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An ancient alliance: Matching evolutionary patterns of cartilaginous fishes (Elasmobranchii) and chloromyxid parasites (Myxozoa)

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ABSTRACT

Myxozoa is a group of endoparasitic cnidarians covering almost 2600 species but merely 53 species, mostly from the genus *Chloromyxum*, have been reported from sharks, rays, and skates (Elasmobranchii). Elasmobranchs play a key role in the study of evolutionary trajectories of myxozoans as they represent ancestral vertebrate hosts. Our study provides new data on *Chloromyxum* spp. from 57 elasmobranchs, covering 20 species from geographical regions and host groups not previously investigated, such as Lamniformes and Hexanchiformes, the most basal phylogenetic shark lineage. In total, 28% of elasmobranchs were infected with *Chloromyxum* spp., indicating high diversity. Of the seven distinguished species, six are formally described based on morphological, morphometric, and genetic (18S rDNA) data. Comprehensive co-phylogenetic analyses and ancestral state reconstruction revealed that parasite and host phylogenies are clearly correlated, resulting in a distinct phylogenetic separation of chloromyxids from selachid (shark) vs. batoid (ray and skate) hosts. Species infecting the most ancient elasmobranchs formed a sublineage, branching off in the middle of the *Chloromyxum sensu stricto* clade. Our findings indicate that chloromyxids likely invaded an ancestral elasmobranch prior the time of divergence of shark and batoid lineages. Our analyses did not show a clear phylogeographic pattern of *Chloromyxum* parasites, probably due to the cosmopolitan distribution and migratory behaviour of many elasmobranch hosts, but geographical sampling must be extended to confirm or refute this observation. This study provides a complex view on species diversity, phylogeny, evolution, host-parasite co-phylogeny, and the phylogeographic origin of *Chloromyxum* species from elasmobranchs. Our results highlight the importance of adding missing data from previously un- or undersampled geographical regions and host species which results in a more accurate estimate of myxozoan biodiversity and a better understanding of the evolution of this parasite group in their hosts and in the different oceans of our planet.

1. Introduction

Sharks, rays, skates, and sawfish (Chondrichthyes: Elasmobranchii) are predominantly marine, large-bodied vertebrates with a cosmopolitan distribution. They inhabit different zones of the water column in marine habitats, from the epipelagic zone above 200 m to the bathypelagic zone below 1000 m (Froese and Pauly, 2021). The evolutionary divergence of these vertebrates from ancient chimaeras (Holocephali; Chimaeriformes) dates back to the early Devonian, about 400 million years ago (Myr) with Selachii (sharks) and Batoidea (rays and skates)

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having diverged about 392 Myr (Heinicke et al., 2009). The fossil record of extant elasmobranch genera extends to the Lower Jurassic with the earliest record being the six gill sharks of the genus *Hexanchus* (Cappetta, 2012). Indeed, cowsharks (Hexanchiformes) have been proposed to be the most basal and first group to diverge from the rest of squalean sharks (Squalomorphii) (Musick et al., 2004; Heinicke et al., 2009; Barnett et al., 2012; Naylor et al., 2012). The extant elasmobranch diversity encompasses 1226 species (Serena et al., 2020), 37% of which are threatened with extinction (IUCN, 2022). In fact, elasmobranchs are the vertebrate group with the highest extinction risk in the marine realm (Dulvy et al., 2014; Stein et al., 2018). The main factors affecting the decline of shark and ray populations are overfishing (Stevens et al., 2000) and human-induced habitat destruction (Jackson et al., 2001).

Elasmobranchs have been recognized as ancestral hosts of various groups of parasites (Olson et al., 2010; Rees et al., 2014; Xavier et al., 2018) including the Myxozoa (Kodádková et al., 2015; Holzer et al., 2018; Lisnerová et al., 2020). Due to parallel speciation of myxozoans and their vertebrate hosts, parasite species from such ancient hosts create independent, well-supported lineages mostly positioned at or close to the base of the teleost-infecting lineages (Kodádková et al., 2015; Olson et al., 2010; Rees et al., 2014; Holzer et al., 2018; Xavier et al., 2018; Lisnerová et al., 2020).

Myxozoans are a diverse group of cnidarian endoparasites with a simplified body plan of only a few cells. They are characterized by complex dixenous life cycles with alternating host groups of invertebrates (annelids and bryozoans as final hosts) and vertebrates (mainly fish but also amphibians, reptiles, birds, and mammals as intermediate hosts). Most myxozoan species do not affect host health while some species cause serious disease and lead to significant declines in host populations. The current myxozoan diversity of about 2600 species is believed to be grossly underestimated (Bartošová-Sojková et al., 2014; Hartikainen et al., 2016; Okamura et al., 2018), especially in elasmobranchs (Holzer et al., 2018; Lisnerová et al., 2020). However, the limited myxozoan parasite records in elasmobranchs may be affected by a considerably lower elasmobranch species diversity (about 7%) than that of marine bony fish (Cohen, 1970; Serena et al., 2020; Froese and Pauly, 2021). Moreover, only about 100 of all extant elasmobranch species (about 8%), and of those usually only few individuals, have been screened for myxozoan infections. In this host group, 53 myxozoan species (about 2% of the total myxozoan species diversity) belonging to seven myxosporean genera (Myxozoa: Myxosporea: *Ceratomyxa*, *Chloromyxum, Ellipsomyxa*, *Kudoa*, *Myxidium*, *Sinuolinea* and *Sphaerospora*) have been described. Most commonly, myxosporean infections of elasmobranchs are encountered in the host's gallbladder, much less frequently in kidneys and muscle (Lisnerová et al., 2020, 2022; Elloumi et al., 2021) while they have not been documented from other organs. Such organ preference has recently been hypothesized to be the result of a potential route of entry and mechanism of new host acquisition, whereby infected invertebrates are ingested and the parasite subsequently migrates to and develops in immunologically privileged sites such as gall bladder/bile and bile ducts of elasmobranchs (Lisnerová et al., 2020).

The genus *Chloromyxum* Mingazinni, 1890, originally established based on morphological characters, is a polyphyletic taxon whose species group into independent clades in the myxosporean phylogeny (Bartošová and Fiala, 2011; Jirků et al., 2011). From a total of 150 nominal *Chloromyxum* species, 25 descriptions originate from elasmobranchs, mostly from Australian waters. This number accounts for almost half of so far known myxosporean diversity from this host group (Eiras et al., 2012; Gleeson and Adlard, 2012; Cantatore et al., 2018; Lisnerová et al., 2020). According to the most updated list (Lisnerová et al., 2020), both, sharks and batoids serve as hosts for *Chloromyxum* spp., though numbers may change as several groups have been neglected in previous sampling efforts such as the earliest extant lineage, cow (Hexanchiformes) and bullhead (Heterodontiformes) sharks as well as the more recent bramble (Echinorhiniformes) and mackerel (Lamniformes) sharks (Heinicke et al., 2009; Lisnerová et al., 2020). Chloromyxids from elasmobranchs consistently group in a single phylogenetic clade, including the type species *Chloromyxum leydigi* Mingazzini, 1890, hence called *Chloromyxum sensu stricto*. This clade constitutes the most basal branch of the oligochaete-infecting myxosporean lineage, whose species are mostly reported from freshwater teleost fishes (Cantatore et al., 2018; Holzer et al., 2018; Lisnerová et al., 2020). *Chloromyxum* species parasitizing teleost fishes and amphibians fall outside the *sensu stricto* clade into different groups and are designated to *sensu lato* (Gleeson and Adlard, 2012; Cantatore et al., 2018). All *Chloromyxum* species have bivalved spores with a smooth or ribbed surface containing four pyriform polar capsules and a binucleate, rarely uninucleate sporoplasm (Lom and Dyková, 2006). An exclusive feature of most (68%; 17/25) elasmobranch-infecting chloromyxids is the hair-like filaments that appear on the posterior end of the spores (Lom and Dyková, 2006; Eiras et al., 2012; Lisnerová et al., 2020). These filaments most likely facilitate spore floating and thus enhance the dispersal of myxozoan transmission stages, the spores, over large distances in the marine environment before they sink down to the sea floor.

In order to provide a more complex view on species diversity, evolutionary, co-evolutionary and phylogeographic patterns in the *Chloromyxum sensu stricto* clade we i) conducted an extensive morphological and molecular screening of elasmobranchs for chloromyxid infections in various geographical regions (mainly off South Africa), as well as from previously under/unsampled lamniform and hexanchiform sharks, ii) classified the new *Chloromyxum* species using morphological and genetic data, iii) assessed the phylogenetic relationships of chloromyxids and parallel speciation patterns with their respective elasmobranch hosts, and iv) performed ancestral mapping of the host groups and geography of chloromyxids.

We hypothesized that i) the *Chloromyxum* diversity in elasmobranchs is profound; ii) *Chloromyxum* spp. from the earliest living hexanchiform sharks evolved first, thus would be positioned at the base of the *Chloromyxum sensu stricto* clade; iii) due to co-speciation events, chloromyxid phylogeny reflects the division of parasite taxa into clearly defined shark- and batoid-infecting subgroups; and iv) *Chloromyxum sensu stricto* spp. cluster in subclades according to their geographical origin.

2. Materials and methods

2.1. Sample collection and light microscopy

Overall, 57 individuals of sharks, rays, and skates (20 species) were collected for an ecological conservation study in South Africa ($n = 56$) and Argentina ($n = 1$) between 2018 and 2020 (Supplementary Table 1). The sample set comprised *Hexanchus griseus* (Bonnaterre, 1788) and *Notorynchus cepedianus* (Péron, 1807) as representatives of the most ancient sharks (Hexanchiformes), and thus a crucial host group for addressing the evolutionary trajectories of their myxozoan parasites. Cartilaginous fishes from South Africa were collected at various sampling sites off the coast of the southeastern Atlantic Ocean (Western Cape; $n = 34$ individuals; 9 species) and southwestern Indian Ocean (KwaZulu-Natal; $n = 22$ individuals; 10 species), by long- and handlines and the shark safety gear. After the capture, cartilaginous fishes were dissected or stored at −20 °C until processing in the laboratory. Permits for the collection of elasmobranchs were issued by the South African Department of Agriculture, Forestry and Fisheries (permit numbers: RES2018–58 and RES2019–61 issued to the South African Shark Conservancy, RES2019–77 and RES2020–20 issued to the KwaZulu-Natal Sharks Board and RES2019–105 issued to BCS (Bjoern C. Schaeffner)). One individual of the broadnose sevengill shark was captured as bycatch by commercial trawling in the San Matías gulf, Argentina, and dissected immediately after capture.

The body cavities of elasmobranchs were opened by mid-ventral incision, and gallbladder contents were fixed in pure ethanol and in 1× PBS mixed with 1× Antibiotic Antimycotic Solution (Merck;

Darmstadt, Germany). The present study focusses on the presence of *Chloromyxum* spp. while other taxa and tissues of some host individuals were the focus of an independent study (Lisnerová et al., 2022). Spores and plasmodia were observed by light microscopy (Olympus BX51; Tokyo, Japan) at 400× and 1000× magnification and documented with a digital camera (Olympus DP70; Tokyo, Japan). Permanent slides were prepared as follows: Myxospores were air-dried directly on glass slides, stained with Epredia™ Shandon™ Kwik-Diff™ Stains (Thermo Fisher Scientific; Waltham, Massachusetts) and mounted with DPX non-aqueous mounting medium (Merck; Darmstadt, Germany). Plasmodium length and width, spore/polar capsule length and width (in micrometres) were recorded from digital photographs of thirty fresh spores for each species, except *Chloromyxum carcharhini* n. sp. ($n = 11$), according to guidelines of Lom and Arthur (1989) and using the program ImageJ 1.53e (Schneider et al., 2012). In species descriptions, parasite measurements are provided from spores found in the type host species. Values in the formal descriptions are presented as the average dimension in μm followed by the mean ± standard deviation in μm followed by the range values of each parameter in parentheses. Sampling codes correspond to each elasmobranch individual utilized. If possible, spores were fixed and prepared for scanning electron microscopy (SEM) as described in Alama-Bermejo et al. (2009) and examined using an JEOL JSM-7401F (JEOL Ltd.; Tokyo, Japan) field emission scanning electron microscope.

2.2. DNA extraction and 18S rDNA amplification

All 57 bile samples were kept in 400 μL of TNES urea buffer (10 mM Tris-HCl with pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4 M urea). Genomic DNA was extracted using the phenol-chloroform protocol adjusted for myxozoan samples (Holzer et al., 2004) including an overnight digestion with proteinase K (50 μg mL^{-1}; Serva, Germany) at 55 °C. DNA pellets were dissolved in 100 μL DNAse-free water overnight at 4 °C and extracts stored at −20 °C.

All samples, including microscopically negative ones, were screened by polymerase chain reaction (PCR) amplifying 18S rDNA using Wiz-Pure HS-PCR FDMix (Wizbiosolutions; Seongnam, South Korea), 10 pmol of each primer, 1 μL of DNA and 18 μL of sterile water. To ensure successful amplification of target myxozoan genes, a nested PCR approach has been conducted using universal eukaryotic primers (ERIB1–ERIB10) in the first round (Barta et al., 1997) and the myxozoan specific primers Myxgp2F–ACT1R (Kent et al., 1998; Hallett and Diamant, 2001) in the second round (Supplementary Table 2). PCR cycling parameters were set up as follows: 1st run: denaturation at 95 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and a terminal extension at 72 °C for 10 min; 2nd run: denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 50 s, 58 °C for 50 s, 72 °C for 1 min 30 s, and a terminal extension at 72 °C for 10 min. To complete partial 18S rDNA sequences obtained with Myxgp2F–ACT1R various other primer sets were used (details in Supplementary Table 2). PCR products were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd.; New Taipei City, Taiwan) and sequenced commercially (SEQme s.r.o.; Dobříš, Czech Republic).

2.3. Phylogenetic approaches and character history tracing

The dataset used for the phylogenetic analysis comprised nine newly obtained 18S rDNA sequences of chloromyxids from elasmobranchs, 35 published sequences of the same genus and three outgroup taxa (marine myxosporeans *Auerbachia pulchra*, *Ellipsomyxa gobii*, and *Myxidium gadi*). The alignment was prepared in MAFFT v6.864b (Katoh et al., 2005) using the *E*-INS-i algorithm implemented in Geneious Prime 2019.0.4 (Kearse et al., 2012). The alignment was manually edited, and ambiguously aligned regions removed. The final alignment consisted of 1720 nucleotides. Phylogenetic trees were inferred by maximum likelihood (ML) and Bayesian inference (BI) in RAxML v7.0.3 (Stamatakis, 2006) and MrBayes v3.0 (Ronquist and Huelsenbeck, 2003), respectively, both implemented in Geneious Prime 2019.0.4 (Kearse et al., 2012). The best-fitting model of evolution (GTR + Γ) has been selected in jModelTest (Posada, 2008), and was applied for both analyses. Bootstraps for ML analysis were based on 1000 replicates. Posterior probabilities were calculated over one million generations via two independent runs of four simultaneous Markov Chain Monte Carlo algorithms with every 100th tree saved and the burn-in set to 10% of all generations. Genetic distances between species (see Remarks sections of the *Chloromyxum* taxonomic descriptions), shown as similarities in %, were calculated in Geneious Prime 2019.0.4 from the original alignment with the sequence terminal parts trimmed to the length of the shortest sequence.

Tracing of the character history was performed in Mesquite v2.5 (Maddison and Maddison, 2011) to determine the proportional likelihood of ancestral character states in the nodes of the whole *Chloromyxum sensu stricto* clade and its individual sublineages. Two character states were defined for each tree terminus/*Chloromyxum* species based on the taxonomical categorization of the associated parasitized elasmobranch host: a shark (state 1) and a batoid (state 2). The character states were traced on the topology of the ML tree that was based on an alignment (1714 bp) of 41 *Chloromyxum* taxa (same species as for the phylogenetic analysis but without outgroup taxa). The parasite ML tree was calculated in RAxML v7.0.3, implemented in Geneious Prime 2019.0.4 (see above). Reconstruction of character states at ancestral nodes was performed using likelihood estimation with the Markov k-state 1 parameter model of evolution (Schluter et al., 1997).

The same 41-taxon ML *Chloromyxum* parasite tree was also used for the co-phylogenetic analysis. The phylogenetic tree of corresponding elasmobranch host species was generated by a subset extraction from the DNA supermatrix of Stein et al. (2018) using the vertlife platform (http://vertlife.org/sharktree/). *Chloromyxum*-elasmobranch host co-phylogeny was conducted in the paco (Procrustean Approach to Cophylogeny; Balbuena et al., 2013) package of R (R Core Team, 2019) with 1000 permutations (Hutchinson et al., 2017). In this method, phylogenetic distance matrices and host-parasite associations are used to test for overall fit, which is interpreted as a congruence between host and parasite phylogenies.

The ancestral distribution areas of *Chloromyxum* parasites were reconstructed to the internal nodes of chloromyxid phylogeny (the previously calculated 41-taxon ML tree) using the BioGeoBEARS package (Matzke, 2014) implemented in RASP 4.0 (Yu et al., 2015). Corrected AIC scores were calculated for the dispersal-extinction cladogenesis (DEC; Ree et al., 2005), DIVALIKE (likelihood implementation of dispersal-vicariance; Matzke, 2014), and BAYAREALIKE (likelihood implementation of BayArea; Matzke, 2014) which corresponded to three biogeographic models. Additional three models were created by inclusion of the *j* parameter to the three existing models. The likelihood ratio test was used to evaluate whether each pair of biogeographic models with and without the *j* parameter results in statistically equivalent likelihood values. The geographic areas were defined as coastal zones of continents, i.e. A – Atlantic coast of South America, B – Atlantic coast of Africa, C – Indian Ocean coastline of Africa, D – Atlantic coast of North America, E – Atlantic coast of Europe, F – Pacific coast of Australia, G – Indian Ocean coastline of Australia. The chloromyxid distribution was assigned according to respective elasmobranch host distribution data (Froese and Pauly, 2021, Supplementary Table 3). The resulting probabilities for the ancestral distribution areas of chloromyxid species were plotted as the highest % support for a parasite origin in a given area at each node of the 41-taxon ML *Chloromyxum* tree.

3. Results

3.1. Chloromyxum species diversity in elasmobranchs

Spores and plasmodia of *Chloromyxum* spp. were recognized in gallbladders of 28.1% of dissected elasmobranch individuals (16/57)

and in 45.0% (9/20) of host species. Overall, seven *Chloromyxum* species new to science, six of which are formally described here, were identified in seven shark species (i.e. *Carcharhinus leucas* (Valenciennes in Müller & Henle, 1839), *Carcharias taurus* Rafinesque, 1810, *Hexanchus griseus*, *Mustelus mustelus* (L.), *Notorynchus cepedianus*, *Poroderma pantherinum* (Smith in Müller & Henle, 1838), and *Sphyrna lewini* (Griffith & Smith, 1834), and two batoid species (*Raja straeleni* Poll, 1951 and *Rhinoptera jayakari* Boulenger, 1895) (host details in Supplementary Table 1). *Chloromyxum* infections were absent in the bile of *Aetomylaeus bovinus* (Geoffroy Saint-Hilaire, 1817), *Carcharhinus brevipinna* (Valenciennes in Müller & Henle, 1839), *C. obscurus* (Lesueur, 1818), *Galeocerdo cuvier* (Péron & Lesueur, 1822), *Haploblepharus edwardsii* (Schinz, 1822), *H. fuscus* Smith, 1950, *H. pictus* (Müller & Henle, 1838), *Mobula eregoodootenkee* (Bleeker, 1859), *M. kuhlii* (Müller & Henle, 1841), *Rostoraja alba* (Lacepède, 1803), and *Sphyrna zygaena* (L.).

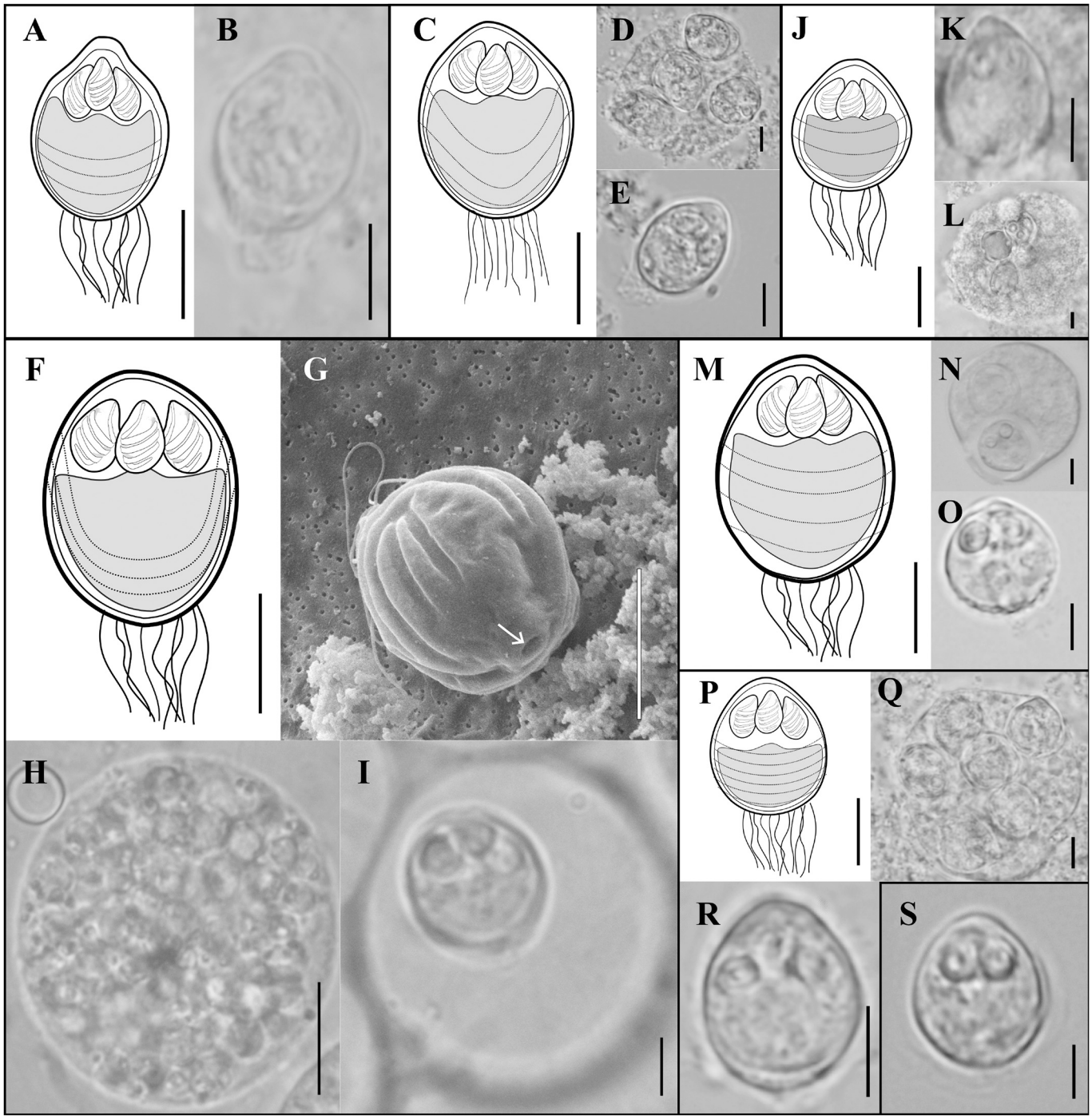


Fig. 1. Light and scanning electron microscopy (SEM) pictures of newly described species from this study. *Chloromyxum acuminatum* n. sp.: **A** line drawing, **B** mature spore; *Chloromyxum africanum* n. sp.: **C** line drawing, **D** tetrasporic plasmodium, **E** mature spore; *Chloromyxum bulliti* n. sp.: **F** line drawing, **G** mature spore with a spore surface striation, hair-like filaments and the pore for polar filament discharge (marked with an arrowhead) as seen by SEM, **H** early plasmodium, **I** monosporic plasmodium; *Chloromyxum carcharhini* n. sp.: **J** line drawing, **K** and **L** mature spores; *Chloromyxum ornamentum* n. sp.: **M** line drawing, **N** disporic plasmodium, **O** mature spore with spore surface striation; *Chloromyxum regularis* n. sp.: **P** line drawing, **Q** polysporic plasmodium, **R** mature spore; *Chloromyxum* sp. ex *Poroderma pantherinum*: **S** mature spore. Scale 5 μm.

3.2. Taxonomic descriptions of *Chloromyxum spp.*

In the present study, we provide formal species descriptions of six *Chloromyxum* parasites of shark, ray and skate hosts based on morphological, morphometrical and molecular characteristics. One species is not formally described due to an insufficient amount of data.

Phylum Cnidaria Hatschek, 1888
Unranked subphylum Myxozoa Grassé, 1970
Class Myxosporea Bütschli, 1881
Order Bivalvulida Schulman, 1959
Suborder Variisporina Lom and Noble, 1984
Family Chloromyxidae Thélohan, 1892
Genus *Chloromyxum* Mingazzini, 1890

***Chloromyxum acuminatum* n. sp. (Fig. 1A–B).**

Type host: *Rhinoptera jayakari* Boulenger, 1895, Oman cownose ray (Myliobatiformes: Rhinopteridae).

Type locality: Richards Bay, South Africa (28° 47′ 42.36″ S, 32° 6′ 37.19″ E).

The site of sporogonic development: Coelozoic in gallbladder (plasmodia and myxospores floating in the bile).

Prevalence of infection: 50% (1/2), host individual KZN20/14.

Etymology: Refers to the Latin word "acuminatus" which means pointed and refers to the pointed spore apex.

ZooBank registration: urn:lsid:zoobank.org:act:2494D275-CED2-441D-9758-69CCDE8404E8.

Molecular data: Complete 18S sequence (1666 bp) obtained from type host.

Material deposited: Extracted DNA and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Pro 69), 18S rDNA sequence (1666 bp for type host) deposited under the GenBank Acc. number ON685871 (isolate KZN20/14).

Description of sporogonic stages: Polysporic plasmodia of round, oval or irregular shape ranging 10.1–39.4 μm ($n = 3$).

Table 1
Comparison of newly described species of *Chloromyxum* with congeners. Abbreviations: SP – spore; PC – polar capsule.

Myxozoan species	Chondrichthyan host	SSU rDNA GenBank Acc. No.	SP length	SP width	PC length	PC width	Reference
***C. acuminatum* n. sp.**	*Rhinoptera javanica*	ON685871	8.5 ± 0.6 (7.5–9.5)	6.4 ± 0.7 (5.5–8.9)	2.5 ± 0.2 (2.1–2.9)	1.5 ± 0.2 (1.2–1.9)	**Present study**
***C. africanum* n. sp.**	*Mustelus mustelus, Carcharias taurus*	ON685872, ON685873, ON685880	10.9 ± 1.0 (9.0–13.49)	8.6 ± 0.6 (7.4–10.6)	2.9 ± 0.5 (1.7–3.7)	1.9 ± 0.3 (1.5–2.4)	**Present study**
C. atlantoraji	*Atlantoraja castelnaui*	MG652633	10.5 ± 0.4 (9.7–11.4)	8.5 ± 0.4 (7.7–9.4)	3.5 ± 0.3 (3.0–4.5)	2.4 ± 0.2 (1.9–3.0)	Cantatore et al. (2018)
***C. bulliti* n. sp.**	*Notorynchus cepedianus, Hexanchus griseus*	ON685874, ON685875	10.1 ± 0.5 (9.1–11.1)	7.7 ± 0.5 (6.7–8.8)	2.9 ± 0.3 (2.1–3.5)	2.0 ± 0.3 (1.2–2.6)	**Present study**
***C. carcharhini* n. sp.**	*Carcharhinus leucas*	ON685876	11.3 ± 1.0 (9.9–12.9)	8.8 ± 0.8 (7.7–12.0)	3.1 ± 0.9 (2.3–4.5)	1.7 ± 0.4 (1.1–2.3)	**Present study**
C. clavatum	*Raja clavata*	JQ793641	14.4 ± 0.5	11.9 ± 0.5	5.5 ± 0.4	2.9 ± 0.5	Rocha et al. (2013)
C. dogieli	*Raja miraletus*	unavailable	10.6–12.0	8.0–8.3	3.3–3.9	2.6	Kovaljova (1988)
C. hemiscyllii	*Hemiscyllium ocellatum*	JN130374	11.8 ± 0.2	9.8 ± 0.5	4.1 ± 0.3	3.2 ± 0.2	Gleeson and Adlard (2012)
C. kuhlii	*Neotrygon kuhlii*	JN130375–6	11.4 ± 0.3	8.8 ± 0.3	4.1 ± 0.3	2.8 ± 0.1	Gleeson and Adlard (2012)
C. levigatum	*Squatina californica*	unavailable	11.0–13.0	8.0–10.0			Jameson (1931)
C. lesteri	*Cephaloscyllium laticeps*	JN130377–8	10.4 ± 0.4	8.4 ± 0.3	3.6 ± 0.3	2.7 ± 0.2	Gleeson and Adlard (2012)
C. leydigi	*Squatina squatina; Centroscymnus coelolepis; Torpedo marmorata* etc.	AY604199; DQ377710	6.0–16.0	5.0–14.0	2.0–5.0	1.0–3.0	Jameson (1929); Fiala and Dyková (2004); Fiala (2006); Rocha et al. (2014)
C. liae	*Prionace glauca*	unavailable	4.4–5.2	3.7	1.48	1.48	Kuznetsova (1977)
C. lissosporum	*Squatina oculata*	unavailable	12.0–13.3	6.7–8.0	5.3–5.6	3.5–4.0	Kovaljova (1988)
C. magnum	*Squalus blainville* as *Acanthias blainville*	unavailable	40.0–48.0	30.0–38.0	12.0–15.0	12.0–15.0	Awerinzev (1913)
C. mingazzinii	*Pristiophorus nudipinnis*	JN130379	11.1 ± 0.3	8.8 ± 0.3	3.8 ± 0.5	2.7 ± 0.2	Gleeson and Adlard (2012)
C. myliobati	*Myliobatis tenuicaudatus*	JN130377–8	11.9 ± 0.6	10.0 ± 0.6	3.9 ± 0.4	3.0 ± 0.2	Gleeson and Adlard (2012)
C. noblei	*Taeniura lymma*	unavailable	8.5 (8.0–10.0)	6.0–7.0	3.5 (2.0–4.0)	2.7 (1.0–3.0)	Moser et al. (1989)
***C. ornamentum* n. sp.**	*Raja straeleni*	ON685879	12.6 ± 0.9 (10.9–14.2)	9.9 ± 0.7 (8.6–11.4)	3.4 ± 0.4 (2.8–4.0)	2.3 ± 0.3 (1.7–2.9)	**Present study**
C. ovatum	*Raja miraletus* and others	unavailable	10.0–14.0	7.0–11.0	4.0–4.5	2.0	Jameson (1929); Kovaljova (1988)
C. parvicostatum	*Bathyraja brachyurops*	unavailable	5.2–5.9	4.4–4.7	2.2–2.3	2.2–2.3	Kuznetsova (1977)
C. pristiophori	*Pristiophorus cirratus*	unavailable	11.0	8.0–9.0	5.0–6.0	3.0	Woolcock (1936)
***C. regularis* n. sp.**	*Sphyrna lewini*	ON685878	10.6 ± 0.6 (9.6–11.8)	8.1 ± 0.6 (7.0–9.1)	3.0 ± 0.4 (2.4–3.7)	2.0 ± 0.2 (1.5–2.4)	**Present study**
C. riorajum	*Rioraja agassizii*	FJ624481; MG652631	11.4 ± 0.3	5.9 ± 0.5	3.2 ± 0.4	2.0 ± 0.3	Azevedo et al. (2009); Cantatore et al. (2018)
C. schulmani	*Raja straeleni*	unavailable	9.7–10.6	6.7–8.0	3.2–4.5	2.0–2.6	Kovaljova (1988)
C. scyliorhinium	*Scyliorhinus torazame*	unavailable	10.6	9.2	3.0	1.9	Noble (1948)
C. sphyrnae	*Sphyrna tiburo*	unavailable	15.0	13.0	4.0	4.0	Gioia and Da Silva Cordeiro (1996)
C. squali	*Squalus acanthias* as *S. fernandinus*	JN130381–3	11.4 ± 0.5	9.4 ± 0.5	3.9 ± 0.3	2.9 ± 0.2	Gleeson and Adlard (2012)
C. striatellus	*Scyliorhinus canicula*	unavailable	10.6–11.2	6.7–10.6	2.7–3.3	2.0	Kovaljova (1988)
C. transversocostatum	*Squalus acanthias* as *S. fernandinus; Bathyraja magellanica*	unavailable	5.8–5.9	3.7	2.9	2.9	Kuznetsova (1977)
C. zearaji	*Dipturus brevicaudatus* as *Zearaja chilensis*	MG652632	11.6 (10.8–12.4)	9.6 (9.0–10.4)			Cantatore et al. (2018)
***Chloromyxum* sp.**	*Poroderma pantherinum*	ON685877	13.9 ± 0.7 (13.0–15.3)	11.3 ± 0.7 (9.7–12.3)	4.4 ± 0.4 (3.8–5.0)	2.7 ± 0.3 (2.2–3.1)	**Present study**

Description of myxospores: Mature spore ellipsoid with a pointed cap-like apex, longer than wide, length 8.5 ± 0.6 (7.5–9.5) μm and width 6.4 ± 0.7 (5.5–8.9) μm (n = 30); four anteriorly pointed, slightly pyriform polar capsules, length 2.5 ± 0.2 (2.1–2.9) μm and width 1.5 ± 0.2 (1.2–1.9) μm (n = 30). Two valves joined at a straight suture, hair-like caudal filaments present, spore surface with a prominent striation. Single sporoplasm irregular in shape.

Remarks: *Chloromyxum acuminatum* n. sp. represents the first record of a myxosporean in *R. jayakari*. The parasite has similar morphological features to other chloromyxids from elasmobranchs (Table 1), however, besides its host spectrum it additionally differs from the morphologically similar species by relatively small spore size and its 18S rDNA sequence. The spores of the newly described species show the highest morphological similarity to *Chloromyxum noblei* Moser et al., 1989 (8.0–10.0 μm × 6.0–7.0 μm), a species described from *Taeniura lymma* (Forsskål, 1775) and additionally reported from *Hemiscyllium ocellatum* (Bonnaterre, 1788), *Lethrinus miniatus* (Forster, 1801) and *Diodon hystrix* L. However, both *Chloromyxum* spp. differ in the size of polar capsules, specifically *C. noblei* has longer and thinner polar capsules (2.0–4.0 μm × 1.0–3.0 μm) than *C. acuminatum* n. sp. (Eiras et al., 2012; Table 1). An additional difference is the absence of hair-like caudal filaments and the ribs on the surface of *C. noblei* spores. Unfortunately, no molecular data of *C. noblei* are available in GenBank to genetically compare the two species. None of the existing 18S rDNA sequence available in GenBank matches the newly described species; the most genetically similar (94.5% similarity, 76-nt difference across 1406 bp) is *Chloromyxum myliobati* Gleeson and Adlard, 2012 (GenBank: JN130380).

***Chloromyxum africanum* n. sp. (Fig. 1C–E)**

Type host: *Mustelus mustelus* (L.), common smooth-hound (Carcharhiniformes: Triakidae).

Additional host: *Carcharias taurus* Rafinesque, 1810, sand tiger shark (Lamniformes: Carchariidae).

Type locality: Schulphoek, Hermanus, South Africa (34° 26′ 401″ S, 19° 12′ 126″ E).

Additional locality: Durban, South Africa (29° 51′ 15.84″ S, 31° 2′ 40.2″ E).

The site of sporogonic development: Coelozoic in gallbladder (plasmodia and myxospores floating in the bile).

Prevalence of infection: Overall 71% (5/7): *Mustelus mustelus* 67% (4/6; host individuals HE18/11, HE18/12, HE18/13, HE18/16), *Carcharias taurus* 100% (1/1; host individual KZN20/24).

Etymology: Refers to the continent of origin, Africa.

ZooBank registration: urn:lsid:zoobank.org:act:B28C56CA-A362-4279-AD59-57E5289C9940.

Molecular data: Four identical partial 18S rDNA sequences: 1354 bp (HE18/11, HE18/12), 1120 bp (HE18/13) and 1341 bp (KZN20/24) and one almost identical sequence length 1120 bp (HE18/16; 99.9% of identity; 2-nt difference) obtained from two host species and five host specimens.

Material deposited: Extracted DNA and slides with spores (for both host species) stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Pro 70), 18S rDNA sequences deposited under the GenBank Acc. numbers ON685872 (1354 bp, type host, isolate HE18/11), ON685873 (1120 bp, isolate HE18/16), and ON685880 (1341 bp, isolate KZN20/24).

Description of sporogonic stages (type host): Polysporic plasmodia of round, oval or irregular shape measuring 19.6–31.3 μm (n = 4) (Fig. 1D).

Description of sporogonic stages (additional host): Polysporic plasmodia of round, oval or irregular shape measuring 38.2–65.4 μm (n = 4).

Description of myxospores: Mature spore ellipsoid, longer than wide, length 10.9 ± 1.0 (9.0–13.9) μm and width 8.6 ± 0.6 (7.4–10.6) μm (n = 30); four anteriorly pointed, slightly pyriform polar capsules, length 2.9 ± 0.5 (1.7–3.7) μm and width 1.9 ± 0.3 (1.5–2.4) μm (n = 30, type host) and polar filaments with 4–5 coils each (n = 5). Two valves joined at a straight suture, hair-like caudal filaments present, spore surface with a prominent striation. Single sporoplasm irregular in shape (Fig. 1C and E).

Description of myxospores (additional host): Mature spore ellipsoid, longer than wide, length 10.8 ± 0.4 (10.1–11.3 μm) and width 8.7 ± 0.6 (7.4–9.4 μm) (n = 10); four anteriorly pointed, slightly pyriform polar capsules, length 3.0 ± 0.3 (2.4–3.4 μm) and width 1.9 ± 0.1 (1.6–2.1 μm) (n = 10). Two valves joined at a straight suture, hair-like caudal filaments present, spore surface with a prominent striation. Single sporoplasm irregular in shape.

Remarks: *Chloromyxum africanum* n. sp. has similar morphological features as other chloromyxids from elasmobranchs (Table 1). Spore features of the newly described species are most similar to *Chloromyxum atlantoraji* Cantatore et al., 2018 (spore 10.5 × 8.5 on average; polar capsules 3.5 × 2.4 on average) and *Chloromyxum lesteri* Gleeson and Adlard, 2012 (spore 10.4 × 8.4 on average; polar capsules 3.6 × 2.7 on average), however, *C. africanum* n. sp. has smaller polar capsules and differs in 18S rDNA sequence from both species (details in Table 1). Though an undescribed *Chloromyxum* sp. has previously been recorded in a lamniform elasmobranch fish, i.e., the megamouth shark *Megachasma pelagios* Taylor, Compagno & Struhsaker, 1983 (Yokoyama, 1997), our finding of *C. africanum* n. sp. in *C. taurus* represents the first formal description of a myxozoan species in Lamniformes. As for *M. mustelus*, the type host of *C. africanum* n. sp., the only other report of a *Chloromyxum* from this fish species is *C. leydigi*, which differs from the newly described species both morphologically and genetically (18S rDNA sequence). *Chloromyxum leydigi* was originally described, without specifying the type host, from numerous elasmobranch species of different genera (*Mustelus* Linck, 1790, *Galeus* Cuvier, 1816, *Raja* L., *Scyliorhinus* Blainville, 1816, *Squatina* Duméril, 1805, *Torpedo* Duméril, 1806 and *Dasyatis* Rafinesque, 1810) (Lom and Dyková, 2006; Rocha et al., 2014). *Torpedo marmorata* Risso, 1810 has only later been assigned as the type host of this species (Rocha et al., 2014). However, *C. leydigi* is likely a species complex (Fiala and Dyková, 2004; Rocha et al., 2014) that is reflected by a large variability in its spore dimensions (Table 1; Eiras et al., 2012), broad host species spectrum and non-matching GenBank 18S rDNA sequences of its isolates. The sequences of *C. africanum* n. sp. also do not match with other chloromyxid sequences available in GenBank (the most similar sequence belongs to *C. hemiscylli* Gleeson and Adlard, 2012 JN130374, 96.7% similarity, 36-nt difference across 1094 bp).

***Chloromyxum bulliti* n. sp. (Fig. 1F–I)**

Type host: *Notorynchus cepedianus* (Péron, 1807), broadnose sevengill shark (Hexanchiformes: Hexanchidae).

Additional host: *Hexanchus griseus* (Bonnaterre, 1788), bluntnose sixgill shark (Hexanchiformes: Hexanchidae).

Type locality: San Matías gulf, Río Negro, Argentina (41° 50′ 000″ S, 64° 50′ 000″ W).

Additional locality: Schulphoek, Hermanus, South Africa (34° 26′ 401” S, 19° 12′ 126″ E).

Site of sporogonic development: Coelozoic in gallbladder (plasmodia and myxospores floating in the bile).

Prevalence of infection: Overall 100% (2/2): *N. cepedianus*: 100% (1/1, host individual NC), *H. griseus*: 100% (1/1; host individual HE18/9).

Etymology: From Latin “bullitus” meaning bubble, due to the spherical shape of plasmodia.

ZooBank registration: urn:lsid:zoobank.org:act:F096CF55-836D-44DD-81B3-E54CC2C06E13.

Molecular data: Almost identical partial 18S sequences (99.9% identity; 1-nt difference; 1633 bp, and 1777 bp) obtained from two host species and two host specimens.

Material deposited: Extracted DNA and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number

IPCAS Pro 71); slide with spores deposited at the Invertebrate Collection of Museo de La Plata (MLP-CRG), La Plata, Buenos Aires, Argentina (Acc. number MLP-Oi 4216); partial 18S rDNA sequences deposited under the GenBank Acc. numbers ON685874 (1777 bp for type host, isolate NC) and ON685875 (1633 bp for *H. griseus,* isolate HE18/9).

Description of sporogonic stages: Round monosporic to polysporic plasmodia ranging 18.1–25.4 μm ($n = 10$) (Fig. 1H, I).

Description of myxospores (type host): Mature spore ellipsoid, longer than wide, length 10.1 ± 0.5 (9.1–11.1) μm and width 7.7 ± 0.5 (6.7–8.8) μm ($n = 30$); four anteriorly pointed, slightly pyriform polar capsules, length 2.9 ± 0.3 (2.1–3.5) μm and width 2.0 ± 0.3 (1.2–2.6) μm (n = 30) and polar filaments with 4–5 coils each (n = 3). Two valves joined at a straight suture, hair-like caudal filaments present. Spore surface with a fine striation containing three main ridges. Single sporoplasm irregular in shape (Fig. 1F, G, H).

Description of myxospores (additional host): Mature spore ellipsoid, longer than wide, length 9.9 ± 0.5 (8.9–10.8) μm ($n = 30$) and width 7.7 ± 0.6 (6.9–9.3) μm (n = 30); four anteriorly pointed, slightly pyriform polar capsules, length 2.6 ± 0.4 (2.2–3.4) μm and width 1.9 ± 0.2 (1.4–2.3) μm (n = 30). Two valves joined at a straight suture, hair-like caudal filaments present. Spore surface with fine striation containing three main ridges. Single sporoplasm irregular in shape.

Remarks: *Chloromyxum bulliti* n. sp. has similar biological (site of infection, host group) and morphological spore features with other chloromyxids from elasmobranchs (Table 1) including typical, fine hair-like filaments on the posterior spore end (Fig. 1H). Morphological features of spores of the newly described species are the most similar to *Chloromyxum atlantoraji* (spore 10.5 × 8.5 on average; polar capsules 3.5 × 2.4 on average) and *Chloromyxum lesteri* (spore 10.4 × 8.4 on average; polar capsules 3.6 × 2.7 on average), however, *C. bulliti* n. sp. has smaller polar capsules and also differs from both species in the 18S rDNA sequence (details in Table 1). The newly described species represents the first myxozoan record in cow sharks (Hexanchiformes) – *N. cepedianus* and *H. griseus* (Table 1), members of the most ancient elasmobranch group. Additionally, 18S rDNA sequence of *C. bulliti* n. sp. is unique in comparison to other *Chloromyxum* species (the most similar sequence belongs to the undescribed species *Chloromyxum* sp. ex *Pseudobatos horkelii* MK937847; 99.1% similarity, 7-nt difference across 790 bp).

***Chloromyxum carcharhini* n. sp. (Fig. 1J–L)**

Type host: *Carcharhinus leucas* (Valenciennes in Müller & Henle, 1839), bull shark (Carcharhiniformes: Carcharhinidae).

Type locality: Zinkwazi, South Africa (29° 16′ 26.76″ S, 31° 27′ 9.22″ E).

Site of sporogonic development: Coelozoic in gallbladder (plasmodia and myxospores floating in the bile).

Prevalence of infection: 100% (1/1), host individual KZN20/23.

Etymology: The species is named after the genus of the host species, *Carcharhinus.*

ZooBank registration: urn:lsid:zoobank.org:act:4E4DA832-88C6-492E-9F6B-0E012E57C939.

Molecular data: Partial 18S sequence (1392 bp) obtained from type host.

Material deposited: Extracted DNA and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Pro 72); partial 18S rDNA sequence deposited under the GenBank Acc. number ON685876 (1392 bp, isolate KZN20/23).

Description of sporogonic stages: Round polysporic plasmodia ranging 37.9–52.2 μm (n = 3) (Fig. 1L).

Description of myxospores: Mature spore ellipsoid with a slightly pointed apex, longer than wide, length 11.3 ± 1 (9.9–12.9) μm and width 8.8 ± 0.8 (7.7–12.0) μm ($n = 11$); four anteriorly pointed, slightly pyriform polar capsules, length 3.1 ± 0.9 (2.3–4.5) μm and width 1.7 ± 0.4 (1.1–2.3) μm ($n = 4$). Two valves joined at a straight suture, hair-like caudal filaments present. Spore surface with a fine striation (Figs. 1J, K).

Remarks: *Chloromyxum carcharhini* n. sp. has similar biological (site of infection, host group) and morphological spore features with other chloromyxids from elasmobranchs (Table 1). By its spore shape and size, the newly described species is most similar to *Chloromyxum kuhlii* Gleeson and Adlard, 2012 (11.4 ± 0.3 μm × 8.8 ± 0.3 μm) which has been described from *Neotrygon kuhlii* (Müller & Henle, 1841). The two parasite species differ from each other by the dimensions of polar capsules, which are larger in *C. kuhlii* (4.1 ± 0.3 μm × 2.8 ± 0.1 μm) (Gleeson and Adlard, 2012; Eiras et al., 2012, Table 1) and genetically (*C. kuhlii* GenBank: JN130375, JN130376; 11.8% difference across 1140 bp). The newly described species is also similar to *C. atlantoraji* (spore 10.5 × 8.5 μm; polar capsules 3.5 × 2.4 μm), *C. lesteri* (spore 10.4 × 8.4 μm; polar capsules 3.6 × 2.7 μm) and *C. africanum* n. sp. (spore 10.9 × 8.6 μm; polar capsules 2.9 × 1.9 μm), however, *C. carcharhini* n. sp. has smaller polar capsules (details in Table 1) and different 18S rDNA sequence (*C. africanum* n. sp. 12.6% difference across 1354 bp; *Chloromyxum lesteri* JN130377 11.3% difference across 1101 bp) than the congeners. Additionally, 18S rDNA sequence of *C. carcharhini* n. sp. is unique in comparison to other *Chloromyxum* species (the most similar sequence belongs to *Chloromyxum* sp. from *Sphyrna tiburo* L. (MK937848, 10.2% difference across 862 bp). *Chloromyxum carcharhini* n. sp. represents the first myxosporean record in Ca. *leucas* and none of the existing 18S rDNA sequence available in GenBank matches the newly described species.

***Chloromyxum ornamentum* n. sp. (Fig. 1M–O)**

Type host: *Raja straeleni* Poll, 1951, spotted skate (Rajiformes: Rajidae).

Type locality: Hawston, South Africa (34° 25′ 091″ S, 19° 15′ 431″ E).

Site of sporogonic development: Coelozoic in gallbladder (plasmodia and myxospores floating in the bile).

Prevalence of infection: 38% (3/8), host individuals HE19/2, HE19/10 and HE19/11.

Etymology: From the Latin word “ornamentum”, which means ornamental and refers to the ornamental/prominently ridged spore surface.

ZooBank registration: urn:lsid:zoobank.org:act:F0437162-DF97-430B-9520-7147E8BDD936.

Molecular data: Partial 18S sequences (1668 bp) obtained from the type host.

Material deposited: Extracted DNA and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Pro 73); partial 18S rDNA sequence (1668 bp) deposited under the GenBank Acc. number ON685879 (isolate HE19/2, type host).

Description of sporogonic stages: Round or oval (disporic or polysporic) plasmodia ranging 9.5–37.5 μm ($n = 16$) (Fig. 1N).

Description of myxospores: Mature spore ellipsoid, longer than wide, length 12.6 ± 0.9 (10.9–14.2) μm and width 9.9 ± 0.7 (8.6–11.4) μm ($n = 30$); four anteriorly pointed, slightly pyriform polar capsules, length 3.4 ± 0.4 (2.8–4.0) μm and width 2.3 ± 0.3 (1.7–2.9) μm (n = 30) and polar filaments with 4–5 coils each ($n = 10$). Spore surface with 3–4 prominent striations and with short caudal filaments (1.5–5.4 μm; $n = 5$). Single sporoplasm irregular in shape (Fig. 1M and O).

Remarks: *Chloromyxum ornamentum* n. sp. has similar biological (site of infection, host group) and morphological spore features with other chloromyxids from elasmobranchs (Table 1). *Raja straeleni* hosts one described *Chloromyxum* species (*C. schulmani* Kovaljova, 1988) which is smaller in spore length and width in comparison to *C. ornamentum* n. sp. (Table 1). Unfortunately, no 18S rDNA data are available for *C. schulmani* for genetic comparison with *C. ornamentum* n. sp. Spores of *C. ornamentum* n. sp. are similar in size to *C. lissosporum* Kovaljova, 1988 from *Squatina oculata* Bonaparte, 1840, however, the latter has narrower spores (6.7–8.0) than the newly described species. In addition, the polar capsules of *C. lissosporum* are longer (5.3–5.6 μm × 3.5–4.0 μm) than those of *C. ornamentum* n. sp. (Eiras et al., 2012; Table 1). No

sequence data are available in GenBank for *C. lissosporum* for comparative analysis. Additionally, 18S rDNA sequence of *C. ornamentum* n. sp. is unique in comparison to other *Chloromyxum* species (the most similar sequence belongs to sequence *C. leydigi* AY604199; 1.6% in the 1342 bp long section and *Chloromyxum* sp. ex *Torpedo torpedo* MN953427; 1.8% in the 1342 bp long section). The new species can be distinguished from other elasmobranch-infecting chloromyxids by a different host species spectrum, 18S rDNA and morphological features (Table 1).

***Chloromyxum regularis* n. sp. (**Fig. 1P–R**)**

Type host: *Sphyrna lewini* (Griffith a Smith, 1834), scalloped hammerhead (Carcharhiniformes: Sphyrnidae).

Type locality: Richards Bay, South Africa (28° 47′ 42.36″ S, 32° 6′ 37.19″ E).

Site of sporogonic development: Coelozoic in the gallbladder (myxospores floating in the bile).

Prevalence of infection: 25% (1/4), host individual HE20/16.

Etymology: From the Latin word "regularis", which refers to the regular shape of spore.

ZooBank registration: urn:lsid:zoobank.org:act:4BF6400B-8181-485F-A7D3-4CC60266D86C.

Molecular data: Partial 18S rDNA sequence (1477 bp) obtained from the type host.

Material deposited: Extracted DNA and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Pro 74); partial 18S rDNA sequence deposited under the GenBank Acc. number ON685878 (1477 bp, isolate HE20/16, type host).

Description of sporogonic stages: Round tetrasporic to polysporic plasmodia ranging 5.7–46.5 μm ($n = 7$) (Fig. 1Q).

Description of myxospore: Mature spore ellipsoid, longer than wide, length 10.6 ± 0.6 (9.6–11.8) μm and width 8.1 ± 0.6 (7.0–9.1) μm; four anteriorly pointed, pyriform polar capsules, length 3.0 ± 0.4 (2.4–3.7) μm and width 2.0 ± 0.2 (1.5–2.4) μm (n = 30); and polar filaments with 3–4 coils each ($n = 4$). Valves joined at a straight suture, short caudal filaments present, spore surface with 7–10 prominent striations. Single sporoplasm irregular in shape (Fig. 1P and R).

Remarks: *Chloromyxum regularis* n. sp. represents the first record of a myxosporean in *S. lewini*. The parasite has similar biological (site of infection, host group - Sphyrnidae) and morphological spore features as other chloromyxids from elasmobranchs (Table 1). The newly described species shows the highest morphological similarity of its spores with *C. atlantoraji* (spore 10.5 × 8.5 μm; polar capsules 3.5 × 2.4 μm), *C. lesteri* (spore 10.4 × 8.4 μm; polar capsules 3.6 × 2.7 μm) and *C. africanum* n. sp. (spore 10.9 × 8.6 μm; polar capsules 2.9 × 1.9 μm). However, *C. regularis* n. sp. differs from the related species by its unique 18S rDNA sequence. The newly described species has highly similar 18S rDNA sequence (4-nt difference across 868 bp) as the chloromyxid sequence from *Chloromyxum* sp. ex *Poroderma pantherinum* obtained in this study (ON685877, 868 bp). Sequence differences between these two species may be much larger as only approximately half of the complete 18S rRNA gene region could have been compared due to incomplete sequence data of *Chloromyxum* sp. and the fact that the remainder of the gene generally contains highly divergent variable regions in myxozoans (Jirků et al., 2011; Bartošová et al., 2013). Moreover, the two parasite taxa differ in morphometry of their spores (*Chloromyxum* sp.: 13.0–15.3 μm × 9.7–12.3 μm) and polar capsules (*Chloromyxum* sp.: 3.8–5.0 μm × 2.2–3.1 μm; details in Table 1). Further, *C. regularis* n. sp. has a similar 18S rDNA sequence (13-nt difference across 559 bp) with *Chloromyxum* sp. ex Ca. *limbatus* (MK937841), however, no morphological data were provided with this sequence.

3.3. Details about an undescribed species of Chloromyxum

Chloromyxum sp. found in the bile of *Poroderma pantherinum* is not formally described as a new species herein due to an insufficient amount of data. However, we provide morphological and sequence data that may be useful for future species investigations.

***Chloromyxum* sp. ex *Poroderma pantherinum* (**Fig. 1S**)**

Host: *Poroderma pantherinum* (Smith in Müller & Henle, 1838), leopard catshark (Carcharhiniformes: Scyliorhinidae).

Localities: Old Harbor (34° 25′ 15.7584″ S, 19° 14′ 37.5576″ E), and New Harbor, Hermanus, South Africa (34° 25′ 59.286″ S, 19° 13′ 32.2536″ E).

Site of sporogonic development: Coelozoic in the gallbladder (myxospores floating in the bile).

Prevalence of infection: 30% (3/10), host individuals HE18/1, HE18/5, HE18/19.

Molecular data: Identical partial 18S rDNA sequences obtained from three host specimens (1113 bp, 1107 bp, 1108 bp).

Material deposited: Extracted DNA and slides with spores stored at the Protistological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice (Acc. number IPCAS Pro 75 (HE18/1); partial 18S rDNA sequence (1113 bp) deposited under the GenBank Acc. number ON685877 (HE18/1).

Description of sporogonic stages: Not observed.

Description of myxospore: Mature spore ellipsoid longer than wide, length 13.9 ± 0.7 (13.0–15.3) μm (n = 7) and width 11.3 ± 0.7 (9.7–12.3) μm ($n = 8$); four anteriorly pointed, pyriform polar capsules, length 4.4 ± 0.4 (3.8–5.0) μm ($n = 5$) and width 2.7 ± 0.3 (2.2–3.1) μm ($n = 6$). Single sporoplasm irregular in shape, short caudal filaments present (Fig. 1S).

Remarks: *Chloromyxum* sp. ex *P. pantherinum* represents the first record of a myxosporean in this host species. The parasite has similar biological (site of infection, host group) and morphological spore features as other chloromyxids from elasmobranchs (Table 1). *Chloromyxum* sp. ex *P. pantherinum* shows the highest morphological similarity of its spores with *C. atlantoraji* (spore 10.5 × 8.5 μm; polar capsules 3.5 × 2.4 μm), *C. lesteri* (spore 10.4 × 8.4 μm; polar capsules 3.6 × 2.7 μm) and *C. africanum* n. sp. (spore 10.9 × 8.6 μm; polar capsules 2.9 × 1.9 μm), however, *Chloromyxum* sp. ex *P. pantherinum* differs from the related species by a unique 18S rDNA sequence. The newly found species has a similar 18S rDNA sequence as *C. regularis* n. sp. (ON685878; 4-nt difference across 868 bp) and with *Chloromyxum* sp. ex Ca. *limbatus* (GenBank: MK937841; 17-nt difference across 786 bp). However, interspecific sequence differences may be much larger as only approximately half of the complete 18S rRNA gene region could be compared and the rest of the gene exhibits highly divergent variable regions (Jirků et al., 2011; Bartošová et al., 2013). Moreover, the two parasite taxa differ in morphometry of their spores (details in Table 1). For *Chloromyxum* sp. ex Ca. *limbatus* no morphological data were provided.

3.4. Chloromyxum sensu stricto phylogeny and tracing character history

The species discovered in this study clustered with other *Chloromyxum* spp. parasitising elasmobranchs within the robustly supported *Chloromyxum sensu stricto* clade (ML/BI = 100/1). This clade is split into five main sublineages (Fig. 2) with all lineages but the most basal one being characterized predominantnly by one host group, i.e. either sharks or batoids (Fig. 3). The first, most basal sublineage contained both the sublineages of shark- (*C. carcharhini* n. sp. and *Chloromyxum* sp. ex *Sphyrna tiburo*) and batoid-infecting chloromyxids (*C. dasyatidis*, *C. myliobati* and *C. acuminatum* n. sp.). The second sublineage was represented by a single *Chloromyxum* parasite of the shark *Squatina guggenheim*. Interestingly, the parasite of the earliest extant shark group Hexanchiformes, *Chloromyxum bulliti* n. sp., was not positioned at the base of the tree but it grouped with several rajiformes-infecting species in the third, internal sublineage of the *sensu stricto* clade. The fourth well-supported sublineage was created by several isolates of *C. clavatum*, a rajiformes-infecting parasite (Fig. 2). The fifth sublineage mostly encompassed parasites of sharks including the newly described chloromyxids *C. regularis* n. sp., *Chloromyxum* sp. ex *P. pantherinum* and

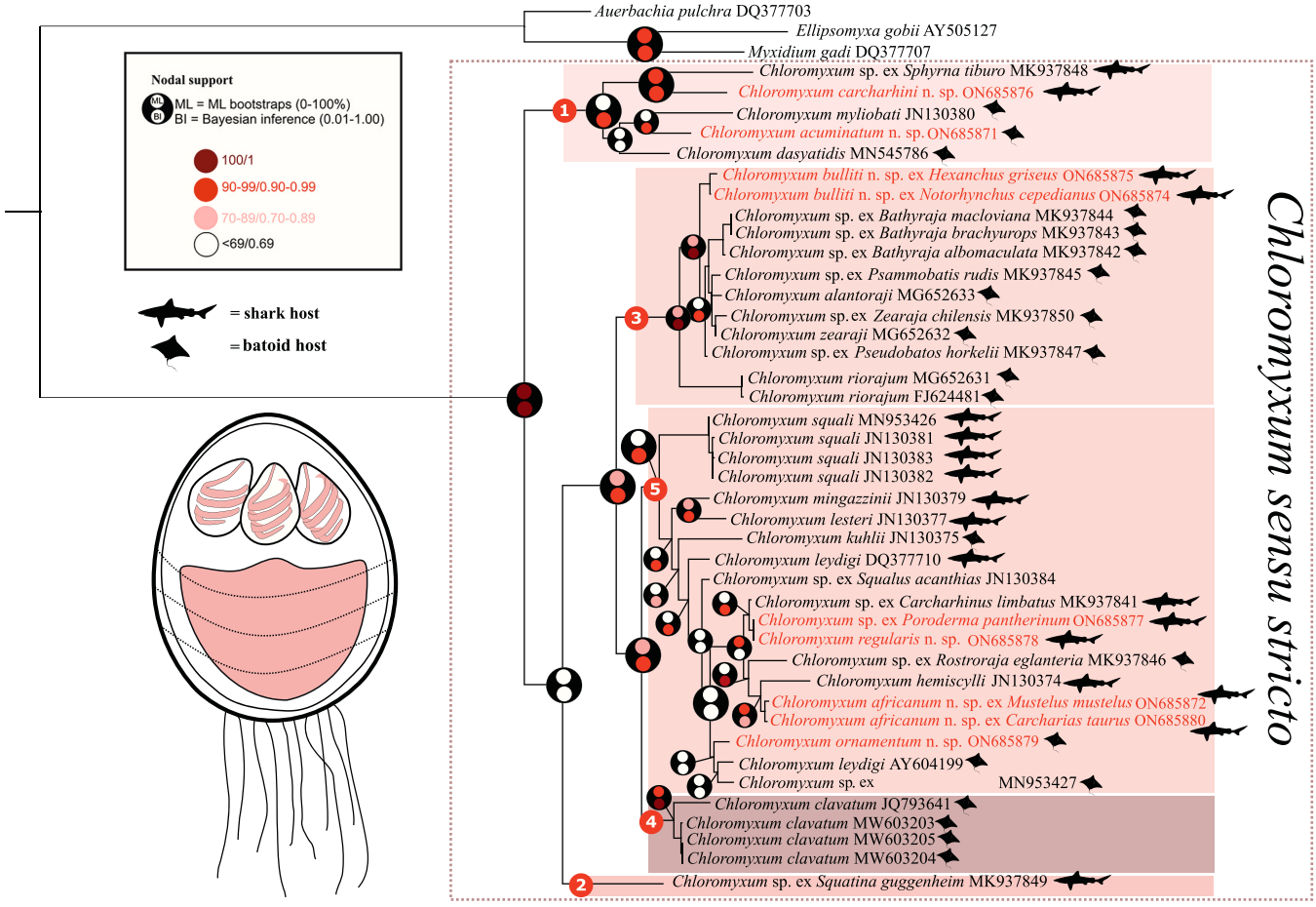


Fig. 2. The 18S rDNA-based phylogenetic tree including all elasmobranch-infecting *Chloromyxum* spp. Teleost-infecting species *Auerbachia pulchra, Myxidium gadi* and *Ellipsomyxa gobii* were used as the outgroup. Newly described species in this study are marked in red. Maximum likelihood/Bayesian inference nodal supports are shown at each node by the coloured circle with scaling as shown in the top left legend. Icons indicate a shark and a batoid host of particular *Chloromyxum* species. Numbers at nodes (1–5) indicate the main phylogenetic sublineages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

C. africanum n. sp. that grouped with the few batoid-infecting species, i. e., *C. ornamentum* n. sp., *C. leydigi*, *Chloromyxum* sp. ex *Torpedo torpedo*, and *Chloromyxum* sp. ex *Rostroraja eglanteria* (Fig. 2).

Character state reconstruction (Fig. 3) suggested sharks to be the most likely ancestral host group of *Chloromyxum sensu stricto*. However, the proportional character state likelihood for the shark character state in this ancestral node was relatively low (0.69) similarly as for the shark ancestor of the most basal chloromyxid sublineage 1 (0.65). Sharks were also traced to be most likely the ancestral hosts for the chloromyxid sublineages 2 and 5 (1.0; 0.87) while a batoid was most likely an ancestral host for sublineages 3 and 4 (0.73; 0.94) (Fig. 3).

3.5. Host-parasite co-phylogeny

Paco analysis revealed that the phylogeny of *Chloromyxum* parasites was significantly correlated with the phylogeny of their hosts ($P < 0.001$, $n = 1000$). In detail, ten interactions with a relatively strong co-phylogenetic signal appeared between *Chloromyxum* spp. and elasmobranchs in the co-phylogenetic network of host and parasite phylogeny, namely: i) four associations of batoid species and their myxozoan parasites (*Rhinobatos horkelii/ Chloromyxum* sp. ex *Rhinobatos horkelii; Raja clavata/C. clavatum; Myliobatis australis/C. myliobati; Rhinoptera jayakari /C. acuminatum* n. sp.); and ii) six associations of shark species and their myxozoan parasites (*Squalus acanthias/C. squali; Squalus blainville/C. squali; Hexanchus griseus/C. bulliti* n. sp.*; Pristiophorus nudipinnis/C. mingazzinii; Sphyrna lewini/C. regularis* n. sp.*; Cephaloscyllium laticeps/C. lesteri)* (Fig. 4).

3.6. Geographic origin of elasmobranch-infecting myxozoans

Pairwise likelihood ratio tests of the biogeographic models showed better AIC scores of the models with the *j* parameter included in the DIVALIKE and BAYAREALIKE analyses ($p < 0.0001$). The AIC model comparison revealed the BAYAREALIKE + *j* model as the best fit for the BioGeoBEARS analysis (Fig. 5; Supplementary Table 4). Globally, a combination of 109 dispersals and seven vicariances explained the geographical distribution of parasites while no extinction events were calculated. The highest amount of speciation events within areas was found in the Atlantic coast of South America (area A; 21). The highest number of dispersals occurred from the Atlantic coast of South America (area A; 11), followed by the Atlantic coast of Africa (area B; 9). The BioGeoBEARS biogeographical analyses showed that chloromyxids from skates and guitarfish *Bathyraja macloviana, B. brachyurops, B. albomaculata, Psammobatis rudis, Pseudobatos horkelii, Atlantoraja castelani* and *Zearaja chilensis* most likely originated on the Atlantic coast of South America (100% probability), however this result may strongly be impacted by a limited data availability. Otherwise, there was no clear phylogeographic pattern for the ancestral parasite distribution (Fig. 5).

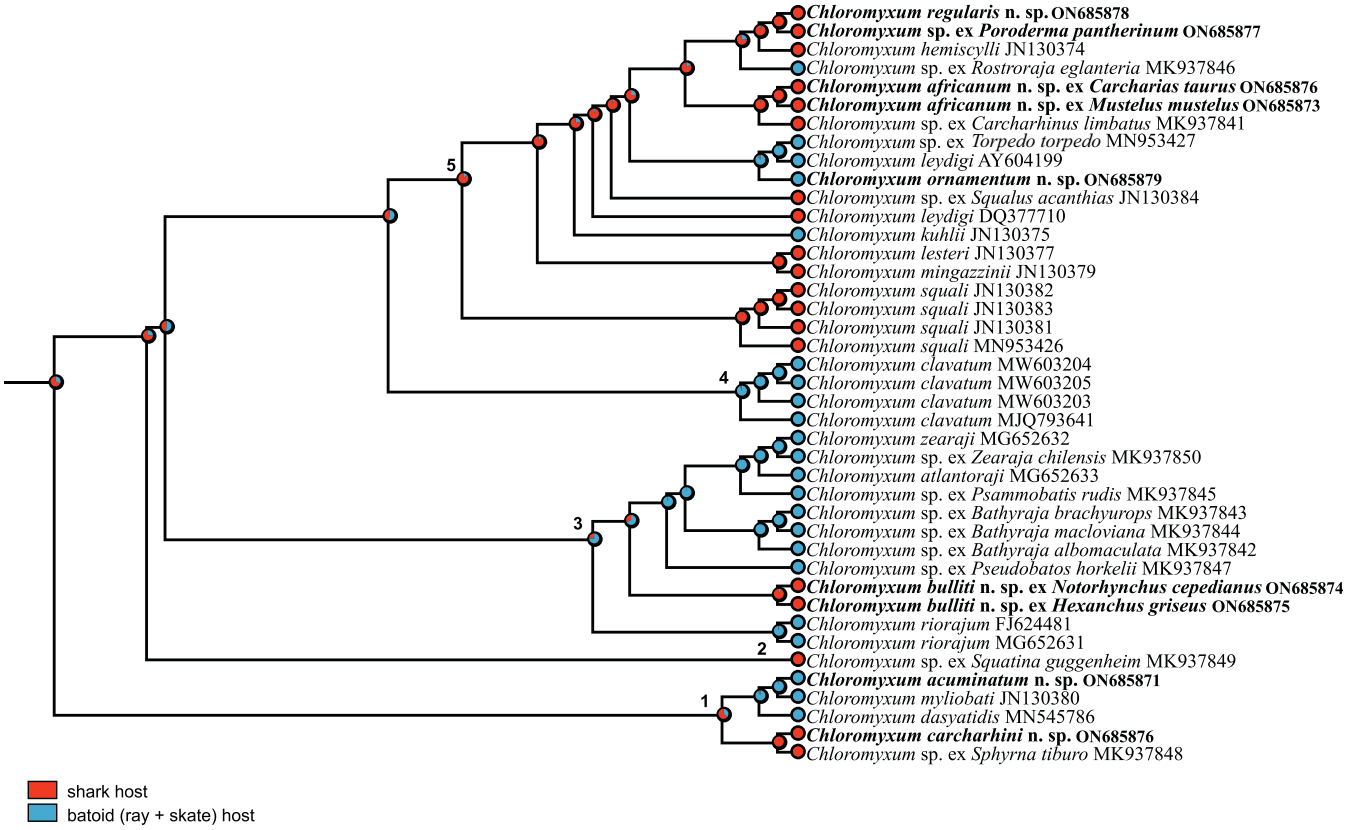


Fig. 3. Tracing ancestral state evolution of the shark- and batoid-associated parasites onto the phylogenetic tree of *Chloromyxum sensu stricto* species. Numbers at nodes (1–5) indicate the main phylogenetic sublineages. Newly found species in the present study are in bold.

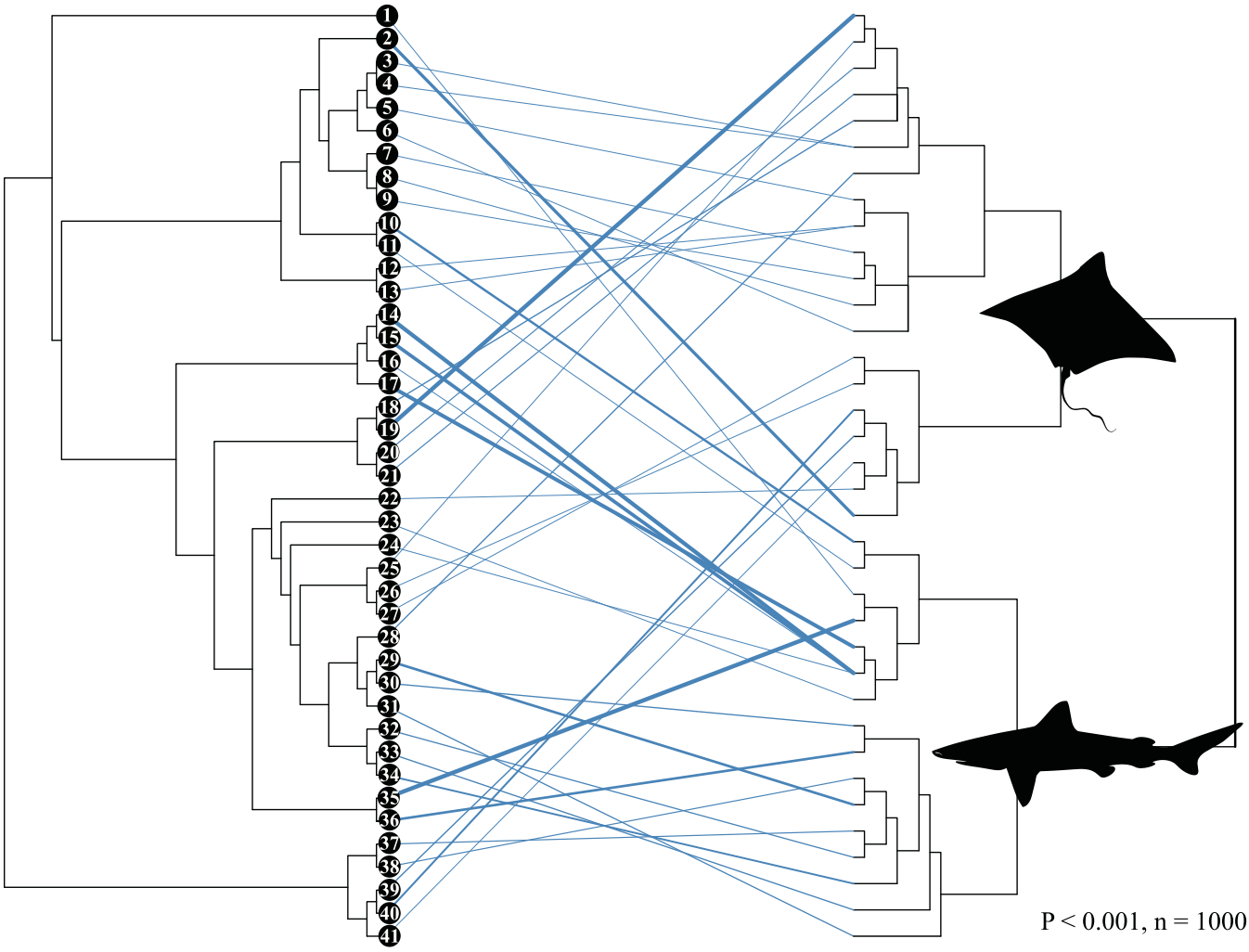
4. Discussion

Here we present new data on the diversity and evolution of *Chloromyxum* spp. in elasmobranchs, an evolutionary ancient and ancestral host group of myxozoan parasites (Kodádková et al., 2015; Holzer et al., 2018; Lisnerová et al., 2020). Our sampling efforts mainly targeted the sparsely explored areas off Southern Africa where we found that chloromyxid infections are relatively common (28%). This further supports our belief that myxozoan diversity in elasmobranchs is only superficially known, a view in accordance with previous studies on other myxozoan genera (Bartošová-Sojková et al., 2014; Hartikainen et al., 2016; Holzer et al., 2018; Okamura et al., 2018). We extend the known host range for chloromyxid myxozoans to include the previously unexplored shark orders Lamniformes and Hexanchiformes. Our discovery of a novel *Chloromyxum* species from hexanchiform sharks has crucial implications for addressing the evolutionary history of the Myxozoa, as these are the most ancient sharks on Earth (Cappetta, 2012).

As expected, all new chloromyxids found in elasmobranch hosts clustered in the *Chloromyxum sensu stricto* clade. One result of this study contradicted our hypotheses as *C. bulliti* n. sp. from a very ancient shark host group, cow sharks (*H. griseus, N. cepedianus*) grouped with chloromyxids from Rajiformes, a host group considered to be more recent than Hexanchiformes and belonging to the first batoid lineages (Heinicke et al., 2009). Although discovered in the earliest shark and batoid hosts, *C. bulliti* n. sp. and its close relatives clustered in a sublineage branching off in the middle of the *Chloromyxum sensu stricto* clade, while the most basal *Chloromyxum* sublineage was associated with younger elasmobranch groups Carchariniformes and Myliobatiformes (Heinicke et al., 2009; Naylor et al., 2012), despite strong support for overall cophylogeny of elasmobranchs and *Chloromyxum* spp. This phylogenetic pattern could be due to relatively recent host switches of early myxozoan lineages to younger elasmobranch lineages. We know that many elasmobranch lineages went extinct (Maisey, 2012) and assume that their parasites went extinct with them. This may lead to unstable phylogenies with low support values, especially in the deeper nodes or nodes connecting undersampled lineages. Similarly, the mapping of character evolution may be strongly biased by limited taxon sampling. Increased taxon sampling may change the topology of the tree and recover the currently earliest lineages of *Chloromyxum* as younger nodes or alternatively reveal early chloromyxid lineages in early hosts.

Kodádková et al. (2015) suggested that the *Chloromyxum sensu stricto* clade diverged off the rest of Myxozoa between 389 and 334 Myr, which overlaps with the divergence of sharks and batoids between 431 and 354 Myr (Heinicke et al., 2009) and especially with the fossil record dating the shark–ray divergence to have happened between 330 and 380 Myr (Janvier and Pradel, 2016). Given this timeline as well as the internal phylogenetic positioning of chloromyxids of hexanchiform sharks and inconclusive proportional likelihood of the ancestral host group for the whole *Chloromyxum sensu stricto* group, it is conceivable that chloromyxids invaded elasmobranchs just prior or at the time of divergence of sharks and batoids, i.e., the origin of chloromyxids slightly predated or overlapped with the division of elasmobranchs into two lineages.

By including new data for an as of yet poorly studied group of myxozoans into a co-phylogenetic analysis we were able to support the hypothesis that myxozoan lineages generally are linked to host phylogeny (Holzer et al., 2018; Patra et al., 2018; Lisnerová et al., 2020) even at lower taxonomic levels and in ancient hosts. Due to a tight co-evolutionary history, a tendency to cluster into shark- vs. batoid-infecting clades as observed here has also been reported for other parasite groups such as tapeworms (Olson et al., 2010) or coccidians (Xavier et al., 2018). The existing incongruences between the host and parasite phylogenies have previously been shown to be caused by host switches from sharks to rays and vice versa (Lisnerová et al., 2020) that may be ecologically driven primarily through overlap in the niches of



1. *Chloromyxum* sp. ex *Squatina guggenheim*
2. *Chloromyxum* sp. ex *Rhinobatos horkelii*
3. *Chloromyxum* sp. ex *Zearaja chilensis*
4. *C. zearaji* / *Zearaja chilensis*
5. *C. atlantoraji* / *Atlantoraja castelnaui*
6. *Chloromyxum* sp. ex *Psammobatis rudis*
7. *Chloromyxum* sp. ex *Rhinoraja albomaculata*
8. *Chloromyxum* sp. ex *Bathyraja brachyurops*
9. *Chloromyxum* sp. ex *Rhinoraja macloviana*
10. *C. bulliti* n. sp. / *Hexanchus griseus*
11. *C. bulliti* n. sp. / *Notorhynchus cepedianus*
12., 13. *C. riorajum* / *Rioraja agassizii*
14., 15., 16. *C. squali* / *Squalus acanthias*
17. *C. squali* / *Squalus blainville*
18. *C. clavatum* / *Raja miraletus*
19. *C. clavatum* / *Raja clavata*
20. *C. clavatum* / *Raja asterias*
21. *C. clavatum* / *Raja montagui*
22. *C. kuhlii* / *Neotrygon kuhlii*
23. *C. leydigi* / *Centroscymnus coelolepis*
24. *Chloromyxum* sp. ex *Squalus acanthias*
25. *C. ornamentum* n. sp. / *Raja straeleni*
26. *C. leydigi* / *Torpedo marmorata*
27. *Chloromyxum* sp. ex *Torpedo torpedo*
28. *Chloromyxum* sp. ex *Raja eglanteria*
29. *C. regularis* n. sp. / *Sphyrna lewini*
30. *Chloromyxum* sp. ex *Poroderma pantherinum*
31. *C. hemiscylli* / *Hemiscyllium ocellatum*
32. *Chloromyxum* sp. ex *Carcharhinus limbatus*
33. *C. africanum* n. sp. / *Carcharias taurus*
34. *C. africanum* n. sp. / *Mustelus mustelus*
35. *C. mingazzinii* / *Pristiophorus nudipinnis*
36. *C. lesteri* / *Cephaloscyllium laticeps*
37. *C. carcharhini* n. sp. / *Carcharhinus leucas*
38. *Chloromyxum* sp. ex *Sphyrna tiburo*
39. *C. myliobati* / *Myliobatis australis*
40. *C. acuminatum* n. sp. / *Rhinoptera javanica*
41. *C. dasyatidis* / *Dasyatis pastinaca*

Fig. 4. The co-phylogenetic analysis of chloromyxids and their elasmobranch hosts, analyzed using the Procrustean Approach to Cophylogeny (paco) in R, with significant congruence ($P < 0.001$, $n = 1000$) in host-parasite associations. The interactions between parasites and hosts are shown as their contribution to the overall phylogenetic congruence where thicker lines mark a smaller residual distance or an interaction with a stronger support of the co-phylogenetic signal. Newly found species in the present study are in bold.

their shark or ray host (Olson et al., 2010) or may additionally be combined with other ecological parameters such as host diet, distribution depth, host size, and geographical location (Beer et al., 2019) or the existence of generalist parasite species that can infect a broad spectrum of elasmobranchs (Olson et al., 2010). Therefore, the paucity of our knowledge about the host spectrum, which appears relatively broad in some chloromyxids, e.g. *C. africanum* n. sp. and *C. bulliti* n. sp. but also other species (Snene et al., 2021), could skew results of phylogenetic and co-phylogenetic analyses as well as of character history mapping. Consequently, increased taxon sampling is necessary to gain further insights into the host specificity of chloromyxids.

Species collected in the Indian Ocean are included for the first time to an analysis of the geographic origin of elasmobranch-infecting myxozoans. Strong support for a common parasite origin was discovered only for chloromyxids from the Southwestern Atlantic Ocean likely due to the isolation of the South American shelf maintained over evolutionary time and the high degree of endemism in this area (Cantatore et al., 2018). Otherwise, the addition of the new samples overall showed limited impact on meaningfulness of phylogeographic analyses, as suggested before (Lisnerová et al., 2020). Given the cosmopolitan distribution of elasmobranchs and their high migration rates (Schaeffner and Smit, 2019; Lisnerová et al., 2020) it appears plausible that there are very

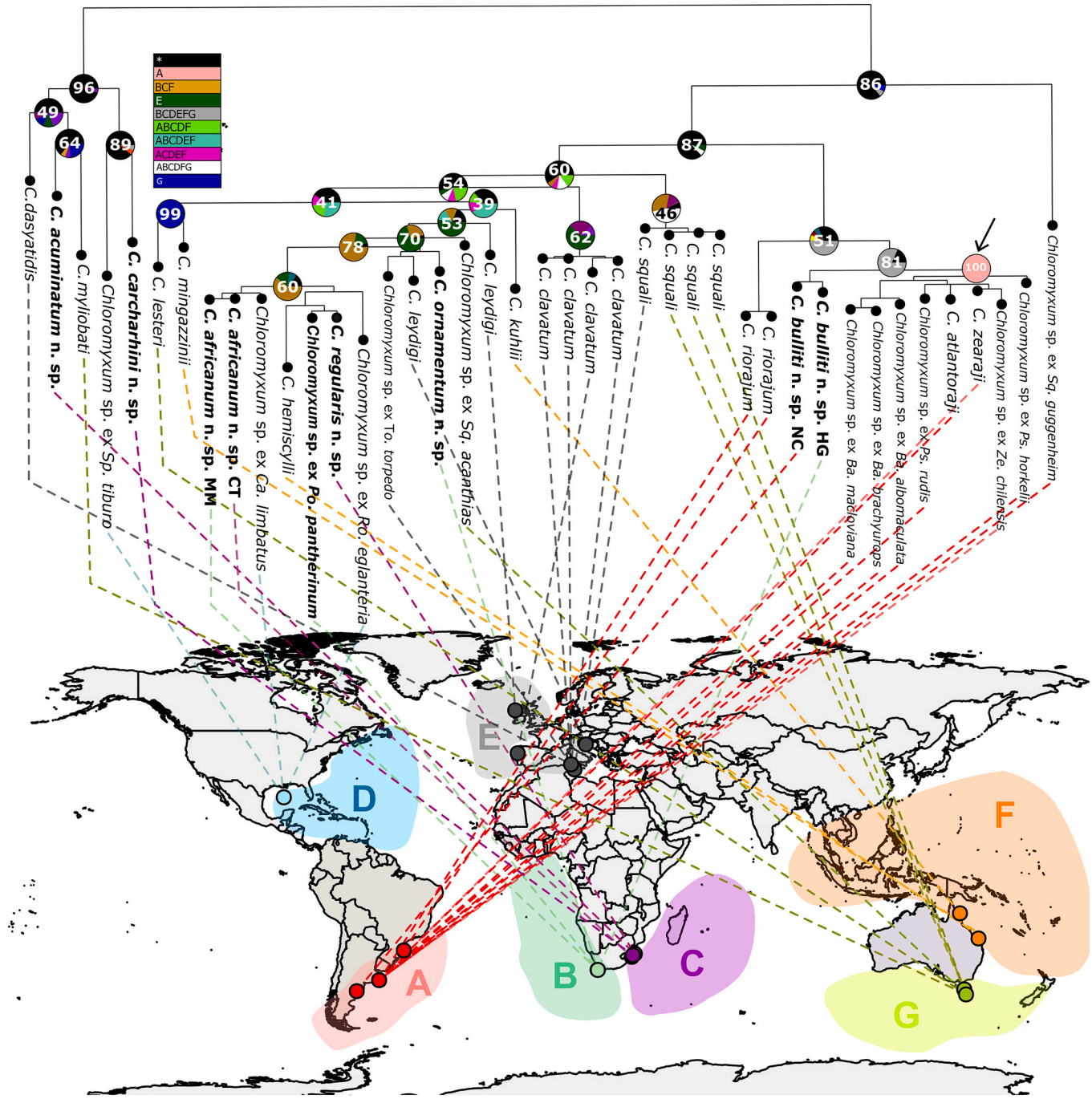


Fig. 5. Ancestral state reconstruction of the geographic distribution of *Chloromyxum sensu stricto* spp. Ancestral areas of each node of the *Chloromyxum* phylogenetic tree were determined by the best-fit model in BioGeoBEARS (BAYAREALIKE + *j*). Geographical areas in the world's coastal zones are designated by letters A–G and by colours which correspond to the colorful lines connecting these areas with associated *Chloromyxum* spp. (new species in bold). The numbers in the circles at each node indicate the highest % support for a parasite origin in a given area; each area is determined in a colorful box legend in the top left corner. Asterisk: No area could have been determined for a given ancestral node. The phylogenetic group with highest probability for the common geographic origin is marked with an arrow.

limited links between geography and phylogenetic relationships in chloromyxids. This raises additional questions about the vertebrate (intermediate) and especially invertebrate (definitive) hosts. Which invertebrates do these chloromyxids use as definitive hosts? Are they specific or do they use a broad range of definitive hosts? Have these hosts a large geographic range or are host migration and reproductive cycles of the parasite synchronized? The geographical sample size is still rather limited and practically nothing is known about the invertebrate host range in this lineage of Myxozoa.

Many elasmobranchs are already listed as threatened (IUCN, 2022) and other factors such as increasing water temperatures, habitat destruction and hunting pressure add further stress to the system which could lead to currently benign parasite infections having greater detrimental health impacts in the future. Parasites play critical roles in ecosystems by contributing to biomass flow, food web connectivity, and population control, and by driving the evolution of other species (Sato et al., 2011; Dunne et al., 2013). Also, parasites of rare and endangered hosts face the highest co-extinction risk and might be the most poorly described and studied fauna, given host rarity. Thus, knowledge of myxozoan biodiversity and their protection remains an important

challenge (Windsor, 1990; Carlson et al., 2020).

5. Conclusion

Chloromyxum sensu stricto is an evolutionary interesting group that appears at the base of the myxozoan oligochaete-infecting lineage and, so far, has only been noticed in ancient fish hosts. Analyses of chloromyxid evolution and radiation is severely hampered by the paucity of records. Increased taxon and geographical sampling, including also Holocephali, are of paramount importance to i) estimate chloromyxid and also myxozoan diversity, ii) gain deeper knowledge about phylogenetic and co-phylogenetic relationships, iii) determine host-range and specificity of *Chloromyxum* spp., and iv) analyse ancestral phylogeographical patterns and their link with migratory vs. local (geographically restricted) host species. These analyses will allow a better assessment of the impact of this group of parasites in an ancient host group, where, most likely, >37% of species are categorized as endangered (IUCN, 2022) and further threatened by climate change, pollution, and other anthropogenic impacts.

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CRediT authorship contribution statement

Martina Lisnerová: Investigation, Data curation, Formal analysis, Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Inga Nicole Martinek:** Investigation, Funding acquisition, Writing – review & editing. **Gema Alama-Bermejo:** Investigation, Resources, Writing – review & editing. **Kateřina Bouberlová:** Investigation. **Bjoern C. Schaeffner:** Resources, Writing – review & editing. **Nomfundo Nkabi:** Resources, Writing – review & editing. **Astrid S. Holzer:** Formal analysis, Funding acquisition, Writing – review & editing. **Pavla Bartošová-Sojková:** Supervision, Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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