biodiversity hot spots. Such a result may arise from an incomplete sampling of Neotropical parasites, although a study on helminth parasite diversity in tropical freshwater fish also suggested a weak trend that helminth species richness may be higher in certain areas in the north temperate region, and that overall helminth species richness in tropical freshwater fish appears to be relatively poor (Choudhury and Dick, 2000). However, general inferences of such patterns must be tempered by appreciation that further sampling in the Amazon could reveal a different picture.

Evolutionary scenarios

The detection of *Buddenbrockia* sp. E in the freshwater stingray is the first report of a malacosporan infection in a cartilaginous fish. If this is a true host, facilitating parasite spore production, the position of *Buddenbrockia* sp. E in our molecular phylogeny may reflect a relatively recent acquisition of cartilaginous fish hosts. Thus, stingrays may have been incorporated as hosts at some point after their inferred Eocene invasion of the Amazon Basin (Bloom and Lovejoy, 2017). Similarly, croakers would have been infected following their invasion. This pattern of host use suggests that the *Buddenbrockia* sp. E lineage could have

Table 3. Dissimilarity matrix for small subunit ribosomal DNA (ssrDNA) of *Buddenbrockia* sp. E sequenced from fish species 1–7

Fish host	1	2	3	4	5	6	7
(1) <i>Plagioscion squamosissimus</i>	–	1/814	0/755	1/783	1/815	0/827	0/759
(2) <i>Cichla pinima</i>	0.1	–	1/755	2/783	2/814	1/814	1/759
(3) <i>Curimata inornata</i>	0.0	0.1	–	1/737	1/755	0/755	0/737
(4) <i>Calophysus macropterus</i>	0.1	0.3	0.1	–	2/783	1/783	1/759
(5) <i>Mylossoma aureum</i>	0.1	0.2	0.1	0.3	–	1/814	1/759
(6) <i>Pimelodina flavipinnis</i>	0.0	0.1	0.0	0.1	0.1	–	0/759
(7) <i>Potamotrygon motoro</i>	0.0	0.1	0.0	0.1	0.1	0.0	–

Fish species identified in the first column. The upper triangle shows nucleotide differences in relation to the number of bases compared. The lower triangle shows % pairwise distances.

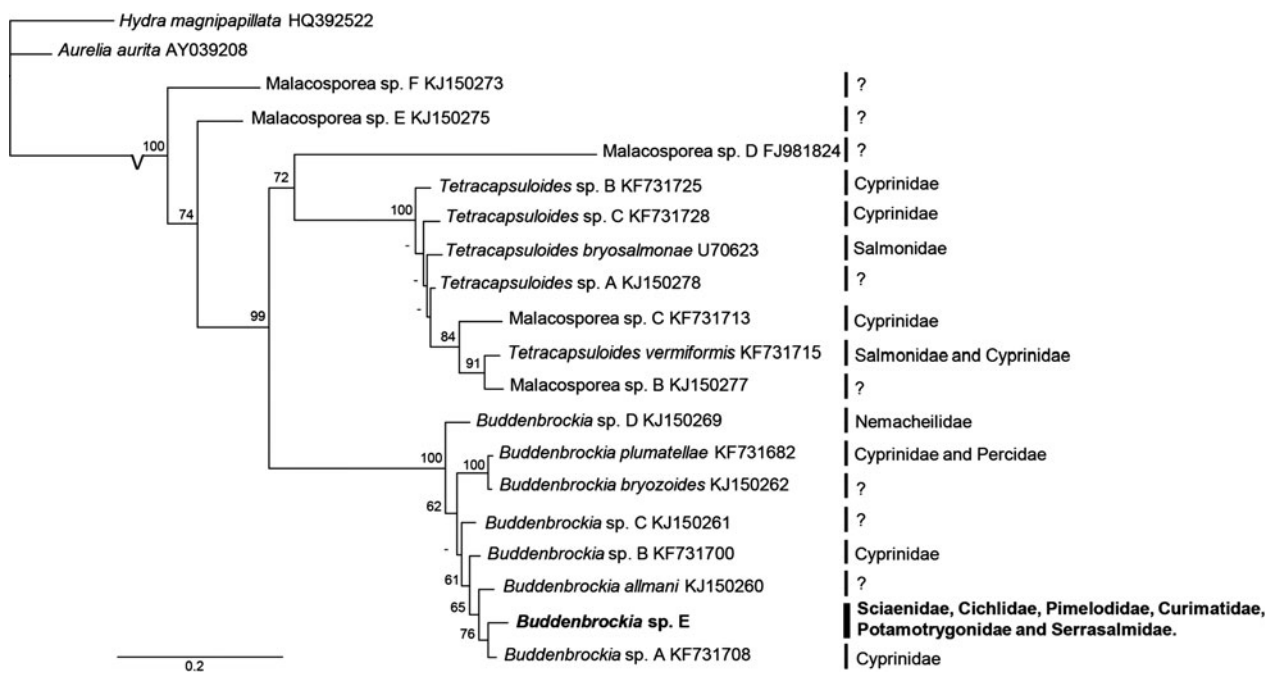


Fig. 4. Maximum likelihood tree showing the relationship between *Buddenbrockia* sp. E and all malacosporean species reported in Fiala et al. (2015) plus *Tetracapsuloides vermiformis* and *Buddenbrockia bryozoides* described in Patra et al. (2018) using sequences deposited in GenBank based on partial small subunit ribosomal DNA (ssrDNA). Fish host families are listed on the right for each malacosporean species when known. Information for *Buddenbrockia* sp. E in bold. Numbers above nodes indicate bootstrap confidence levels from maximum likelihood. Values for weakly supported nodes (<50) are not shown.

been present in South America long before marine incursions introduced host lineages now utilized.

There are, however, alternative explanations to the scenario proposed above. These include a relatively recent introduction and spread of a generalist parasite. It could also be the case that stingrays are accidental hosts, which would then demonstrate a capacity to infect fish characterized by substantially differing physiologies. These issues could variously be resolved with broader sampling, the use of more general primers and more in-depth profiling of genetic variation of *Buddenbrockia* sp. E.

We stress that evolutionary scenarios based on the patterns of host use in the present day must always be tempered with appreciation that parasites may undergo unseen shifts in host use over time that can muddy the waters (De Baets et al., 2015; Okamura and Gruhl, 2019). A better understanding of myxozoan diversities and patterns of host use can help to evaluate, add perspective to and refine proposed evolutionary scenarios regarding early host use (e.g. Kodádková et al., 2015; Holzer et al., 2018; Lisnerová et al., 2008).

Is malacosporean diversity really low in the Amazon Basin?

It was surprising to detect sequences characterising only one malacosporean species infecting fish species sampled from a diversity of fish families in a biodiverse region. One explanation is that many Neotropical malacosporeans may have diverged sufficiently to preclude amplification using the primers employed in this study, despite the primers being suitable for characterising broad malacosporean diversity in temperate regions (Supplementary Fig. S1). This could be investigated by histology and ultrastructure to confirm the presence of malacosporean stages in kidney material not amplified by the primers employed here. Characterising kidney metagenomes might then prove helpful. The alternate explanation, that malacosporeans are indeed species poor in the Neotropics, is supported by the apparent absence of closely related species that might have amplified in concert with *Buddenbrockia* sp. E. The broad host range of *Buddenbrockia* sp. E provides some support for the hypothesis that host heterogeneity may limit the evolution of specialist parasite lineages (Gibson et al., 2020) which is based on theoretical

expectations regarding the evolution of niche width. Nevertheless, many parasite radiations are apparently linked with relative host diversities (e.g. Kamiya *et al.*, 2014). Indeed, this pattern has been noted for myxozoans with the species-rich myxosporeans using diverse polychaete (some 8000 extant species; Ruppert *et al.*, 2004) and oligochaete (some 3500 and extant species; Ruppert *et al.*, 2004) hosts and the species-poor malacosporeans exploiting depauperate freshwater bryozoan hosts (<100 species; Massard and Geimer, 2008; Okamura *et al.*, 2018). The invertebrate host use of *Buddenbrockia* sp. E is entirely unknown. Wood and Okamura (2017) identify nine freshwater bryozoan species that could serve as hosts in the Amazon Basin with further sampling revealing some four new species and one previously unrecorded from the region (Wood and Okamura, unpub. data). Finally, it is entirely possible that other malacosporeans were simply not sampled in view of low sample sizes, biased sampling (e.g. poor sampling of younger fish by fishermen) and the limited period of sampling.

Further insights and caveats

For such a limited study, our data highlight various issues and questions in addition to those raised above. Notably, evidence that malacosporeans may take advantage of fish invading freshwater habitats (stingrays and croakers) from a marine environment suggests a considerable facility for incorporating new vertebrate hosts – a scenario further supported by myxosporeans that utilize non-fish hosts (e.g. shrews, mole and ducks) and the repeated adoption of diverse amphibians (Hallett *et al.*, 2015; Hartigan *et al.*, 2016). Possibly this is promoted if species already have a wide host range or a rather unspecific host invasion strategy.

It is important to note that ascribing fish hosts based on molecular detection of *Buddenbrockia* sp. E infections is potentially confounded. Such detection identifies putative hosts but confirmation of hosts requires demonstration of spore development or undertaking transmission trials. Naldoni *et al.* (2019) gained evidence for spore development (by ultrastructure) in fish belonging to three of the seven species from the River Stour in which malacosporean infections were detected by molecular methods. Bartošová-Sojková *et al.* (2014) report detecting malacosporean developmental stages and/or spores in 36% of all fish samples and that malacosporean DNA was detected in half of these microscopically positive results. Microscopic detection of spores can be variously compromised, e.g. if infections are immature or spores are sparse. These results suggest that a reasonable proportion of fish (if not all) identified in this study to harbour *Buddenbrockia* sp. E infections act as true hosts. In theory, infecting a broad range of dead-end hosts should be selected against. Notably, spore development has been observed even in non-native fish when infected by a generalist strain of the myxosporean *Ceratonova shasta* (Stinson *et al.*, 2018).

Conclusions and future work

This study highlights our limited knowledge of malacosporean diversity and patterns of host use – in keeping with our generally poor knowledge of parasite diversity (Dobson *et al.*, 2008; Poulin, 2014; Okamura *et al.*, 2018) and parasite host range (Dallas *et al.*, 2017). Improving such knowledge for malacosporeans is likely to become ever more difficult due to the many challenges faced by residents of freshwater environments, including overexploitation, pollution, flow modification, habitat destruction or degradation, and invasion by exotic species (Dudgeon *et al.*, 2006). As a result of such challenges, some 28% of freshwater fish are at risk of extinction and on the IUCN Red List (IUCN, 2019), and affiliate taxa (parasites and symbionts) are therefore threatened with co-extinction along with their endangered freshwater hosts

(Dunn *et al.*, 2009; Carlson *et al.*, 2017). Amplicon sequencing applied to environmental samples and archived material (e.g. museum specimens) across global scales may be one means of retrieving insights on the hidden diversity of malacosporeans and other parasites before many vanish (e.g. Hartikainen *et al.*, 2016; Harmon *et al.*, 2019; Murphy *et al.*, 2020).

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Conflict of interest. None.

Ethical standards. The methodology of the present study was approved by the Ethics Research Committee of the Federal University of São Paulo (CEUA N 92090802140), in accordance with Brazilian law (Federal Law No. 11794, dated 8 October 2008).

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