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Phylogenetic relationships amongst *Chloromyxum* Mingazzini, 1890 (Myxozoa: Myxosporea), and the description of six novel species from Australian elasmobranchs

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ABSTRACT

Six novel species of *Chloromyxum* Mingazzini, 1890 are described using a whole evidence approach combining morphometric and molecular data, together with features of their biology. Elasmobranchs were collected in Australian waters, from the Great Barrier Reef, Queensland, off Lizard and Heron Islands; from Moreton Bay, southeast Queensland; off Hobart, Tasmania; and from the Tamar River, Launceston, Tasmania. The novel species proposed here are: *Chloromyxum hemiscyllii* n.sp. from *Hemiscyllium ocellatum*; *Chloromyxum kuhlii* n.sp. from *Neotrygon kuhlii*; *Chloromyxum lesteri* n.sp. from *Cephaloscyllium laticeps; Chloromyxum mingazzinii* n.sp. from *Pristiophorus nudipinnis*; *Chloromyxum myliobati* n.sp. from *Myliobatis australis*; and *Chloromyxum squali* n.sp. from *Squalus acanthias*. A seventh species from *Squalus acanthias* is also reported but due to limited material is not formally described. Molecular phylogenetic analyses revealed that the genus *Chloromyxum* is polyphyletic, and species from elasmobranchs form a well-supported sister clade, with the type species *Chloromyxum leydigi*, to all other congeneric species clustering within the freshwater myxosporean clade. Morphological analysis showed that elasmobranch-infecting species are predominantly pyriform shaped, have clearly thickened spore apex and possess caudal filaments, compared to other *Chloromyxum* species which are generally spherical or subspherical, and lack caudal filaments. These morphological and phylogenetic data provide further support for the erection of new genera, but we conservatively consider the species described in this study and other elasmobranch-infecting *Chloromyxum* species as *Chloromyxum* sensu strictu, whilst the freshwater teleost infecting and amphibian infecting species we will assign as *Chloromyxum* sensu lato, until more comprehensive data are available.

1. Introduction

Members of the genus *Chloromyxum* Mingazzini, 1890 (Bivalvulida: Myxosporea: Myxozoa) are coelozoic parasites found commonly in the gall bladder of freshwater and marine fishes, with approximately 120 nominal species described to date [1]. In elasmobranchs, 17 *Chloromyxum* spp., including the type species of the genus *Chloromyxum leydigi* Mingazzini, 1890 have been described (see Table 1). Recent studies on the myxosporean fauna of Australian elasmobranchs [2,3] have shown that myxosporeans are encountered more frequently than previous records would suggest. Nonetheless, only two *Chloromyxum* species have been reported from Australian elasmobranchs: *Chloromyxum noblei* Moser, Kent & Dennis, 1989 from *Hemiscyllium ocellatum* and *Taeniura lymma*, and *Chloromyxum pristiophori* Woolcock, 1936 from *Pristiophorus cirratus*.

Molecular data have advanced our understanding of relationships within the Myxozoa but even after a decade of effort few DNA sequences are currently available for *Chloromyxum* species. Three of the genetic datasets relate to two species of *Chloromyxum* infecting marine elasmobranchs, *Chloromyxum leydigi* (see [4,5]) and *Chloromyxum riorajum* (see [6]). Analysis of the phylogenetic position of these two species places them as a basal clade to the other species of *Chloromyxum* and to a variety of other freshwater myxosporean species [1,4-6]. The potential difference in the phylogenetic position of marine elasmobranch and freshwater *Chloromyxum* species has been highlighted previously by Fiala and Dyková [4] and Azevedo et al. [6]. They have suggested that it may relate to a morphological difference in spores and host preference, with elasmobranch-infecting *Chloromyxum* spores predominantly having posterior caudal filaments present, whereas freshwater *Chloromyxum* do not. Nevertheless, there are some descriptions of elasmobranch-infecting *Chloromyxum* species that do not explicitly define the presence of caudal filaments, but are either obvious in the associated figures e.g. *C. multicostatum* and others (see [7]), *C. dogieli* and others (see [8]); or in fact lack the filaments altogether e.g. *C. levigatum* (see [9]), *C. noblei* (see [10]).

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Table 1
Mean spore dimensions including range in μm, for elasmobranch-infecting *Chloromyxum* species from their respective hosts.

Chloromyxlm species	Host	Location	Sp length	Sp thickness	Sp width	PCL	PCW	Reference
Chloromyxum dogieli	*Raja miraletus*	Africa	10.6–12	8.0–8.3		3.3–3.9	2.6	[8]
Chlorornvxum hemiscvii n.sp.	*Hemiscyllium ocellatum*	Heron Island, Australia	11.8 (11.4–12.2)	9.8 (8.8–10.2)	9.5 (8.8–10.2)	4.1 (3.6–4.7)	3.2 (2.9–3.4)	This Study
Chloromyxum kuhlii n.sp.	*NeoPygon kuhlii*	Lizard Island, Australia	HA (10.9–11.8)	88 (8.2–9.8)	9.1 (8.7–10.0)	4.1 (3.5–4.7)	2.8 (2.5–3)	This Study
Chloromyxum testeri n.sp.	*Cephaloscy llium laticeps*	Tasmania, Australia	10.4 (94–11.2)	8.4 (7.8–9.8)	81 (7.3–8.9)	3.6 (2.9–4.1)	2.7 (2.3–3.3)	This Study
Chloronxum levigarum	*Squarina calfornica*	U.S.A.	11–13	8–10				[9]
Chloromyxum leydtgi	*Centroscymnus coelolepzs & 20 others*	Brazil, U.S.A., Germany, Italy, North Atlantic, Risso	6–16	5–*13.75*		2–4.38	1–3.13	[4–5,24,26–29]
Chloromycum liae	*Prionace glauca*	Argentina	4.4–5.2	3.7		1.48		[7]
Chloromyxum lssosporunz	*Squatina oculata*	Africa	12.0–13.3	6.65–8.0		5.3–5.6	3.5–4.0	[8]
Chloronyxwn ?nagnum	*Acanthias blainvtllei*	Africa	40–48	30–38		12–15		[30]
Chloromyxum mingazjill n.sp.	*Prisriophorus nudpinnis*	Tasmania, Australia	11.1 (10.3–11.6)	8.8 (7.8–9.2)	8.7 (8.4–9.0)	3.8 (3.1–4.9)	2.7 (2.2–3.2)	This Study
Chlorom.xum muiricosratum	*Squarina squatina*	Argentina	5.9–7.4	4.44–*5.2*		2.2		[7]
Chloromixum mvliobati n.sp.	*Miliobarus australis*	Tasmania. Australia	11.8 (10.8–12.9)	10.0 (8.9–11.0)	9.6 (9.1–10.5)	3.9 (3.3–4.7)	3.0 (2.6–3.5)	This Study
Chloronnxum noblei	*Hemiscvllium ocellatwn, Taenzura lymma*	Heron Island, Australia	*8.5* (8.0–10.0)	6.0–7.0	8.5 (8.0–10.0)	3.5 (2.0–4.0)	2.7 (1.0–3.0)	[10]
Chloromvcum ovarum	*Galeorhinus zygopterus & 6 others*	U.S.A., Argentina & Africa	10–13.6	7.3–10.9		4–4.4	2	[7–9,24]
Chlorompumparvicostatum	*Bathyraja brachyurops & B. magellanica*	Argentina	5.2–*5.9*	*4.4*–4.7		2.2–2.3		[7]
Chloromyxumpristiophori	*Pristiophorus cirratus*	Port Philip Bay, Australia	11	8–9		5–6	3	[19]
Chloromvxum riorgium	*Rioraja agassizit*	Brazil	11.41 ± 0.31	5.92 ± 0.54	8.48 ± *0.45*	3.2 0.4	2.4 ± 0.3	[6]
Chloromyxum scylorhinum	*Scyliorhinus torazame*	Korea	10.6	9.2		3	1.9	[31]
chloromyxum shulmani	*Raja streleni*	Africa	9.7–10.6	6.65–8.0		3.2–*4.5*	2.0–2.6	[8]
Chloromyxum sp. ex *S. acanthias*	*Squalus acanthias*	Tasmania, Australia	12.2 (11.1–13.5)	9.2 (8.6–9.9)	9.3 (8.6–10.2)	4.0 (3.1–4.7)	2.6 (2.1–3.1)	This Study
Chloronxwn sphyrn.ae	*.Sphyrna tiburo*	Brazil	15	13		4	4	[32]
Chloromyxum squali n.sp.	*Squatus acanthias*	Tasmania, Australia	11.4 (10.7–12.4)	9.4 (8.5–10.4)	9.3 (8.8–10. 1)	3.9(3.2–4.4)	2.9(2.5–3.3)	This Study
Chloronxum striatellus	*Sciliorhinus canicula*	Africa	10.6–11.2	6.7–10.6		2.7–3.3	2	[8]
Chloromixum transversocostatum	*Squalus acanthias*	Argentina	5.8–5.9	3.7		2.9		[7]

SP, Spore; PCL, Polar Capsule Length; PCW, Polar Capsule Width.

This study characterizes six novel *Chloromyxum* species, and provides small subunit (SSU) ribosomal DNA (rDNA) sequences to evaluate their systematic relationship and phylogenetic position amongst the broader myxosporean group.

2. Materials and methods

2.1. Host and parasite collection

Elasmobranchs were collected by line fishing, seine netting, and spear fishing techniques from: off Lizard Island (14°40′S, 145°27′E) in the northern Great Barrier Reef; off Heron Island (23°27′S, 151°55′E) in the southern Great Barrier Reef; from Moreton Bay (27°50′S, 152°50′E); the Tamar River, Launceston (41°14′S, 146°57′E); and off Hobart (42°58′S, 147°34′E), Tasmania. A total of 56 individuals from five orders, six families and six species of elasmobranchs were collected. The elasmobranchs were euthanized immediately after capture by neural pithing. The bile from the gall-bladders was removed by syringe and placed in a cavity block. A drop of bile was then placed on a microscope slide and examined using a compound light microscope at 400× magnification. Infected samples were preserved in 100% ethanol and frozen in vertebrate physiological saline, for molecular and morphological analysis, respectively.

2.2. Morphological analysis of spores

Frozen or fresh bile was used in morphological characterization of spores with the measurements following the guidelines proposed by Lom and Arthur [11] for species descriptions of Myxosporea. Digital photographs of the spores were taken using a Nikon Digital Sight DS-L1 (Nikon Corporation, Japan) camera/capture image device mounted on an Olympus BH2 compound microscope. Thirty different spores were measured from digital images taken at 1000× magnification. Measurements (calibrated using a micrometre slide) were then used to calculate a mean and standard deviation (SD) for each spore dimension to allow morphological comparison between isolates. All measurements are given in micrometres. Type specimens were deposited in the collections of the Queensland Museum, Brisbane, Australia.

2.3. SSU rDNA extraction, amplification and sequencing

DNA of the bivalvulidans was extracted from 300 μl of infected, ethanol preserved bile. The sample was pelleted at 15,700***g*** for 10 min and the ethanol removed. DNA was then extracted from the pellet using a QIAgen DNeasy® Blood & Tissue Kit (Qiagen Inc., Valencia, California) according to the manufacturer's protocol. A partial section of the SSU rDNA was amplified by PCR using the primers MyxospecF 5-TTC TGC CCT ATC AAC TWG TTG [5] and 18R 5-CTA CGG AAA CCT TGT TAC G [12]. Standard 25 μl PCR reactions were performed using 15.1 μl of autoclaved Millipore water, 2 μl of template DNA (undetermined concentration), 2.5 μl of 10× Hotmaster Taq Buffer (Eppendorf, Hamburg, Germany), 2 μl of 10 mM dNTP's, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 1.25 μl of dimethyl sulfoxide (Sigma, France), and 0.15 μl of 5U Hotmaster Taq (Eppendorf, Hamburg, Germany). PCR amplification was performed in a cp2-01 thermocycler (Corbett Research, Sydney, Australia) using the cycling parameters of an initial denaturation at 94 °C for 21/2 min, followed by 35 cycles of: denaturation at 94 °C for 20 s; annealing at 46 °C for 30 s; extension at 65 °C for 70 s, with a final extension at 65 °C for 10 min.

Amplified PCR products were purified by standard submarine agarose gel electrophoresis using a PerfectPrep Gel Cleanup Kit (Eppendorf, Hamburg, Germany) or QIAquick PCR Purification Kit (Qiagen Inc., Valencia, California). Sequencing reactions were performed in both directions following standard sequencing protocol for ABI Big

Dye® Terminator (Applied Biosystems) as described by Heiniger et al. [13].

2.4. Phylogenetic analysis

Sequences were assembled and aligned using BioEdit version 7.0.5.3 [14], together with all *Chloromyxum* species, and representatives of the freshwater myxosporean clade as defined by Fiala [5], available on GenBank. Representative species of *Myxobolus*, *Henneguya*, *Myxidium*, *Zschokkella*, *Sphaeromyxa*, *Sphaerospora* and *Hoferellus* were used. The alignment also included species that lie outside the freshwater myxosporean clade, *Auerbachia* species and *Coccomyxa* species, and these were used as outgroups in analyses. Sequences were aligned using default parameters of Clustal W [15], and when edited in BioEdit for best fit, a 1296 base alignment was produced. Maximum parsimony analyses were performed using PAUP* 4.0b10 [16] and relationships tested by bootstrapping with 1000 replicates. The optimal evolutionary model of nucleotide substitution using Akaike Information Criterion was determined using Modeltest 3.06 [17], which identified the general time reversible model (GTR + I + Γ), estimated from the data. Bayesian analyses were conducted using MrBayes [18], using the aforementioned evolutionary model, with 2 million generations, trees sampled every 100 generations, with a burn-in of 4000 trees. A second alignment only containing members in the *Chloromyxum* clade was used to produce a distance matrix.

3. Results

3.1. Characterisation of Chloromyxum infections of elasmobranchs

Phylum Myxozoa Grasse, 1970
Class Myxosporea Butschli, 1881
Order Bivalvulida Schulman, 1959
Family Chloromyxidae Thélohan, 1892
Genus *Chloromyxum* Mingazzini, 1890

3.1.1. Chloromyxum hemiscyllii n.sp. (Table 1 and Fig. 1)

Description: Spores typical of the genus *Chloromyxum*. Mature spores are ovoid with a slightly pointed anterior apex, 11.8 ± 0.2 long in lateral view; 9.5 ± 0.4 wide and 9.8 ± 0.5 thick (n = 16) in apical view. Two equal sized valves with no visible surface ridges. Sutural line is narrow but distinct. A bundle of posterior caudal filaments or tails (4.6 ± 0.2 long) originate out of the basal portion of the two valves. Four anteriorly pointed, equal-sized, ellipsoidal to slightly pyriform polar capsules, with a slightly pointed apex, (4.1 ± 0.3 long, 3.2 ± 0.2 wide; n = 16) were located in the anterior section of the spore; each contained 5 (rarely 6) obliquely coiled turns of the polar filament. Sporoplasm irregular in shape and comprises approximately 30% of the spore.

Type material: Syntype G465474 (Air-dried slide stained with Giemsa); Voucher G465475 (bile in absolute ethanol), deposited in Queensland Museum, South Brisbane, Australia.

Type Host: Hemiscyllium ocellatum (Bonnaterre, 1788), Epaulette shark (Elasmobranchii, Orectolobidae) adult.

Prevalence: 1 of 3.

Type Locality: off Heron Island (23°27′S, 151°55′E).

Site of infection: within gall bladder.

Etymology: specific name refers to species of host.

Taxonomic affinities: Chloromyxum hemiscylli n.sp. most closely resembles *Chloromyxum* spp. described from elasmobranch hosts. *Chloromyxum hemiscylli* n.sp. is morphologically similar to *Chloromyxum levigatum* Jameson, 1931. *C. hemiscylli* n.sp. can be distinguished through having ellipsoidal to slightly pyriform polar capsules compared to that of *C. levigatum* which are long and narrow. Furthermore, *C. hemiscylli* n.sp. has the presence of caudal filaments, which are absent in plates for the original description of *C. levigatum*, however this is not explicitly confirmed in the description of this species.

Remarks: a total of 1355 bases of SSU rDNA was generated from *Chloromyxum hemiscylli* n. sp. (GenBank accession no: JN130374). The sequence of *C. hemiscylli* n. sp. differs from the aligned sequences of *Chloromyxum* species at 44–447 of the 1756 nucleotide alignment (similarity matrix) and has maximum genetic similarity of 96.6% with *C.* sp. ex. *Squalus acanthias* (GenBank accession no: JN130384).

3.1.2. Chloromyxum kuhlii n.sp. (Table 1 and Fig. 1)

Description: spores typical of the genus *Chloromyxum*. Mature spores are ellipsoidal to pyriform, 11.4 ± 0.3 long in lateral view; 9.1 ± 0.3 wide and 8.8 ± 0.3 thick (n = 30) in apical view. Two equal sized valves with 4 (rarely 5) surface ridges parallel to the sutural line in each. The ridges become fainter towards the anterior end if the spore. Sutural line is narrow but distinct. A bundle of posterior caudal filaments or tails (4.5 ± 0.5 long) originate out of the basal portion of the two valves. Four anteriorly pointed, equal-sized, ellipsoidal to slightly pyriform polar capsules, with a slightly pointed apex, (4.1 ± 0.3 long, 2.8 ± 0.1 wide; n = 30) were located in the anterior section of the spore; each contained 5 (rarely 6) obliquely coiled turns of the polar filament. Sporoplasm irregular in shape and comprises approximately 40% of the spore.

Type material: syntype G465476-77 (Air-dried slide stained with Giemsa); Voucher G465478 (bile in absolute ethanol), deposited in Queensland Museum, South Brisbane, Australia.

Type Host: Neotrygon kuhlii (Müller & Henle, 1841), Bluespotted stingray (Elasmobranchii, Dasyatidae) adult.

Prevalence: 13 of 35 (5 of 7 from off Lizard Island; 8 of 28 from Moreton Bay).

Type Locality: Off Lizard Island (14°40′S, 145°27′E).

Other Localities: Moreton Bay (27°50′S, 152°50′E).

Site of infection: within gall bladder.

Etymology: specific name refers to species of host.

Taxonomic affinities: Chloromyxum kuhlii n.sp. most closely resembles *Chloromyxum* spp. described from elasmobranch hosts. *Chloromyxum kuhlii* n.sp. is morphologically similar to *Chloromyxum leydigi* Mingazzini, 1890, *Chloromyxum lesteri* n.sp. and *Chloromyxum squali* n.sp.. *C. kuhlii* n.sp. can be distinguished by having larger average spore width, thickness, and length (9.1 × 8.8 × 11.4) compared to that of *C. lesteri* n.sp. (8.2 × 8.4 × 10.4). However, *C. kuhlii* n.sp. cannot be separated through comparison of morphometrics with those of *C. leydigi* or *C. squali* n.sp., but significant variation in SSU rDNA sequences exist between these species.

Remarks: Two identical sequences of 1,386 bases of SSU rDNA were generated for *Chloromyxum kuhlii* n. sp. from two different *Neotrygon kuhlii* hosts (GenBank accession no: JN130375 and JN130376). The sequence of *C. kuhlii* n. sp. differs from the aligned sequences of *Chloromyxum* species at 38–439 of the 1,756 nucleotide alignment (similarity matrix) and has maximum genetic similarity of 97.1% with *C. leydigi* (GenBank accession no: AY604199).

3.1.3. Chloromyxum lesteri n.sp. (Table 1 and Fig. 1)

Description: Spores typical of the genus *Chloromyxum*. Mature spores ovoid, 10.4 ± 0.4 long in lateral view; 8.2 ± 0.3 wide and 8.4 ± 0.3 thick (n = 30) in apical view. Two equal sized valves with 4 to 5 surface ridges running parallel to the sutural line in each. Ridges become diminished towards the anterior end of spore. Sutural line narrow but distinct. A bundle of posterior caudal filaments or tails (3.9 ± 0.2 long) originate from basal portion of the two valves. Four anteriorly pointed, equal-sized, ellipsoidal to slightly pyriform polar capsules (3.6 ± 0.3 long, 2.7 ± 0.2 wide; n = 30) located in the anterior hemisphere of the spore; each contained 5 (rarely 6) obliquely coiled turns of the polar filaments. Sporoplasm irregular in shape comprising approximately 30% of the spore.

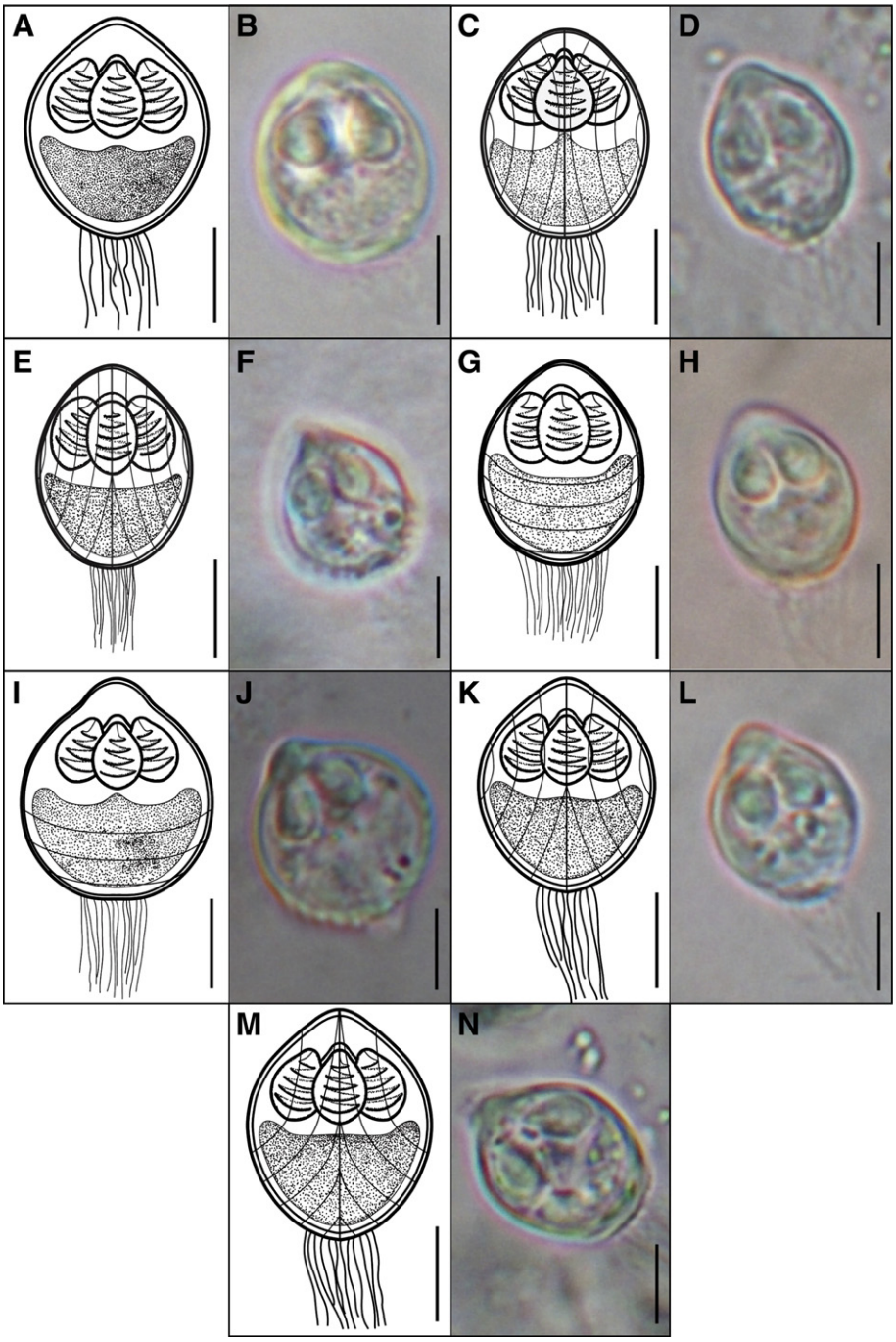

Fig. 1. Phase-contrast micrographs and diagrammatic illustrations of novel *Chloromyxum* spores. (A–B) *Chloromyxum hemiscylli* n.sp. ex. *Hemiscyllium ocellatum*; (C–D) *Chloromyxum kuhlii* n.sp. ex. *Neotrygon kuhlii*; (E–F) *Chloromyxum lesteri* n.sp. ex. *Cephaloscyllium laticeps*; (G–H) *Chloromyxum mingazzinii* n.sp. ex. *Pristiophorus nudipinnis*; (I–J) *Chloromyxum myliobati* n.sp. ex. *Myliobatis australis*; (K–L) *Chloromyxum squali* n.sp. ex. *Squalus acanthias*; (M–N) *Chloromyxum* sp. ex. *Squalus acanthias*. Scale bars: 5 μm.

Type material: Syntypes G465479-80 (Air-dried slide stained with Giemsa); Voucher G465481 (bile in absolute ethanol), deposited in Queensland Museum, Australia.

Type host: Cephaloscyllium laticeps (Duméril, 1853), Australian swellshark (Elasmobranchii, Scyliorhinidae) adult.

Prevalence: 3 of 3 (2 of 2 from off Hobart; 1 of 1 from off Launceston).

Type Locality: off Hobart, Tasmania (42°58′S, 147°34′E).

Other Localities: Tamar River, Launceston, Tasmania (41°14′S, 146°57′E).

Site of infection: within gall bladder.

Etymology: specific name in honour of Emeritus Prof R.J.G. Lester in recognition of his contributions to marine parasitology.

Taxonomic affinities: Chloromyxum lesteri n.sp. most closely resembles *Chloromyxum* spp. described from elasmobranch hosts. *Chloromyxum lesteri* n.sp. is morphologically similar to *C. leydigi* Mingazzini, 1890, *C. kuhlii* n.sp. and *C. squali* n.sp.. *C. lesteri* n.sp. can be distinguished by having smaller average spore width, thickness, and length ($8.2\times8.4\times10.4$), compared to that of *C. kuhlii* n.sp. ($9.1\times8.8\times11.4$) and that of *C. squali* n.sp. ($9.3\times9.4\times11.4$). However, *C. lesteri* n.sp. cannot be separated through comparison of morphometrics with those of *C. leydigi*, but significant variation in SSU rDNA sequences exist between these species.

Remarks: Two identical sequences of 1,379 bases of SSU rDNA were generated for *Chloromyxum lesteri* n. sp. from two different *Cephaloscyllium laticeps* hosts (GenBank accession no: JN130377 and

JN130378). The sequence of *C. lesteri* n. sp. differs from the aligned sequences of *Chloromyxum* species at 22–437 of the 1,756 nucleotide alignment (similarity matrix) and has maximum genetic similarity of 98.3% with *C. mingazzinii* n.sp. (GenBank accession no: JN130379).

3.1.4. *Chloromyxum mingazzinii* n.sp. (Table 1 and Fig. 1)

Description: spores typical of the genus *Chloromyxum*. Mature spores are ellipsoidal to pyriform, 11.1 ± 0.3 long in lateral view; 8.7 ± 0.2 wide and 8.8 ± 0.3 thick (n = 19) in apical view. Two equal sized valves with 3 to 4 surface ridges in the posterior half of the spore. Ridges are located on the last half of the spore and run roughly parallel to the basal portion of the sutural ridge. Sutural line is narrow but distinct. A bundle of posterior caudal filaments or tails (4.1 ± 0.7 long) originate out of the basal portion of the two valves. Four anteriorly pointed, equal-sized, ellipsoidal to slightly pyriform polar capsules (3.8 ± 0.5 long, 2.7 ± 0.2 wide; n = 19) located in the anterior hemisphere of the spore; each contain 5 (rarely 6) obliquely coiled turns of the polar filament. Sporoplasm irregular in shape comprising approximately 40% of the spore.

Type material: Syntypes G465482 (air-dried slide stained with Giemsa); Voucher G465483 (bile in absolute ethanol), deposited in Queensland Museum, South Brisbane, Australia.

Type Host: Pristiophorus nudipinnis Günther, 1870, Shortnose sawshark (Elasmobranchii, Pristiophoridae) adult.

Prevalence: 1 of 3. (1 of 2 from off Hobart; 0 of 1 from Tamar River, Launceston).

Type Locality: off Hobart, Tasmania (42°58′S, 147°34′E).

Site of infection: within gall bladder.

Etymology: specific name in honour of P. Mingazzini who erected the genus *Chloromyxum*, and for contributions to marine parasitology.

Taxonomic affinities: Chloromyxum mingazzinii n.sp. most closely resembles *Chloromyxum* spp. described from elasmobranch hosts. *Chloromyxum mingazzinii* n.sp. is morpholgically similar to *C. pristiophori* Woolcock, 1936 and *C. myliobati* n.sp.. *C. mingazzinii* n.sp. can be distinguished from *C. pristiophori*, a species described from *Pristiophorus cirratus* (see [19]), in having a smaller polar capsule length and width (3.8 × 2.7) than that of *C. pristiophori* (5–6 × 3), and in having 3 (sometimes 4) ridges, compared to 5 ridges on each spore valve in *C. pristiophori*. Furthermore, *C. mingazzinii* n.sp. can be distinguished from *C. myliobati* n.sp. in having a smaller spore width, thickness and length (8.7 × 8.8 × 11.1) compared to that of *C. myliobati* n.sp. (9.6 × 10.0 × 11.9).

Remarks: a total of 1366 bases of SSU rDNA was generated from *Chloromyxum mingazzinii* n. sp. (GenBank accession no: JN130379). The sequence of *C. mingazzinii* n. sp. differs from the aligned sequences of *Chloromyxum* species at 22–438 of the 1756 nucleotide alignment (similarity matrix) and has maximum genetic similarity of 98.3% with *C. lesteri* n.sp. (GenBank accession no: JN130377 and JN130378).

3.1.5. *Chloromyxum myliobati* n.sp. (Table 1 and Fig. 1)

Description: Spores typical of the genus *Chloromyxum*. Mature spores are ovoid with a slightly pointed anterior apex, 11.9 ± 0.6 long in lateral view; 9.6 ± 0.4 wide and 10.0 ± 0.6 thick (n = 26) in apical view. Two equal sized valves with 3 to 4 surface ridges in the posterior half of the spore. Ridges are located on the last half of the spore and run roughly parallel to the basal portion of the sutural ridge. A bundle of posterior caudal filaments or tails (3.0 ± 0.4 long) originate out of the basal portion of the two valves. Four anteriorly pointed, equal-sized, ellipsoidal to slightly pyriform polar capsules (3.9 ± 0.4 long, 3.0 ± 0.2 wide; n = 26) were located in the anterior section of the spore; each contained 4 (rarely 5) obliquely coiled turns of the polar filament. Sporoplasm irregular in shape and comprises approximately 40% of the spore.

Type material: Syntype G465484 (Air-dried slide stained with Giemsa); Voucher G465485 (bile in absolute ethanol), deposited in Queensland Museum, South Brisbane, Australia.

Type Host: Myliobatus australis Macleay, 1881, Australian bull ray (Elasmobranchii, Myliobatidae) adult.

Prevalence: 1 of 1.

Type Locality: Off Hobart, Tasmania (42°58′S, 147°34′E).

Site of infection: within gall bladder.

Etymology: specific name refers to the genus of host.

Taxonomic affinities: Chloromyxum myliobati n.sp. most closely resembles *Chloromyxum* spp. described from elasmobranch hosts. *Chloromyxum myliobati* n.sp. is superficially similar to *C. pristiophori* Woolcock, 1936 and *C. mingazzinii* n.sp.. *C. myliobati* n.sp. can be distinguished by having larger average spore thickness and length (10.0 × 11.9) compared to that of *C. pristiophori* (8–9 × 11) and that of *C. mingazzinii* n.sp. (8.8 × 11.1).

Remarks: a total of 1400 bases of SSU rDNA was generated from *Chloromyxum myliobati* n. sp. (GenBank accession no: JN130380). The sequence of *C. myliobati* n. sp. differs from the aligned sequences of *Chloromyxum* species at 22–452 of the 1,756 nucleotide alignment (similarity matrix) and has maximum genetic similarity of 91.6% with *C. lesteri* n.sp. (GenBank accession no: JN130377 & JN130378).

3.1.6. *Chloromyxum squali* n.sp. (Table 1 and Fig. 1)

Description: Spores typical of the genus *Chloromyxum*. Mature spores are ellipsoidal to pyriform with a slightly pointed anterior apex, 11.4 ± 0.5 long in lateral view; 9.3 ± 0.3 wide and 9.4 ± 0.5 thick (n = 30) in apical view. Two equal sized valves with 4 to 5 surface ridges run parallel to the sutural line in each. The ridges become fainter towards the anterior end if the spore. Sutural line is narrow but distinct. A bundle of posterior caudal filaments or tails (5.1 ± 0.6 long) originate out of the basal portion of the two valves. Four anteriorly pointed, equal-sized, ellipsoidal to slightly pyriform polar capsules, with a slightly pointed apex, (3.9 ± 0.3 long, 2.9 ± 0.2 wide; n = 30) were located in the anterior section of the spore; each contained 5 (rarely 6) obliquely coiled turns of the polar filament. Sporoplasm irregular in shape and comprises approximately 35% of the spore.

Type material: Syntype G465486-87 (Air-dried slide stained with Giemsa); Voucher G465488 (bile in absolute ethanol), deposited in Queensland Museum, South Brisbane, Australia.

Type Host: Squalus acanthias Linnaeus, 1758, Picked dogfish (Elasmobranchii, Squalidae) adult.

Prevalence: 5 of 11 (4 of 7 from off Launceston; 0 of 4 from off Hobart).

Type Locality: off Launceston, Tasmania (41°14′S, 146°57′E).

Site of infection: within gall bladder.

Etymology: specific name refers to genus of host.

Taxonomic affinities: Chloromyxum squali n.sp. most closely resembles *Chloromyxum* spp. described from elasmobranch hosts. *Chloromyxum squali* n.sp. is morphologically similar to *C. leydigi* Mingazzini, 1890, *C. kuhlii* n.sp. and *C. lesteri* n.sp.. *C. squali* n.sp. can be distinguished in having larger average spore width, thickness and length (9.3 × 9.4 × 11.4) compared to that of *C. lesteri* n.sp. (8.2 × 8.4 × 10.4). *C. squali* n.sp. can also be distinguished from *C. transversocostatum* Kuznetsova, 1977 described from *Squalus acanthias*, in having a larger spore thickness and length (9.4 × 11.4) than that of *C. transversocostatum* (3.7 × 5.8–5.9). However, *C. squali* n.sp. cannot be separated through comparison of morphometrics with those of *C. leydigi* or *C. kuhlii* n.sp., but significant variation in SSU rDNA sequences exist between these species.

Remarks: Three sequences of 1381 bases of SSU rDNA were generated for *Chloromyxum squali* n. sp. from three different *Squalus acanthias* hosts (GenBank accession no: JN130381, JN130382 & JN130383). The sequence of *C. squali* n. sp. differs from the aligned sequences of *Chloromyxum* species at 22–444 of the 1756 nucleotide

alignment (similarity matrix) and has maximum genetic similarity of 97.3% with *C.* sp. ex. *Squalus acanthias* and *C. leydigi* (GenBank accession no: JN130384 and DQ377710, respectively). Furthermore, intraspecific variation was noted between *C. squali* n.sp. isolates but at only a 1–2 nucleotide (0.1%) difference (see Table 2).

3.1.7. Chloromyxum sp. ex. Squalus acanthias (Table 1 and Fig. 1)

Description: spores typical of the genus *Chloromyxum*. Mature spores are ovoid, 12.2 ± 0.6 long in lateral view; 9.3 ± 0.3 wide and 9.2 ± 0.4 thick (n = 30) in apical view. Two equal sized valves with 5 (sometimes 6) ridges parallel to the sutural line in each. The ridges become fainter towards the anterior end if the spore. Sutural line is narrow but distinct. A bundle of posterior caudal filaments or tails (5.1 ± 0.5 long) originate out of the basal portion of the two valves. Four anteriorly pointed, equal-sized, ellipsoidal to slightly pyriform polar capsules, with a slightly pointed apex, (4.0 ± 0.4 long, 2.6 ± 0.3 wide; n = 30) were located in the anterior section of the spore; each contained 5 (rarely 6) obliquely coiled turns of the polar filament. Sporoplasm irregular in shape and comprises approximately 50% of the spore.

Material: Voucher G465489 (Air-dried slide stained with Giemsa); Voucher G465490 (bile in absolute ethanol), deposited in Queensland Museum, South Brisbane, Australia.

Host: Squalus acanthias Linnaeus, 1758, Picked dogfish (Elasmobranchii, Squalidae) adult.

Prevalence: 1 of 11.

Type Locality: Off Hobart, Tasmania (42°58′S., 147°34′E.).

Site of infection: within gall bladder.

Taxonomic affinities: Chloromyxum sp. ex. *Squalus acanthias* most closely resembles *Chloromyxum* spp. described from elasmobranch hosts. *Chloromyxum* sp. ex. *Squalus acanthias* is morphologically similar to *C. leydigi* Mingazzini, 1890, *C. squali* n.sp., *C. kuhlii* n.sp. and *C. lesteri* n.sp.. *C.* sp. ex. *S. acanthias* can be distinguished in having larger average spore width, thickness and length (9.3 × 9.2 × 12.2) compared to that of *C. lesteri* n.sp. (8.2 × 8.4 × 10.4). *C.* sp. ex. *S. acanthias* can also be distinguished from *C. transversocostatum* Kuznetsova, 1977 described from *Squalus acanthias*, in having a larger spore thickness and length (9.2 × 12.2) than that of *C. transversocostatum* (3.7 × 5.8–5.9). However, *C.* sp. ex. *S. acanthias* cannot be separated through comparison of morphometrics with those of *C. leydigi*, *C. squali* n.sp. or *C. kuhlii* n.sp.. Significant variation in SSU rDNA sequences exist between *C.* sp. ex. *S. acanthias* and that of *C. squali* n.sp. and that of *C. kuhlii* n.sp., but only slight variation (18–20 base pairs) exist between *C.* sp. ex. *S. acanthias* and *C. leydigi* (see Table 2). On the basis of morphometric and SSU rDNA differences we believe the species from *Squalus acanthias* is not *C. kuhlii* n.sp., *C. lesteri* n.sp., *C. squali* n.sp., or *C. transversocostatum*, however, due to a limited sample of only one isolate we take a conservative approach to not assign the species to either *C. leydigi*, nor to propose the erection of a new species.

Remarks: A total of 1399 bases of SSU rDNA was generated from *Chloromyxum* sp. ex. *S. acanthias* (GenBank accession no: JN130384). The sequence of *C.* sp. ex. *S. acanthias* differs from the aligned sequences of *Chloromyxum* species at 16–443 of the 1,756 nucleotide alignment (similarity matrix) and has maximum genetic similarity of 98.8% with *C. leydigi* n.sp. (GenBank accession no: AY604199).

3.2. Phylogenetic analyses

An initial BLAST search conducted through GenBank demonstrated that sequences with closest homology all belonged to *Chloromymum* spp. Molecular analyses included 61 myxozoan sequences, of which 22 were *Chloromyxum* sequences, from 16 species. Maximum parsimony and Bayesian analyses produced trees with similar tree topologies and consistent with those produced in the phylogeny of Jirků et al. [1], Fiala [5], and that of Azevedo et al. [6] (see Fig. 2). Some variation of clade topologies occurred, but the overall positions in each analysis showed that the genus *Chloromyxum* was polyphyletic, with strong support for an elasmobranch-infecting *Chloromyxum* clade being sister to all other congeneric species clustering within the freshwater myxosporean clade. Two well-supported node-based *Chloromyxum* clades were evident: a clade containing *Chloromyxum* species from elasmobranch hosts including *Chloromyxum hemiscylli* n.sp., *Chloromyxum kuhlii* n.sp., *Chloromyxum lesteri* n.sp., *Chloromyxum leydigi* (see [4,5]), *Chloromyxum mingazzini* n.sp., *Chloromyxum myliobati* n.sp., *Chloromyxum riorajum* (see [6]), *Chloromyxum squali* n.sp., and *Chloromyxum* sp. ex. *S. acanthias*; and a second clade containing *Chloromyxum* species from freshwater teleosts including *Chloromyxum cyprini* (see [4]), *Chloromyxum auratum* (see [20]), *Chloromyxum fluviatile* (see [21]), and *Chloromyxum truttae* (see [22]). Furthermore, phylogenetic analysis showed that *Chloromyxum trijugum*, *C. careni*, *C. legeri* and *C. schurovi* did not cluster in these clades or with one another.

Table 2
Similarity in SSU rDNA sequences of all *Chloromyxum* species. (Column 2 identifies host isolate. Lower triangle shows actual nucleotide difference, while the upper triangle shows the percentage of nucleotide difference).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	*C hemiscylli* n.sp.		5.6	5.6	4.7	4.7	4.6	9.3	4.9	4.9	4.9	3.4	3.7	3.5	7.0	21.0	20.9	21.1	21.3	26.6	26.9	44.6	36.8
2	*C. kuhlii* n.sp. 1	72		0	3.1	3.1	3.3	9.1	4.1	4.1	4.1	3.0	3.3	2.9	5.6	21.5	20.1	20.1	21.2	26.2	27.2	43.7	35.0
3	*C. kuhlii* n.sp. 2	72	0		3.1	3.1	3.3	9.1	4.1	4.1	4.1	3.0	3.3	2.9	5.6	21.5	20.1	20.1	21.2	26.2	27.2	43.7	35.0
4	*C. lesteri*n.sp. 1	61	41	41		0	1.7	8.4	3.5	3.4	3.5	2.5	2.8	2.9	5.5	21.5	19.9	19.9	20.9	25.3	26.4	43.5	34.5
5	*C. lesteri*n.sp. 2	61	41	41	0		1.7	8.4	3.5	3.4	3.5	2.5	2.8	2.9	5.5	21.5	19.9	19.9	20.9	25.3	26.4	43.5	34.5
6	*C. mingazzinii* n.sp.	59	43	43	22	22		8.8	3.0	2.9	3.0	1.7	2.4	2.3	5.3	21.2	19.8	19.6	20.5	24.6	26.4	43.6	35.7
7	*C. myliobati* n.sp.	117	115	115	106	106	111		9.0	8.9	9.0	8.7	9.1	8.8	9.7	22.4	21.9	22.1	23.3	27.7	29.3	44.6	35.9
8	*C. squali* n.sp. 1	64	54	54	46	46	39	114		0.1	0.1	2.8	2.8	2.9	4.9	20.9	19.3	19.5	20.7	25.4	26.3	43.8	37.2
9	*C. squali* n.sp. 2	63	53	53	45	45	38	113	1		0.1	2.7	2.7	2.8	4.8	20.8	19.2	19.4	20.6	25.3	26.2	44.0	36.9
10	*C. squali* n.sp. 3	64	54	54	46	46	39	114	2	1		2.8	2.8	2.9	4.9	20.9	19.3	19.5	20.7	25.4	26.3	44.1	36.9
11	*C.* sp. ex *S. acanthias*	44	40	40	33	33	23	110	37	36	37		1.4	1.2	4.3	21.2	20.1	19.9	20.6	26.2	26.0	44.1	37.3
12	*C. leydigi* 1	48	43	43	37	37	31	115	37	36	37	18		1.8	5.1	21.6	20.1	20.0	20.8	25.9	26.6	43.6	36.5
13	*C. leydigi* 2	46	38	38	38	38	31	111	38	37	38	16	24		4.7	21.1	19.7	19.8	20.7	26.0	26.5	43.7	35.5
14	*C. riorajum*	89	72	72	71	71	68	122	63	62	63	56	66	61		21.4	20.4	20.4	21.2	26.2	26.0	43.8	36.8
15	*C. trijugum*	241	245	245	245	245	242	255	240	239	240	243	246	242	244		23.7	24.3	22.9	30.4	34.4	47.4	38.1
16	*C. cyprini*	242	234	234	232	232	231	255	227	226	227	235	234	230	237	302		3.4	10.1	27.7	31.3	45.4	25.7
17	*C. auratum*	244	234	234	231	231	228	256	229	228	229	232	233	231	237	308	53		9.7	28.1	31.3	45.2	26.3
18	*C. fluviatile*	247	245	245	242	242	238	268	241	240	241	240	242	241	246	290	148	143		26.1	31.9	46.0	27.5
19	*C. truttae*	238	234	234	227	227	222	250	230	229	230	235	232	233	235	304	298	302	283		37.4	34.4	29.8
20	*C. legeri*	299	301	301	293	293	293	323	293	292	293	291	295	295	289	408	371	372	377	356		51.6	38.0
21	*C. careni*	447	439	439	437	437	438	452	442	443	444	443	439	440	439	535	538	538	539	369	563		22.6
22	*C. schurovi*	165	158	158	155	155	159	165	166	165	165	166	163	159	163	188	132	134	137	158	186	130	

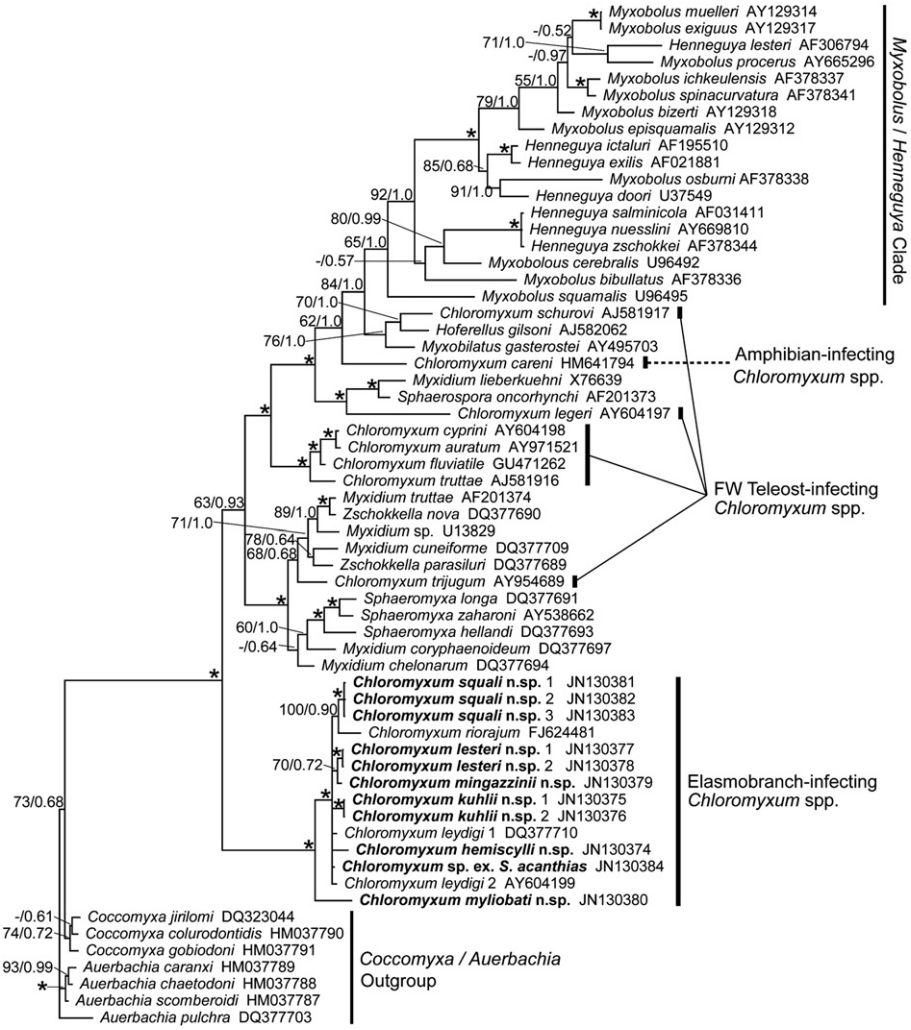

Fig. 2. Phylogenetic tree resulting from Bayesian inference analysis inferred from the SSU rDNA dataset. Support values at branching points are listed as: bootstrap values from maximum parsimony analyses/clade credibility values (CCV) from Bayesian analysis. Where support values exceeded 95% in each analysis, a star is shown. Any values below 50% are indicated by a dash. GenBank Accession numbers follow the species name. (Note: FW = Freshwater).

4. Discussion

In this study bivalvulidan species from the genus *Chloromyxum* were identified from five orders, six families and six species of elasmobranchs, and from all regions covering tropical (Lizard Island, 14°40′S) to temperate (Hobart, 42°58′S) localities in Australia. Six novel species are proposed, as well as a record for a seventh species that is not assigned at this time.

The taxonomy of myxosporeans was traditionally based on spore morphology, while now, a combination of genetic, morphological and biological correlates (e.g. geographic locality, host species, tissue type) have enabled more informed taxonomic discrimination at the species level. Some myxosporean species are indistinguishable through morphometrics alone, but using a combined approach novel species have been identified and cryptic species discovered (see examples in [3,21,23]). In the genus *Chloromyxum*, two species have been described from a number of elasmobranch hosts based on morphological characters, *Chloromyxum leydigi* Mingazzini, 1890, has been described from 21 species of sharks and rays, whilst *Chloromyxum ovatum* Jameson, 1929 has been described from 7 species of sharks and rays. Having such a broad specificity is rather unusual amongst myxosporeans, since many have high host specificity (i.e. some even to a single host species), hence the suggestions that these *Chloromyxum* species are in fact an assemblage of several species [7,24,25].

In this study we collected four species which are not distinguishable, using morphological features, from *C. leydigi*. Three of these species are considered novel, *C. kuhlii* n.sp., *C. lesteri* n.sp. and *C. squali* n.sp., and the other an undescribed *Chloromyxum* sp. ex *Squalus acanthias*. Their novelty is based, in descending order of significance, on genotypic differences, subtle variations in morphometric characters, the identity of the host and the geographic locality in which infected hosts were found. To strengthen support for the proposed novelty of species, replicates were examined to compare intra- and inter-specific variation. Intra-specific variation has been observed for *Chloromyxum leydigi* from its two sequenced isolates (i.e. a 24 nucleotide, 1.8% difference), whilst variation is also observed in sequences of a freshwater *Chloromyxum* species, with up to 10 nucleotides (0.45%) variation in isolates sequenced from *C. fluviatile* (see [21]). In this study the sequences of *C. kuhlii* n.sp. and *C. lesteri* n.sp. showed no intra-specific variation, while only a 1–2 nucleotide (0.1%) difference was observed between isolates of *C. squali* n.sp.. In terms of inter-specific variation, *C. cristatum* and *C. cyprini* have a 0.56% difference in SSU rDNA, however recently this was thought to be intra-specific variation and a suggestion to synonymise these species has been proposed [21]. Therefore, the smallest difference in SSU rDNA sequence exist between the two similar morphological, and previously sequenced elasmobranch *Chloromyxum* species, *C. riorajum* and *C. leydigi* which had a 2.1% difference (see [6]). Based on the knowledge of both intra- and inter-specific relationships amongst *Chloromyxum* species, *C. kuhlii* n.sp., *C. lesteri* n.sp. and *C. squali*

n.sp. have significant variation (>2.7%) in SSU rDNA sequence between each species, and when compared with that of *C. leydigi* (see Table 2). Additionally, slight morphological variation and presence in different host species, provides confidence to propose these species as novel. Meanwhile, only a single sequence was isolated for *C.* sp. ex. *S. acanthias* and it differed at 16–18 nucleotides (1.2-1.4%) with that of *C. leydigi*, which by previous species accounts is within the range of intraspecific variation. Nevertheless, we took the conservative approach not to assign *C.* sp. ex. *S. acanthias* to *C. leydigi* until further molecular data are generated from hosts of *C. leydigi*, as we believe like previous studies (e.g. [24] etc), that *C. leydigi* may represent a species complex.

Phylogenetic relationships observed in this study (see Fig. 2) are in congruence with other studies [1,5,6]. *Chloromyxum* is polyphyletic, and our analyses show strong support for an elasmobranch-infecting *Chloromyxum* clade, despite them having different geographic origins (i.e. from Australia, North Atlantic, Southern Brazil, and Mediterranean), being sister to all other congeneric species clustering within the freshwater myxosporean clade. In addition, the *Chloromyxum* characterised in this study were found to clade with the type species, *Chloromyxum leydigi*. Morphological analysis showed that elasmobranch-infecting species are predominantly pyriformed shaped, have clearly thickened spore apex and possess caudal filaments, compared to other *Chloromyxum* species (i.e. from freshwater teleosts and amphibians) which are generally spherical or subspherical, and lack caudal filaments. These morphological and phylogenetic data provide further support for the elasmobranch-infecting *Chloromyxum* species to be assigned as *Chloromyxum* sensu strictu.

Concerning the other *Chloromyxum* species, and similar to other studies (see [1,5,6]), a few individual lineages are evident from phylogenetic analyses. Analyses show that a well-supported node-based *Chloromyxum* clade exists and includes the species *C. cyprini*, *C. auratum*, *C. fluviatile* and *C. truttae*, which are known to infect the gall bladder of their respective freshwater teleost hosts (see [5]). Meanwhile, the other two freshwater teleost-infecting gall bladder species, *C. trijugum* and *C. legeri*, did not cluster with these species or one another. Interestingly, *Chloromyxum schurovi*, which inhabits the kidney tubules of salmonids, was observed clustering with other myxosporean species infecting the kidney tubules and urinary bladder. In addition, *C. careni*, described from an amphibian host, was a sole representative of an independent lineage sister to a predominantly *Myxobolus*/*Henneguya* clade. The combination of morphological, biological and phylogenetic data provides further evidence for the erection of new genera to house *Chloromyxum* species infecting freshwater teleosts and amphibians, however we conservatively assigned these species as *Chloromyxum* sensu lato, until further phylogenetic and morphological analysis of chloromyxids from a broad range of hosts is investigated.

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