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Morphology and molecular taxonomy of *Gyrodactylus jennyae* n. sp. (Monogenea) from tadpoles of captive *Rana catesbeiana* Shaw (Anura), with a review of the species of *Gyrodactylus* Nordmann, 1832 parasitising amphibians

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Abstract *Gyrodactylus jennyae* n. sp. is described from the body surface and mouthparts of tadpoles of the bullfrog *Rana catesbeiana* Shaw imported presumably from Missouri, USA, into a federal government facility in Moncton, New Brunswick, Canada. Its morphology resembles most closely that of *G. chologastris* Mizelle, Whittaker & McDougal, 1969 described from two amblyopsids (blind cave fishes) in Kentucky and North Carolina. Both species have long slender hamuli, a ventral bar with a relatively long membrane and small anterolateral processes, a cirrus with two rows of small spines and marginal hooks with a well-developed sickle heel and short handle. The two species differ morphologically; *G. jennyae* has a marginal hook sickle with a more pronounced heel than that found in *G. chologastris*. A BLAST search using a 945 base pair sequence that included the nuclear ribosomal DNA internal transcribed spacers 1 and 2 and the 5.8S rRNA gene from *G. jennyae* n. sp. showed that the overall similarity with other *Gyrodactylus* sequences on GenBank was relatively low. The ITS1 region was similar to that of *G. misgurni* Ling, 1962; however, no ITS2 and 5.8S rRNA sequences are available for that species. A separate search using 5.8S sequences revealed that *G. markakulensis* Gvosdev, 1950 and *G. laevis* Malmberg, 1957 were the closest to *G. jennyae* (1 and 2 bp differences, respectively). These species are parasites of cyprinids (or their predators) and are similar to *G. jennyae* and *G. chologastris* in having a double row of small hooks on the cirrus and overall similar morphologies of the haptoral hard parts. There are now five species of *Gyrodactylus* described exclusively from amphibians and this appears to have involved at least three separate host-switches from fishes.

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Introduction

Species of *Gyrodactylus* Nordmann, 1832 primarily parasitise fishes, with 450 nominal species being reported from hosts in freshwater and marine habitats worldwide (Bakke et al., 2007). Four species,

G. ambystomae Mizelle, Kritsky & McDonald, 1969, *G. aurorae* Mizelle, Kritsky & McDonald, 1969, *G. ensatus* Mizelle, Kritsky & Bury, 1968 and *G. catesbeiana* Wootton, Ryan, Demaree & Critchfield, 1993 have been described from frogs and salamanders in North America (Tables 1, 2). Several more recent accounts of gyrodactylids found on larval amphibians surveyed in Canada and the USA could reveal additional species upon their identification beyond the level of the subgenus *Gyrodactylus* (Table 1) (Crawshaw, 1997; Dodd et al., 2004; Gunzburger et al., 2005; Green & Dodd, 2007). One species, *G. arcuatus* Bychowsky, 1933, was reported on *Hyla arboria* (L.) tadpoles from the Danube delta, Romania (Tables 1, 2) (Volgar-Pastukhova, 1959; Vojtkova, 1989; R. Bray, personal communication), but its type host is the three-spined stickleback (*Gasterosteus aculeatus* L.) (Geets et al., 1999), which suggests that the infected tadpoles were acting as transient hosts (Prudhoe & Bray, 1982). The present study describes a new species of *Gyrodactylus* from the body surface of captive bullfrogs *Rana catesbeiana* Shaw imported into Canada from a commercial frog farm believed to be located in Missouri, USA.

Materials and methods

The new species was first found on tadpoles in April, 2007 at a federal government research facility in

Table 1 Reports of *Gyrodactylus* spp. infecting amphibians[a]

Gyrodactylus species	Host species	Place of host collection (Reference/Source)
Gyrodactylus sp.	*Rana catesbeiana* (Bullfrog)	Southern New Jersey, USA (Stunkard & Dunihue, 1933; Andrews et al., 1992)
Gyrodactylus arcuatus	*Hyla arborea*[b] (European tree frog)	Danube Delta, Romania (Volgar-Pastukhova, 1959; Vojtkova, 1989)
Gyrodactylus ensatus	*Dicamptodon ensatus* (Pacific coast giant salamander)	Humboldt County, California, USA (stream) (Mizelle et al., 1968)
Gyrodactylus aurorae	*Rana aurora aurora* (Northern red-legged frog)	Del Norte County, California, USA (pond) (Mizelle et al., 1969)
Gyrodactylus ambystomae	*Ambystoma macrodactylum* (Long-toed salamander)	Lassen County, California, USA (pond) (Mizelle et al., 1969)
Gyrodactylus catesbeiana	*Rana catesbeiana* (Bullfrog)	Glenn County, California, USA (creek) (Wootton et al., 1993)
Gyrodactylus jennyae n. sp.	*Rana catesbeiana* (Bullfrog)	Frog farm, Missouri, USA (This study)
Gyrodactylus sp.	*Rana catesbeiana* (Bullfrog)	Algonquin Park, ON, Canada (Crawshaw, 1997)
Gyrodactylus sp.	*Rana sphenocephala* (Southern leopard frog)	St-Mark's National Wildlife Refuge, FL, USA (Dodd et al., 2004; D.E. Green, personal communication)
Gyrodactylus sp.	*Rana grylio* (Pig frog)	St-Mark's National Wildlife Refuge, FL, USA (Dodd et al., 2004; D.E. Green, personal communication)
Gyrodactylus sp.	*Hyla gratiosa* (Barking treefrog)	Welaka National Fish Hatchery, FL, USA (D.E. Green, personal communication)
Gyrodactylus sp.	*Rana sphenocephala* (Southern leopard frog)	Welaka National Fish Hatchery, FL, USA (Gunzburger et al., 2005; Green & Dodd, 2007; D.E. Green, personal communication)
Gyrodactylus sp.	*Rana clamitans* (Green frog)	Warm Springs National Fish Hatchery, GA, USA (Green & Dodd, 2007; D.E. Green, personal communication)
Gyrodactylus sp.	*Hyla cinerea* (Green treefrog)	Warm Springs National Fish Hatchery, GA, USA (D.E. Green, personal communication)
Gyrodactylus sp.	*Rana sphenocephala* (Southern leopard frog)	Upper Ouachita National Fish Hatchery, LA, USA (D.E. Green, personal communication)
Gyrodactylus sp.	*Rana aurora aurora* (Northern red-legged frog)	Near Redwood National Park, CA, USA (Nieto et al., 2007)

[a] All host records involve only the larval stages of the amphibian

[b] The tadpoles likely were accidental hosts of *G. arcuatus* (see Prudhoe & Bray, 1982)

Table 2 Species-group and host family of the *Gyrodactylus* spp. described from amphibians (adapted from Malmberg, 1970)

Gyrodactylus species	Host family	Species-group
Gyrodactylus arcuatus[a,b]	Gasterosteidae[a]	*G. arcuatus*
Gyrodactylus ensatus[a]	Urodela, Ambystomatidae[a]	*G. eucaliae*
Gyrodactylus aurorae[a]	Anura, Ranidae[a]	*G. eucaliae*
Gyrodactylus ambystomae[a]	Urodela, Ambystomatidae[a]	*G. eucaliae*
Gyrodactylus catesbeiana	Anura, Ranidae	Unknown
Gyrodactylus jennyae n. sp.	Anura, Ranidae	*G. elegans*

[a] From Malmberg (1970)

[b] Found on *Rana catesbeiana* tadpoles, which likely were accidental hosts (Prudhoe & Bray, 1982)

Montreal, Quebec, which had obtained the amphibians from a stock of tadpoles housed by a federal government research facility in Moncton, New Brunswick. These tadpoles originally came from a commercial frog farm located in the southern USA, believed to be in Missouri, via an international commercial distributor. Following the discovery of the parasite, more tadpoles were ordered from the same distributor the following year and they also arrived infected, suggesting that the farm stock was likely the original source of the parasite.

In Montreal, the tadpoles were held in filtered, aerated and dechlorinated water, at a density of 1 tadpole/litre, and exposed to a 16 h–8 h light–dark cycle and a temperature of 21 (±1.3)°C. Clinical symptoms, including skin erosion, scoliosis, lethargy and emaciation, first appeared 5 weeks after their arrival at the Montreal facility and microscopical inspection showed that they were infected with a species of *Gyrodactylus*.

For morphological studies, live specimens were frozen and then fixed in 10% formalin. Specimens were stained briefly in Masson's trichrome and mounted in a 50% solution of glycerine for clearing and study. The holotype and paratype specimen slides were soaked overnight in tap-water to remove the glycerine, dehydrated in ethanol, cleared in xylene and mounted in Canada balsam. The descriptive terminology follows You et al. (2008). Measurements are presented in micrometres for the holotype, followed in parentheses by the mean ± SD, the range and the number of specific measurements taken from 10 additional paratype specimens. The holotype of *G. chologastris* Mizelle, Whittaker & McDougal, 1969 (USNM Accession Number 70461) was examined for comparative purposes because, morphologically, this was the most similar species.

For genomic DNA extraction, seven individual specimens were briefly air-dried to remove the ethanol, placed in 5 µl water and stored at −20°C. For three specimens, the haptor was separated from the body before drying, enabling simultaneous morphological and molecular analyses of these individuals. The body was placed in 5 µl of milli-Q water, while the haptor was mounted in ammonium picrate glycerine, as described by Malmberg (1970). All specimens were digested by the addition of 5 µl of lysis solution consisting of 1× PCR buffer (Eurogentec), 0.45% (v/v) Tween 20, 0.45% (v/v) NP 40 and 60 µg/ml of proteinase K (Sigma). The samples were incubated at 65°C for 25 min, followed by 10 min at 95°C to inactivate the proteinase. The primer pairs ITS1A (5′-GTAACAAGGTTTCCG-TAGGTG-3′) and ITS2 (5′-TCCTCCGCTTAGTG ATA-3′) (Matějusová et al., 2001) were used to amplify a fragment spanning the 3′ end of the 18S rRNA gene, the internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, ITS2 and the 5′ end of the 28S rRNA gene. The amplification reactions (20 µl) consisted of 1× PCR buffer (Eurogentec), 1.5 mM $MgCl_2$ (Eurogentec), 200 µM of each dNTP (Amersham Pharmacia Biotech, Sweden), 1 µM of each primer (Eurogentec), 2 µl lysate, 1 unit *Taq* polymerase (Eurogentec) and milli-Q water. The mixtures were heated for 4 min at 96°C and subjected to 35 cycles of 1 min at 95°C, 1 min at 50°C and 2 min at 72°C, followed by final extension at 72°C for 7 min. The PCR products were visualised using ethidium bromide on a 1.2% agarose gel. The products were then purified by means of GFX columns according to the manufacturer's instructions (Amersham Pharmacia). Both DNA strands were sequenced using a Big Dye Chemistry Cycle Sequencing Kit (version 1.1) in a 3130 DNA Analyzer (Applied Biosystems).

The PCR primers and 2 internal primers, ITS1R (5′-ATTTGCGTTCGAGAGACCG-3′) and ITS2F (5′-TGGTGGATCACTCGGCTCA-3′) (Ziętara & Lumme, 2002), were used for sequencing.

We first compared sequences of specimens obtained from the bullfrog tadpoles. These were identical. We then searched for similar sequences among other species of *Gyrodactylus* in Genbank using BLAST (available at www.ncbi.nih.gov/BLAST/). Where possible, the entire ITS1-5.8S-ITS2 sequences were compared. Unfortunately, only ITS1 or ITS2 sequences exist for certain species, so these were compared separately. We also compared the 5.8S sequences separately. Previous work has shown that the 5.8S sequences are useful in distinguishing between the six subgenera of *Gyrodactylus*, as proposed by Malmberg (1970; see Ziętara et al., 2002; Huyse et al., 2003). This classification into subgenera is based on characteristics of the excretory system studied in living animals (Malmberg, 1970). The selected sequences were downloaded and aligned in Clustal W implemented in MEGA 4 (Tamura et al., 2007).

Gyrodactylus jennyae n. sp.

Type-host: *Rana catesbeiana* Shaw (Anura: Ranidae), bullfrog larvae.
Type-locality: Unknown, but believed to be a bullfrog farm in Missouri, USA.
Site: Body surface, mainly on the head, around the oral region and at the base of the tail.
Type-material: The holotype and paratype slides have been deposited in the Harold Manter Laboratory of Parasitology (Accession numbers HWML 49087), The University of Nebraska, Lincoln, Nebraska, USA.
Etymology: This helminth is named for Dr Jenny Cook, a long time supporter of parasitology research and expert on immigration patterns into New Brunswick, Canada.

Description (Figs. 1–3)

Coverslip-flattened specimens 508 (461 ± 63.5, 360–540, 11) long, 104 (114 ± 21.0, 92.5–166, 11) wide at mid-body. Prohaptor with distinct spike sensilla. Pharynx large, 46.4 (52.4 ± 8.0; 45–63, 6) long. Intestinal caeca typically containing large amounts of black pigment granules. Cirrus bulb small, 15 (16.4 ± 1.2, 15–17, 3) in diameter, with single large spine and 2 rows of smaller spines. Haptor oval in ventral view, 72 (73.7 ± 9.3, 62–87, 7) long, 95 (68.0 ± 17.3, 53–95, 5) wide. Hamuli slender, 62 (60.1 ± 2.5, 55.5–63.5, 10) long; root 24.1 (21.4 ± 1.8, 19.5–24.0, 7); shaft 43.5 (45.5 ± 2.2, 43–48, 7); point 31.8 (29.5 ± 2.5, 26–33, 7). Ventral bar sub-rectangular, 18 (18.1 ± 1.3, 16–20, 7) wide, 7.7 (6.5 ± 1.6, 4.5–8.5, 7) long medially; ventral bar membrane expanded distally, 16.7 (16.8 ± 1.8, 13.5–18.5, 6) long; ventral bar anterolateral processes 2.0 (1.9 ± 0.4, 1.5–2.0, 5) long. Dorsal bar simple, 1.8 (1.75 ± 1.8, 13.5–18.5, 6) long medially, 10.9 (12.6 ± 2.5, 11–15, 2) wide. Marginal hook 23.4 (23.1 ± 0.4, 22.5–23.5, 5) long; handle 14.5 (15.8 ± 0.9, 14.5–16.5, 4) long; sickle with prominent heel, length 8.4 (8.4 ± 0.8, 8–9.5, 4), distal width 2.7 (2.8 ± 0.5, 2.5–3.5, 4), proximal width 3.2 (3.5 ± 0.4, 3–4, 4); filament 15.1 (12.4 ± 2.4, 10.5–15.5, 4).

Comments

The morphological features of *G. jennyae* n. sp. (Figs. 1–3) place it within the subgenus *Gyrodactylus* and the *G. elegans* species-group of Malmberg (1970). Morphologically *G. jennyae* most closely resembles *G. chologastris* Mizelle, Whittaker & McDougal, 1969 from blind cave fish *Chologaster agassizi* (Putman) in Kentucky and *C. cornutus* (Agassiz) in North Carolina. Both species of parasite have a cirrus armed with small spines in multiple rows, long slender hamuli, a ventral bar with a relatively long membrane and small anterolateral processes, and marginal hooks with a well-developed sickle heel and a short handle. All of these characters are consistent with the subgenus *Gyrodactylus*. *G. jennyae* has a marginal hook sickle with a much more pronounced heel than *G. chologastris*, as confirmed by our examination of the holotype slide of *G. chologastris*. Unfortunately, no sequence data are available for this species.

The molecular data further supports that *G. jennyae* n. sp. fits the *G. elegans* species-group of Malmberg (1970). The amplified rRNA fragment was 945 bp long and consisted of the 3′ end of the SSU gene (34 bp), the ITS1 spacer (383 bp), the 5.8S gene (157 bp), the ITS2 (319 bp) and the 5′ end of the LSU gene (52 bp). The sequences of all seven specimens of *G. jennyae* were identical (GenBank Accession No.

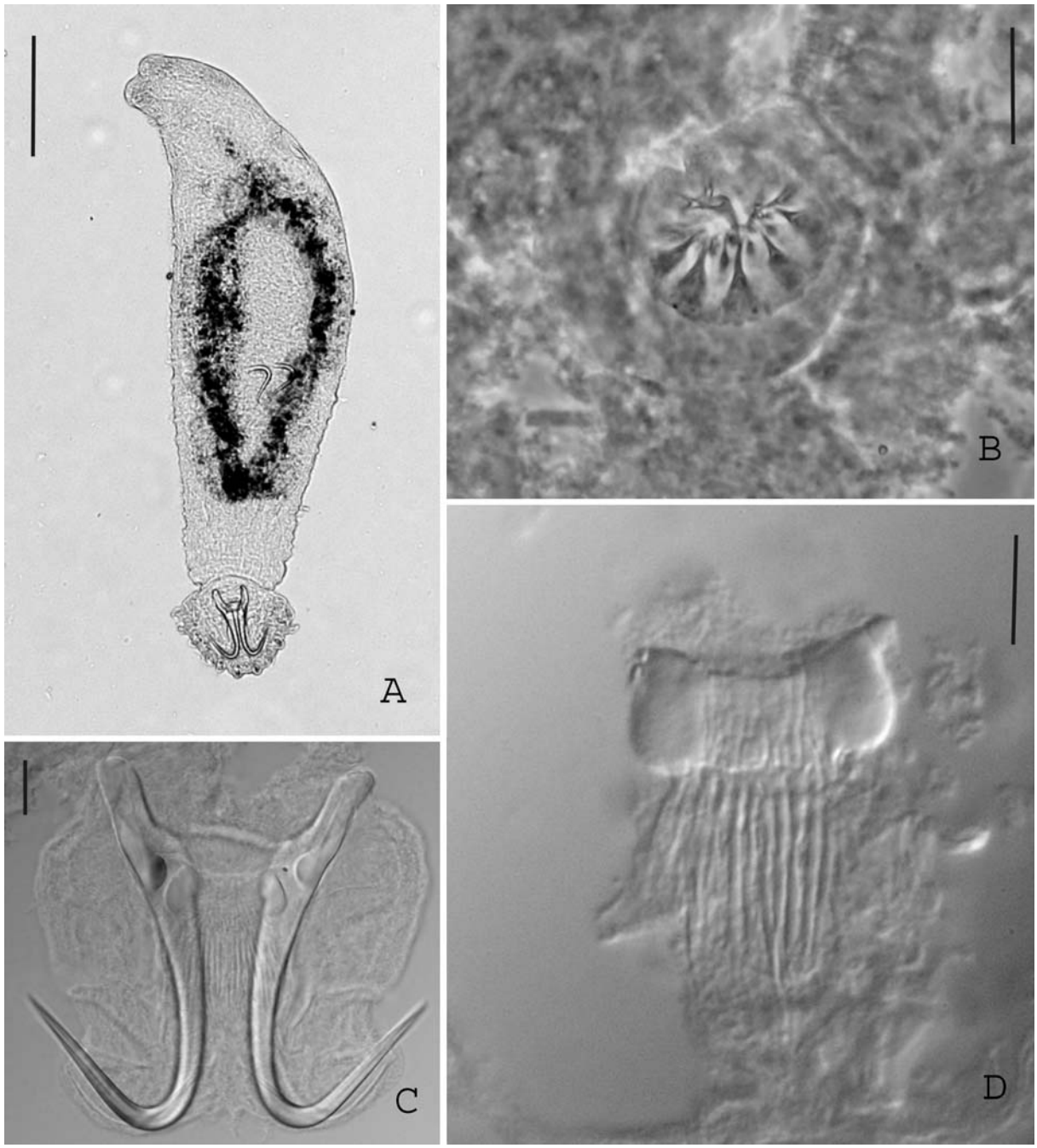

Fig. 1 Wholemount of *Gyrodactylus jennyae* n. sp. in ventral view showing: the accumulation of host pigment granules within the intestinal caeca (A); the cirrus with the double row of small hooks (B); a ventral view of the haptor with the relatively thin hamuli (C); and the ventral bar with a characteristic sub-rectangular membrane (D). *Scale-bars*: A, 100 μm, B–D, 10 μm

EU678357). Based on BLASTN searches on GenBank (Altschul et al., 1997), the ITS1 appeared most similar to that of *G. misgurni* Ling, 1962 (host: *Misgurnus anguillicaudatus* Cantor). Unfortunately, only ITS1 is available for *G. misgurni*. We aligned these sequences with Clustal W as implemented in Mega 4.0 (Tamura et al., 2007) and calculated the p-distance (15%). As all other BLAST hits were too distant, a reliable

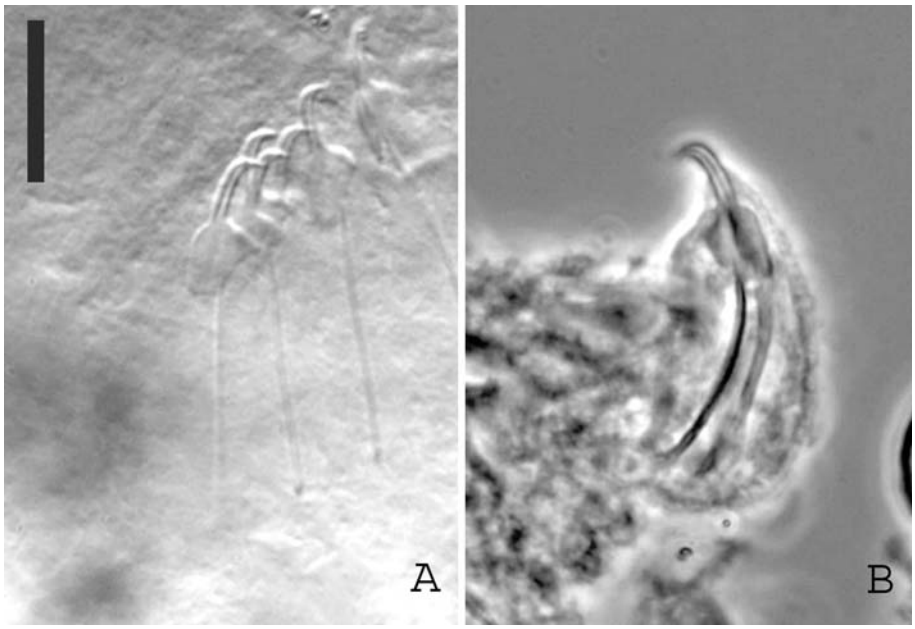

Fig. 2 Photomicrographs of the marginal hooks of *Gyrodactylus jennyae* n. sp. showing the characteristic shape of the marginal hook sickle with a pronounced heel and relatively short handle. Glycerine mounts. Differential interference contrast (A) and bright field optics (B). *Scale-bar*: 10 μm

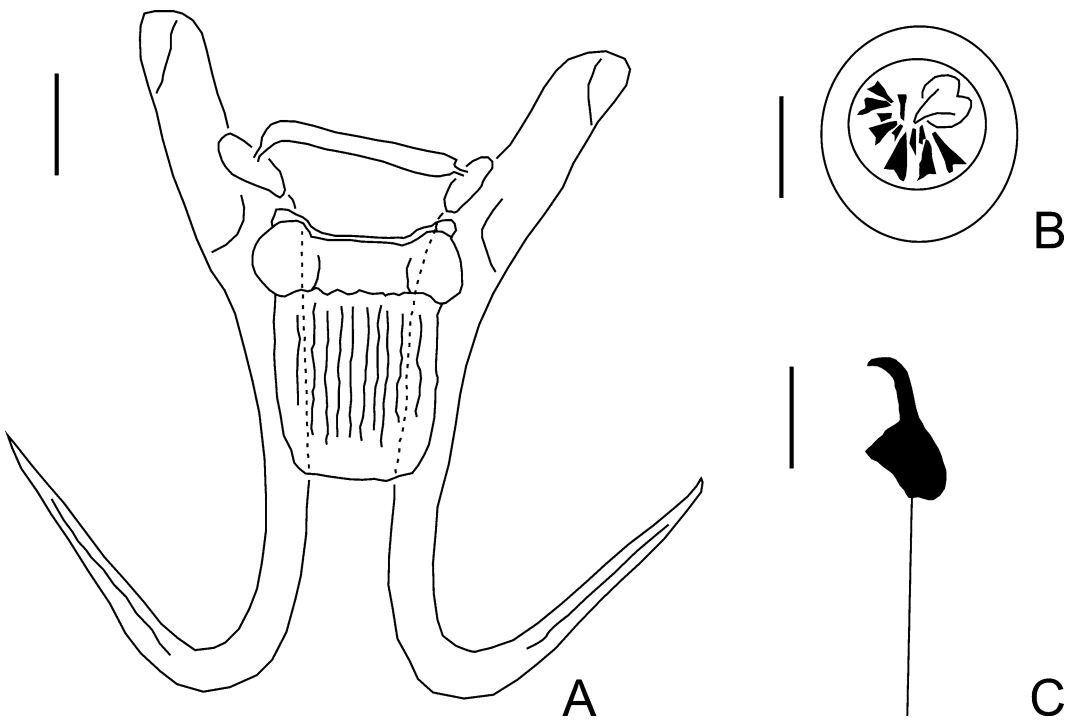

Fig. 3 Line drawings of the haptoral hard parts (A), cirrus (B) and marginal hook (C) of *Gyrodactylus jennyae* n. sp. *Scale-bars*: 10 μm

alignment was not possible and phylogenetic reconstruction was not attempted. The 5.8S sequence of the *G. jennyae* subunit was most similar to *G. markakulensis* Gvosdev, 1950 (host: *Gobio gobio* L.; 1 bp difference) and *G. laevis* Malmberg, 1957 (host: *Phoxinus phoxinus* L.; 2 bp difference). Comparisons with *G. prostae* Ergens, 1963, *G. phoxini* Malmberg 1957, *G. magnificus* Malmberg, 1957, *G. neili* Leblanc, Hansen, Burt & Cone, 2006, *G. elegans* Nordmann, 1832 and *G. carassii* Malmberg, 1957 revealed differences of 3 bp. These species are all members of the subgenus *Gyrodactylus*. The ITS2 sequences proved too difficult to align reliably, but BLASTN searches showed maximum identity of 70–80% with these last six species.

Pathology

Bullfrog tadpoles were the host of this parasite (Fig. 4). Like most monopisthocotylean monogeneans, *G. jennyae* n. sp. was observed to be an epidermal forager and possibly also ingests dermal tissue of the host (Cable et al., 1997; Bakke et al., 2007).

During the early stages of infection, the parasites were concentrated around the hosts' eyes and tail base, but, over time, *G. jennyae* could be observed throughout the entire body surface of the tadpoles, as well as on their oral papillae and inside their buccal cavity (Nieto et al., 2007). In heavy infections, tadpoles hosted hundreds of parasites, in one case carrying 104 worms around the oral region alone. After a period of time, the infected areas of the tadpoles' skin became paler in colour. Thus, the most prominent clinical symptoms of infection were the development of a characteristic light-coloured 'facial mask' (Fig. 4A) and a 'patchy' tail base. Lethargy and emaciation (Fig. 4B) followed simultaneously. A 10% mortality rate occurred in tadpoles within 2 weeks of the onset of symptoms.

Discussion

To date, there are 16 known cases of amphibian infection with *Gyrodactylus* spp., all but one of which were found in North America (Table 1). The majority of infections were observed in California and Florida and in only seven cases were the parasites identified to species. All but two cases involve tadpoles of anurans, with bullfrogs being the most common hosts, followed by southern leopard frogs.

Morphological and molecular results place *G. jennyae* n. sp. within Malmberg's (1970) subgenus *Gyrodactylus* and the *G. elegans* species-group (Table 2), which is typically found on cyprinid fishes in freshwaters of the northern hemisphere (Malmberg, 1970), implying that there has been a host-switch within the lineage from fish to amphibians. Host-switching is common among gyrodactylids (Bakke et al., 2002) and it may be an important mode of speciation (Bakke et al., 2007), despite strong barriers. When a switch is successful, the hyperviviparous reproduction used by gyrodactylids may lead to the

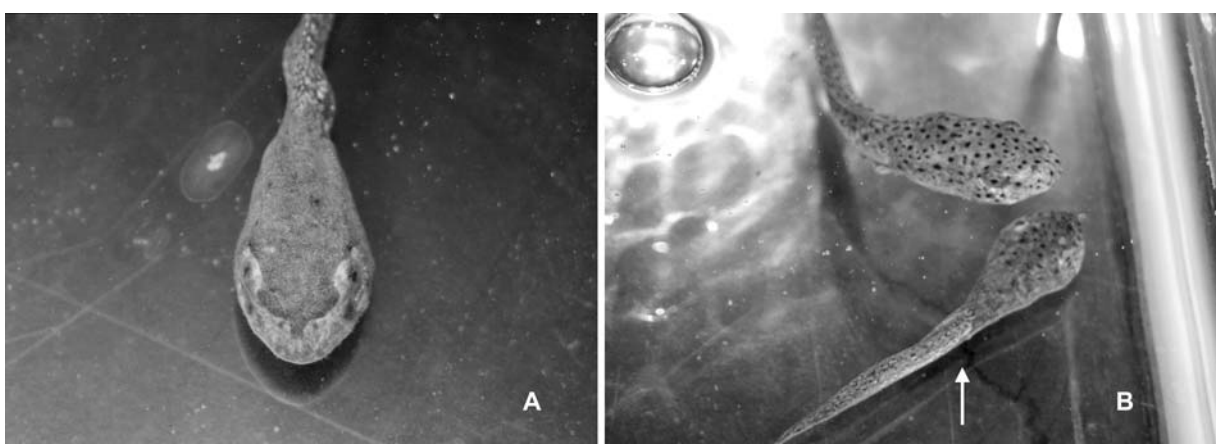

Fig. 4 Bullfrog tadpoles exhibiting clinical symptoms associated with infection by *Gyrodactylus jennyae* n. sp., including the pale 'facial mask' (A) and a great degree of emaciation that is most visible on the tail (B, *arrow*)

evolution of a new species by way of genetic isolation on the host (Ziętara & Lumme, 2002). Such a host-switch amongst members of *Gyrodactylus* is thought to have occurred between cyprinids and piscivorous escocid predators (pike and pickerel), giving rise to *G. neili* Leblanc, Hansen, Burt & Cone, 2006 and *G. fryi* Cone & Dechtiar, 1984 (Cone & Dechtiar, 1984; Leblanc et al., 2006).

Two additional host-switches likely occurred to produce the other *Gyrodactylus* spp. that have been described exclusively from amphibians: one switch may have produced *G. aurorae* Mizelle, Kritsky & McDonald, 1969, *G. ambystomae* Mizelle, Kritsky & McDonald, 1969 and *G. ensatus* Mizelle, Kritsky & Bury, 1968, which are members of Malmberg's (1970) subgenus *Metanephrotus* Malmberg, 1964 and the *G. eucaliae* species group (Table 2) described from various gasterosteid and centrarchid fishes. Another switch appears to have given rise to *G. catesbeiana* Wootton, Ryan, Demaree & Critchfield, 1993, which is of unknown subgenus placement, but which morphologically resembles *G. stunkardi* Kritsky & Mizelle, 1968 and *G. spathulatus* Mueller, 1936 (Wootton et al., 1993) from cyprinid and catostomid fishes. Thus, parasitism of amphibian larvae has resulted from at least three separate host-switches from fishes to amphibian tadpoles, and, along with the previous description of *G. catesbeiana*, the present study suggests that these have involved switches to bullfrog tadpoles at least twice.

The captive bullfrog tadpoles infected with *G. jennyae* n. sp. developed clinical symptoms, including emaciation and lethargy, before death, although these could not be attributed to the presence of the parasite due to the lack of an uninfected tadpole control group. The pale-coloured 'facial masks' and 'patchy' tail bases observed on the tadpoles, however, were possibly caused by the superficial grazing of the parasites. Indeed, the intestinal caeca of many of the parasites were filled with pigment granules, potentially of host integumental origin. On fish, it is believed that gyrodactylids secrete proteolytic enzymes onto the host epithelium and then ingest the mucus and partly-digested epithelial cells for further digestion and that, occasionally, the worms may ingest dermal cells of the fish, as well as their melanocytes (Bakke et al., 2007). In fact, Cable et al. (1997) confirmed that the pigment granules present in the gut of *Macrogyrodactylus polypteri* Malmberg, 1957 were of host origin. In amphibians, melanophores are present in both layers of the amphibian integument: they are individually scattered in the epidermis as melanocytes and form a chromatophore unit with other pigment cells in the dermis (Herman, 1992; Zug, 1993). Thus the decolourised regions that we observed on the tadpoles were potentially due to the destruction or ingestion of their melanophores by the parasites in one or both layers of the integument.

The mortality of tadpoles coinciding with gyrodactylid infections has only been observed on bullfrog tadpoles maintained in laboratory facilities (Crawshaw, 1997; Wootton et al., 1993; this study). Certainly, captive conditions can affect normal host-parasite relationships and lead to disease (Barber, 2007). But *Gyrodactylus* spp. have already gained notoriety for the widespread disease epidemics they have caused when they apparently spread among both cultured and wild fish populations, not to mention the enormous economic impacts and conservation threats that have ensued (Malmberg, 1993; Bakke et al., 2007). Thus the possibility exists that gyrodactylids could also cause extensive mortality among amphibian larvae in natural conditions, further adding to the suite of factors that are globally threatening the existence of these vertebrates (Stuart et al., 2004).

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