Global parasite trafficking: Asian *Gyrodactylus* (Monogenea) arrived to the U.S.A. via invasive fish *Misgurnus anguillicaudatus* as a threat to amphibians

Florian B. Reyda 1

Florian.Reyda@oneonta.edu

Scott M. Wells 2

Alexey V. Ermolenko 3

Marek S. Ziętara 4

Jaakko I. Lumme 5

1. Biology Department and Biological Field Station, State University of New York at Oneonta, College at Oneonta, Oneonta, NY, U.S.A.;

2. New York State State Department of Environmental Conservation, Region 4 Bureau of Fisheries Stamford, NY, U.S.A.;

3. Institute of Biology and Soil Science 159, Prospekt Stoletiya, 690022 Vladivostok, Russia;

4. Department of Molecular Evolution, Faculty of Biology, University of Gdańsk, Wita Stwosza St., 59, PL-80-308 Gdańsk, Poland;

5. Ecology and Genetics , University of Oulu, Oulu, Finland.

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Abstract

A monogenean flatworm *Gyrodactylus jennyae* Paetow, Cone, Huyse, McLaughlin & Marcogliese, 2009 was previously described as a pathogen on bullfrog *Lithobates catesbeianus* Shaw, 1802, in a Canadian captive population originating in Missouri, U.S.A. The ITS barcoding of *G. jennyae* showed relatedness to Asian *Gyrodactylus macracanthus* Hukuda 1940, a parasite of the Asian loach *Misgurnus anguillicaudatus* Cantor, 1842. The resulting suggestion that the globally invasive pet-trade of fish may be a mechanism for arrival of *Gyrodactylus* species to North America provided the framework for the current study. The present study was undertaken following the discovery of two other species of *Gyrodactylus* in a population of illegally introduced *M. anguillicaudatus* in New York State. Here the invasion hypothesis was tested via DNA sequencing of the ITS of the two *Gyrodactylus* species obtained from *M. anguillicaudatus* from New York, termed *Gyrodactylus* sp. A and *Gyrodactylus* sp. B. Both *Gyrodactylus* sp. A and *Gyrodactylus* sp. B were closely related to *G. jennyae* and *G. macracanthus*, and all belong to a molecularly well-supported monophyletic Asian freshwater group. In conclusion, this invasive fish has trafficked at least three parasite species to the U.S.A., one of them also found on frog. This route from the Asian wetlands to other continents is similar to that of amphibian chytrid fungi of genus *Batrachochytrium* Longcore, Pessier & Nichols, 1999.

Keywords

Intercontinental parasite dispersal

Molecular systematics

Aquatic invasive species

Chytrid fungus

Introduction

Introduced and invasive species are increasingly recognized as a threat to local ecosystems. The interactions of the invaders and the native populations are often simplified as resource competition of the organisms at the same trophic level. Perhaps biological invasions are more complicated, and the harmful effects caused by new intruders surpass simple competition. Introduced organisms may shed their native parasites and diseases and gain a competitive advantage (Enemy release hypothesis, Torchin et al. 2003) in new ecosystems. The invaders may also introduce diseases which are new to the target ecosystem, and therefore harm the native populations of interacting species (Verneau et al. 2011). Disturbed ecosystems may in turn spread pathogens to humans and livestock and increase the number of zoonoses (EIDs or emerging infectious diseases; Daszak et al. 2000). One drastic example of harmful invasion in line with the focus of this paper was the transport of Baltic strains of Atlantic salmon (*Salmo salar* L.) to Norway. With clinically healthy (tolerant) Baltic fish, the monogenean flatworm parasite *Gyrodactylus salaris* Malmberg, 1957 arrived in Norway in 1975 and destroyed 45 Atlantic salmon river populations in just a few decades (Johnsen and Jensen 1991; Bakke et al. 2002, 2007). In contrast, the long-isolated Baltic salmon (Lumme et al. 2015) is tolerant and co-adapted with *G. salaris*, so that the spatially and genetically differentiated host-parasite combinations remain stable despite the physical mobility (Lumme et al. 2016a). The parasite introduced to the susceptible Atlantic salmon populations spread rapidly, with fatal consequences.

Gurevitch and Padilla (2004) asked whether species invasions are a major cause of extinctions, and claimed that data supporting this widely-accepted view often are anecdotal, speculative, and based on limited observation. A problem common with such observations about "change" is that many global changes occur in the same time axis and are temporally associated. This leads to skepticism against invoking explanations of "change". Pimentel et al.

(2005) estimated that there are 50,000 foreign plant or animal species in the United States alone, and that 42% of endemic species are endangered or threatened because of aliens. The authors concluded that the total economic damages and losses caused by invasive species add up to an estimated $120 billion per year in the U.S.A. alone.

This study maps a complex case of an introduced and rapidly spreading Asian fish in North America, accompanied by East Asian parasites. The starting point of this narrative was as follows. A monogenean flatworm species *Gyrodactylus jennyae* Paetow, Cone, Huyse, McLaughlin and Marcogliese 2009 was described as a new pathogenic parasite on the American bullfrog *Lithobates catesbeianus* Shaw, 1802 in North America. The bullfrog itself is widely trafficked (Mata-López et al. 2010). Paetow et al. (2013) also demonstrated that *G. jennyae* infection increased the mortality of the tadpoles associated with fungal disease *Batrachochytrium dendrobatidis* Longcore, Pessier & Nichols, 1999, making matters worse. This fungal disease is considered one of the main reasons for the contemporary decline of global amphibian populations (Collins and Crump 2009). The question was raised about the origin of this newly described *Gyrodactylus* species, which are monogenean ectoparasites of fish, seldom found on amphibians. Records on amphibians were reviewed in Paetow et al. 2009.

Fortunately, the formal description of *G. jennyae* was accompanied with the DNA sequence of complete ribosomal Internal Transcribed Spacer segments (ITS1-5.8S rDNA–ITS2), hereafter ITS. This fragment of DNA is the most popular in *Gyrodactylus* studies (e.g., Paetow et al. 2009), and consequently the most useful DNA segment for “barcoding” in the genus (Mendoza-Palmero et al., 2019). The parasites of genus *Gyrodactylus* are very small (<0.5 mm), living on the surface or gills of the fish, and most often clinically more or less asymptomatic. In fact, they are seldom detected without targeted search. Emphasis of research has been on fish farms, especially those of salmonids (Paladini 2012). A minimum number of

Gyrodactylus species diversity has been estimated at 20,000 (Bakke et al. 2002), with some 417 formally described (Harris et al. 2004), and only 200 barcoded by ITS.

Paetow et al. (2009) conducted a BLAST search of *G. jennyae* among the published ITS sequences of *Gyrodactylus* spp., and confirmed closest matches of ITS1 and ITS2 segments to species of the nominal subgenus *G.* (*Gyrodactylus*) which are mainly Eurasian parasites on cyprinids. The genetic distance of *G. jennyae* to *G. neili* LeBlanc, Hansen, Burt & Cone 2006, a North American member of the subgenus *G.* (*Gyrodactylus*) is $d_{K2P} = 0.320$. However, in a small unpublished collection of ITS sequences from Far East Asia maintained by the authors, a much closer match $d_{K2P} = 0.085$ was found between *G. jennyae* and *G. macracanthus* Hukuda, 1940, described in the Han River system, Korea. *G. macracanthus* is a parasite of the Asian pond loach (*Misgurnus anguillicaudatus* Cantor, 1842).

Because *M. anguillicaudatus* is an invasive freshwater species trafficked by the aquarium pet trade (Chang et al. 2009) with established non-native populations in Australia, North and South America, and Europe (Belle et al. 2017 and references therein), we hypothesized that it might be a mechanism for transport of East Asian parasites to North America. A focused search was conducted in New York State (Wells 2014).

Methods

The Far East and U.S.A. samples of Gyrodactylus

The new ITS sequences used here were of *Gyrodactylus macracanthus* taken from *M. anguillicaudatus* collected from the wild near Vladivostok, Russia (43.25º N, 131.98º E) by hand net. The ITS samples from Topmouth gudgeon (a.k.a. Stone moroko) *Pseudorasbora parva* (Cyprinidae) were from the same geographical area, from the River Bol'shaya Ussurka (45.98º N, 134.03º E), and were included in this study because of the relatedness of the ITS.

The Russian fish were killed immediately by a blow to head and the fins and gills were removed and stored in 96 % ethanol.

In central New York State, U.S.A., specimens of *M. anguillicaudatus* were collected by fish traps baited with dog food in the Manor Kill watershed near Conesville, New York (42.402 º N, -74.30 º W) on 21 October 2013 and 28 November 2015.

The North American fish were maintained in aquaria for up to one month until examination for parasites. Fish were killed by cervical transection following submersion in Tricaine-S (Tricaine methanesulfonate, Western Chemical, Inc., Ferndale, Washington) in accordance with the guidelines of SUNY Oneonta (State University of New York College at Oneonta) IACUC protocol 201303. Gills were subsequently examined for monogeneans. Monogeneans encountered were preserved in 95–100% ethyl alcohol and examined with a light microscope and/or subjected to DNA sequencing (see below).

Morphological recording of the parasites

A subset of the monogeneans encountered in New York State were photographed with a Leica DM2500 equipped with a DFC295 digital camera (Leica Microsystems, Buffalo Grove, Illinois). The photos of complete live specimens are provided here to serve as photo vouchers (Supplementary material).

The monogenean specimens chosen for analysis of ITS sequence were stored in ethanol and cut in two parts. The opisthaptor was placed on a microscope slide for morphological inspection. When the ethanol was evaporated, a drop of proteinase K digestion mix (see below) was added and the digestion was followed under a microscope (100x phase contrast, Zeiss Axiolab). When the hard parts of the haptor were cleared, the sample was embedded in saturated ammonium picrate in glycerol under a coverslip, and fixed with nail polish. The samples were photographed during and after the digestion process with a Nikon

Coolpix 995 digital camera at 100x and 200x magnifications with phase contrast optics. Calibration for measurements was done with photos of a 0.01 mm scale (Graticules Ltd, Tonbridge, Kent, England). The microscope slides were deposited as vouchers or hologenophores (sensu Pleijel et al., 2008) in the Finnish National collection in Helsinki (MZH118018-19 for *Gyrodactylus* sp. *A*, #8 and #4, and MZH118018-20 for *Gyrodactylus* sp. *B*, #11).

DNA sequencing of the ITS segment

The isolation of DNA, primary PCR and sequencing of the ITS of the anterior portions of ethanol fixed worms was conducted as described in previous *Gyrodactylus* studies (Ziętara and Lumme, 2002, 2003; Ziętara et al. 2006, 2008). The ethanol was evaporated. DNA was released by digesting single parasite halves in 10 µl of lysis solution consisting of 1 × PCR buffer, 0.45% (v/v) Tween 20, 0.45% (v/v) NP 40 and 60 μg/ml proteinase K. The samples were incubated at 65 °C for 25 minutes to allow proteinase K digestion, then at 95 °C for 10 minutes to denature the proteinase and cooled down to 4 °C.

Aliquots of 2 μl of this lysate were used as templates for PCR amplification. The 10 µl PCR mix consisted of 2 µl 5x Phusion HP buffer (7.5 mM $MgCl_2$), 1 µl 2.0 mM dNTP, 0.5 1 µl of each primer stock (20 µM), 0.2 U of Phusion DNA polymerase and 2 µl of digested parasite. The cycling profile was as follows: 98 °C for 30 sec, 35 cycles of 98 °C for 10 s, 50 °C for 20 s and 72 °C 40 s, and a final extension at 72 °C for 5 min, and cooling down to 4 °C. For primary PCR of whole ITS segment, the primers (Oligomer Oy, Helsinki, Finland) were ITS1F 5'- GTTTC CGTAG GTGAA CCT -3', and ITS2R 5'- GGTAA TCACG CTTGA ATC -3'.

The PCR product was checked in agarose gel. The product was purified by ExoSAP procedure: 0.8 µl Fast AP buffer, 0.5 µl Fast AP, 0.1 µl Exo I, 6.6 µl H_2O, and 2 µl PCR product.

The sequencing mix was 1.65 µl BigDye Terminator v 3.1 in 5x sequencing buffer, 0.7 µl Big dye terminator v.3.1 Cycle in Sequencing RR-100, 0.65 µl of ITS1F, ITS2R, or the additional ITS2F or ITS1R primers, and 2 µl of Exosap purified PCR product. The internal sequencing primers were ITS2F 5'- TGGTG GATCA CTCGG CTCA -3' and ITS1R 5'- ATTTG CGTTC GAGAG AGACC -3'. Altogether, the coverage was threefold to fourfold.

The sequencing reactions were purified by the Sephadex method and introduced to an ABI3730 automatic DNA reader. The ABI reads were decoded, edited and aligned by Codon Code Aligner, and transferred to MEGA7 for further analysis.

Sequence comparisons, alignment and phylogenetic tree construction

The ITS sequences vary so much within *Gyrodactylus* that only 5.8S RNA and the terminal parts of the ITS1 and ITS2 sequences can be reliably aligned genus-wide. We selected the set of species to be compared with *G. jennyae* by phylogenetic hypothesis by internal BLAST search, confirmed by secondary structure comparison of the ITS2 (Zuker 2013). We utilized the MUSCLE (Edgar 2004) program as implemented in MEGA7. The hypervariable segment in ITS1 containing a variable number of TAAAAA repeats was hand-edited.

The distance method K2P (Kimura's two parameter) was chosen because it is the most simple model that can be used for this type of DNA sequence data. The sequence evolution of rDNA is strictly constrained for maintaining the secondary structure stems and hairpins, which in ITS are needed for correct splicing of the segments to be removed. The phylogenetic reconstructions were made in MEGA7 by Neighbor Joining and Maximum Likelihood

methods, both tested by 500 rounds of bootstrapping. All methods produced a robust separation between the species and branches, with the same topology.

The new ITS sequences from Far East Asia and North America were deposited in GenBank with accession numbers MH667459-MH667466 (Fig. 1).

Results

In New York State, *M. anguillicaudatus* dominated the catch in the infested headwaters in the Manor Kill watershed, living together with native amphibians, the spotted salamander (*Ambystoma maculatum* Shaw, 1802) and red spotted newt (*Notophthalmus viridescens* Rafinesque, 1820), as described by Wells (2014).

A total of 48 *M. anguillicaudatus* specimens were caught and the gills examined for monogeneans, including 30 from the October 2013 sample and 18 from the November 2015 sample. Eight of the 48 fish examined were found to have *Gyrodactylus* specimens, for an overall prevalence of 16.7%. The maximum number of *Gyrodactylus* specimens found on a single fish was six. The ITS DNA sequence was obtained from a total of seven North American specimens of *Gyrodactylus*, and included in the phylogeny (Fig. 1).

Molecular phylogenetic position of Gyrodactylus *intruders in North America*

The species of *Gyrodactylus* to be compared with *G. jennyae* were selected from available GenBank data by BLAST search and sequence comparison. The group is monophyletic also in the partial global phylogeny by Mendoza-Palmero et al. (2019), supported by Posterior probability/ Bootstrap values 0.93/61. An apomorphic indel influencing the secondary structure folding in ITS2 confirmed the monophyly of the group in Fig. 1.

A phylogenetic hypothesis containing a total of 29 published and new ITS1-5.8S-ITS2 sequences was constructed for placing *G. jennyae* in a plausible systematic position in a monophyletic clade supported by Posterior probability/ Bootstrap values 0.93/61 in the partial

global phylogeny by Mendoza-Palmero et al. (2019), a study which helped us guide our sampling representation here. The phylogenetic hypothesis strongly supports three sister subclades presented in Fig 1: first one from Africa on Siluriformes (Přikrylová et al. 2012), second mostly on European cyprinids described as subgenus *G.* (*Gyrodactylus*) (Malmberg 1970), and the third group in focus in this study, containing the Far East Asian species on Cobitidae and *P. parva*, along with the three found as intruders in North America. This cluster was characterized by a

The branch from Far East Asia in Fig. 1 appears to be a sister clade to the predominantly European subgenus *G.* (*Gyrodactylus*). In the tree of Mendoza-Palmero et al. (2019), *G. jennyae* was paired with *G. granoei* in this clade, which now contains eight species. The two previously unknown *Gyrodactylus* parasites found following examination of *M. anguillicaudatus* from New York State clustered with *G. jennyae* and *G. macracanthus*, but they are clearly separate species differing from one another by a $d_{K2P} = 0.085$. Not presented in the tree is *G. misgurni* Ling, 1962, because only ITS1 sequence (AJ407887) is available. The hypervariable ITS1 of *G. misgurni* is closer to *G. macracanthus* ($d_{K2P} = 0.084$) than to *G. jennyae* ($d_{K2P} = 0.145$). Thus, it belongs to the subclade with the American *G.* sp. B and *G. macracanthus*. *G. misgurni* has been named and described from *Misgurnus anguillicaudatus* from China, but the ITS1 sequence is from European *Misgurnus fossilis* (Matějusová et al. 2001).

Thus, the monophyletic sub-cluster including *G. jennyae* contains at least five parasite species from *Misgurnus* species. This strongly supports a fish origin of *G. jennyae*. The Far East Asia origin of *G. jennyae* and the two undescribed species A and B from New York is supported by relatedness of the sub-clade containing *G. granoei* on cobitids and the three unnamed species on the cyprinid *Pseudorasbora parva*. The combination of the two subclades is 100/100% supported and forms a novel Asian freshwater group of *Gyrodactylus*

species. Comparing it with the subgenus *G*. (*Gyrodactylus*) may suggest a subgeneric status. However, Malmberg's five subgenera are based on the osmoregulatory system, studied in living worms. Constructing new subgenera without this information seems unjustified.

The two most basal species likely belonging to the subgenus *G*. (*Gyrodactylus*) are *G. neili* from chain pickerel (*Esox niger* Lesueur, 1818) from New Brunswick, Canada (LeBlanc et al. 2006), and *G. laevisoides* King, Cone, Mackley & Bentzen, 2013 from Northern redbelly dace (*Chrosomus eos* Cope, 1861) in Nova Scotia (King et al. 2013). Interestingly, these two are the only molecularly-identified North American members of subgenus *G.* (*Gyrodactylus*), and *G. neili* is the only species of this subgenus known from a non-cyprinid host. The phylogenetic analysis suggested that the inclusion of *G. jennyae* in subgenus *G.* (*Gyrodactylus*) is not supported.

Morphological comparisons

The morphological taxonomy of *Gyrodactylus* is based on the form and size of the sclerotized parts of the haptor (opisthaptor) organ on the posterior of the animal, containing two major hooks (hamuli) and sixteen marginal hooks. The species descriptions available in Pugachev et al. (2009) and in GyroBase (http://www.gyrodb.net/) cover the present taxonomical knowledge of *Gyrodactylus* species in Far East Asia. The haptoral morphology of the two potentially new North American species, *Gyrodactylus* sp. A and *Gyrodactylus* sp. B, is presented in Fig. 2.

The marginal hook morphology of *Gyrodactylus* sp. A and *Gyrodactylus* sp. B is characteristic for the molecularly confirmed morpho-group including the three American species, the far East Russian *G. macracanthus*, and the European specimen of *G. misgurni*. The base is hoof-like and looks hollow in light microscopy, but this is not visible in the all-black or outline profile drawings in the literature. The external part of the base is much

enlarged. The hooklet (sickle) is about the same length as the base, and rather straight until the curved tip, versus tightly curved from halfway as in *G. granoei* (You et al. 2010) and the unnamed species hosted by *P. parva* in Fig. 1. Five molecularly tagged taxa belong to this morpho-group parasitizing Cobitidae: *G. jennyae, G. macracanthus* and *G. misgurni* (ITS1 only) and the two species *G.* sp. A and *G.* sp. B depicted and sequenced here. The following unsequenced species also belong to this morpho-group, characterized by specific marginal hooks, on the basis of drawings compiled in Pugachev et al. (2009) and in GyroBase (http://www.gyrodb.net/): *G. micracanthus* Hukuda, 1940 on *M. anguillicaudatus* (Korean Peninsula) and/or on *Cobitis granoei* Rendahl, 1935, (Russian Far East), *G. molnari* Ergens, 1978, on *Cobitis taenia* L. (Hungary), *G. yukhimenkoi* Ergens, 1978, on *Cobitis sibirica* Gladkov, 1935 (Far East Asia), and *G. latus* Bychowsky, 1933, on *Cobitis taenia* (Europe). The *G. macracanthus* from Australian *M. anguillicaudatus* is also a member in this morpho-group, but it is tagged not with ITS, but by 28S ribosomal RNA, Histone 3 and Elongation factor 1 α sequences (Perkins et al. 2009).

Total length of the hamulus (main hook) in *G.* sp. A was 53.2 - 54.5 µm, within the published range of *G. yukhimenkoi* (53-57 µm) and *G. latus* (50-57 µm). In *G.* sp. B, the hamulus was smaller, 48.4 µm, within the range reported for *G. misgurni* (42-51 µm). Hamuli of both *G.* sp. A and *G.* sp. B are clearly smaller than the hamulus of *G. jennyae* (62 µm) or *G. molnari* (63-65 µm). Both *G.* sp. A and *G.* sp. B have longer central hooks than the original Korean *G. macracanthus* (41-47 µm). The *G.* sp. *B* is close to the Australian sample named as *G. macracanthus* by Dove and Ernst (49 µm). The above species have not been molecularly confirmed, and thus may or may not be conspecific with any of the species mentioned in the phylogeny in Fig. 1.

In the description of *G. granoei* (You et al. 2010) the marginal hooks of several of the above species were compared in figures 4-7, and they are clearly different: the hooklet of *G.*

granoei is much more curved. The marginal hooks of the parasites on *Pseudorasbora parva* (data not shown) confirm that they are morphologically related to *G. granoei*, but not to the group of *G. jennyae*. The parasites on *P. parva* presented in Fig. 1 are most probably all undescribed species.

Discussion

The *method* of this report is molecular phylogeography (Avise 2000). The species names are less important than the DNA sequences and phylogenetic hypothesis constructed and overlaid on the continents. We have decided not to describe and name the few supposedly undescribed species in this study, owing to lack of adequate material (specimens), and because doing so is beyond the scope of this study. We report here two species related to *G. jennyae* that were found in North America, but they evidently originated from Far East Asia, together with their invasive host fish *Misgurnus anguillicaudatus*. There are several other morphologically related species described in Eurasia, but without DNA barcodes they cannot be reliably compared. In addition, three undescribed species on *Pseudorasbora. parva* barcoded here are surely novel, because the only species described on this host (*G. parvae* You, Easy & Cone, 2008, EF450249) belongs to subgenus *G.* (*Limnonephrotus*). However, they help in anchoring the clade to Far East Asia.

The strong phylogenetic association of the barcoding ITS sequence of the amphibian parasite *G. jennyae* with *G. macracanthus* on the originally Asian invasive host fish lead to a hypothesis of intercontinental parasite trafficking. Autonomous dispersal is excluded as an explanation for a freshwater host and parasite. *M. anguillicaudatus* belongs to the family Cobitidae (~ 29 genera, ~ 110 species) which is native to freshwaters in Eurasia (> 100 species) and Morocco, North Africa (only 2 species) (Kottelat 2012). Consequently, we predicted that *M. anguillicaudatus* may have also carried *G. jennyae* and perhaps other Asian

Gyrodactylus species into North America. If *G. jennyae* or other relative(s) of *G. macracanthus* could be found in established immigrant fish, they could be taken as direct evidence that the Asian loach carried them to a new continent. It was with this beginning hypothesis that we set out to investigate *Gyrodactylus* parasites on Oriental weatherfish in one of many feral populations now occurring in New York State (Wells 2014).

Despite some gaps in the dataset, a global molecular systematic scaffold for *Gyrodactylus* has developed, supporting and complementing the morphological systematics of the genus based mostly on Eurasian material (Malmberg 1970; Ergens 1985). In a very important study, Boeger et al. (2003) developed a hypothesis that the viviparous genus *Gyrodactylus* originated in South America from an egg-laying predecessor, and later occupied the marine environments and expanded to other continents. Molecular phylogenetic investigations may support this hypothesis. Accumulating data from Europe (Matějusová et al. 2003; Ziętara and Lumme, 2004), North America (Gilmore et al. 2012) and Mexico (Garcia-Vasquez et al. 2015; Mendoza-Palmiero et al., 2019) help to test and further define such hypotheses. Among the marine (coastal) parasite groups, surprisingly distant samples show genetic relatedness. In the large dataset of ITS2 in GenBank, the overall genetic distance $d\text{ITS2}_{\text{K2P}} = 0.449$, even while deflated via comparisons within clades. Two Antarctic (Rokicka et al. 2009) and one Chilean (Ziętara et al. 2012) marine species of *Gyrodactylus* were phylogenetically connected with small clusters of respective Northern species groups. Representative species pairs are the Antarctic *G. coriicepsi* Rokicka, Lumme & Zietara. 2009, *vs.G. groenlandicus* Levinsen, 1881, from British Columbia ($d\text{ITS2}_{\text{K2P}} = 0.046$) and *G. chileani* Zietara, Lebedeva, Muňoz & Lumme, 2012 vs. Mediterranean *G. orecchiae* Paladini, Cable, Fioravanti, Faria, Cave & Shinn, 2009 ($d\text{ITS2}_{\text{K2P}} = 0.158$) A surprising coincidence was that two species collected on Mexican freshwater poecilids, *G. takoke* Garcia-Vásquez, Razo-Mendivil & Rubio-Godoy, 2015 (KM514457; $d\text{ITS2}_{\text{K2P}} = 0.031$) and *G. xalapensis*

Rubio-Godoy, Paladini, Garcia-Vásquez & Shinn, 2010 (KJ621985; dITS2$_{K2P}$ = 0.018) were the nearest relatives of *G. arcuatus* Bychowsky, 1933 (AF328865), a circumpolar parasite on three-spined stickleback *Gasterosteus aculeatus* L. (Lumme et al., 2016b). At this scale, the differences between *G. jennyae* and its invasion partners are moderate (*G.* sp. A; dITS2$_{K2P}$ = 0.035; *G.* sp. B; dITS2$_{K2P}$ = 0.039).

Monogenean parasites are only dispersed by the fish host. They have no known means of independent long-distance dispersal, and they are not known to use vectors; hence such cases of long-distance transfer are assumed to be anthropogenic. The invasive fishes may transport their own parasites to new areas, as shown here for *M. anguillicaudatus*. Some other examples are *Gyrodactylus perccotti* Ergens, Yukhimenko & Yukhimenko, 1973 on Amur sleeper *Perccottus glennii* Dybowski, 1877 from Russia to Eastern Europe (Ondračková et al. 2012); *Gyrodactylus proterohini* Ergens, 1967 on Western tubenose goby *Proterorhinus semilunaris* (Heckel, 1837) from the Ponto-Caspian region to Western Europe, Belgium (Huyse et al. 2015) and *G. salmonis* (Yin & Sproston, 1948) on rainbow trout from boreal North America to Mexico (Rubio-Godoy et al. 2012). In these cases, subsequent spread of *Gyrodactylus* to any new fish (or amphibian) species of the new homeland was not reported, either due to limited examination or because the parasite failed to relocate. In another example of parasite trafficking, the topmouth gudgeon (*P. parva*) has introduced a protistan disease rosette agent (*Sphaerothecum destruens*) to the U.S.A., U.K. and the Netherlands (Ragan et al. 1996; Pinder et al. 2005; Spikmans et al. 2013), but no *Gyrodactylus*, as far as we know.

Conversely, invasive fishes can adopt local parasites in their new localities, e.g. the unnamed *Gyrodactylus* (*Limnonephrotus*) sp. on round goby *Neogobius melanostomus* (Pallas, 1814) in Europe (Huyse et al. 2015). The rainbow trout (*Oncorhynchus mykiss* [Walbaum, 1792]), a global resident now (Fausch 2007) has adopted numerous European parasites, often via hybridization of two strains of *Gyrodactylus* (Lindenstrøm et al. 2003;

Rokicka et al. 2007, Kuusela et al. 2008, Ziętara et al. 2010, Ieshko et al. 2015). As a widely-cultured species, rainbow trout serves as an optimal platform to receive and subsequently serve as a vector for the introduction of opportunistic parasites to new locations.

Of course, both scenarios can be combined. Introduced and local parasites may breed and produce novel combinations and perhaps transfer further, into new hosts and localities. Human trafficking has replaced the geological perturbations as a source of novelty by recombination.

The conclusion based on the phylogenetic comparison in this work is that the clade of parasites related to *G. jennyae* on *Pseudorasbora* and *Misgurnus* appears to be a monophyletic branch originating from Asia. All native loach in the family Cobitidae are freshwater forms in Eurasia and in Morocco, Africa (Kottelat 2012). Consequently, the monogenean parasite species of this evolutionary clade are not expected to be endemic to any North American freshwater fishes, let alone frogs.

Similar invasion histories of Misgurnus, Gyrodactylus *and chytridiomycosis*.

In the amphibian facet of the story, *G. jennyae* forms an intriguing coincidence with the fungal disease chytridiomycosis, caused by pathogenic fungi of genus *Batrachochytrium*, which have apparently invaded global amphibian populations. The global decline of amphibian populations is a major concern for scientists (Storfer 2003). The causes of this decline are diverse, complex, and still under much study and debate (e.g., Collins and Crump 2009 *vs.* Pounds and Masters 2009).

The enzymatic digestion and the hooked haptors of *Gyrodactylus* damage the frog's epidermis (Nieto et al. 2007; Paetow et al. 2009, 2013). Epidermal damage on tadpole lips caused by *G. jennyae* (figs. 4A and 1A in Paetow et al. 2009 and 2013, respectively) appears similar to erosion caused by fungus *Batrachochytrium dendrobatidis* (fig. 6.10 in Collins and

Crump 2009). Such epidermal damage on frogs may facilitate opportunistic fungal infections which can result in osmoregulation failure and even death via heart failure (Voyles et al. 2009; Paetow et al. 2013).

Recently, a molecular phylogenetic framework for the chytrid fungi killing amphibians was generated, first in The Netherlands (Martel et al. 2013), describing a novel species, *Batrachochytrium salamandrivorans* Martel et al., 2013. The globally more widely spread *B. dendrobatidis* was extensively studied (Fisher et al. 2018; O'Hanlon et al. 2018). It was demonstrated that the worldwide pathogenic strains of the fungus originated in the Korean Peninsula and were subsequently spread most probably by the general pet trade, since 1975. The pet trade routinely traffics both amphibians and fishes from areas where the collecting might be an important cottage industry. The worldwide industry is largely unregulated and lacks a central database to assess its impact upon the environment (Lee 2014). The distribution history of *M. anguillicaudatus* demonstrates that it is commonly shipped live worldwide and may be included among the potential vectors of aquatic diseases.

Here we raise the possibility that in their new environment, invasive parasites can move from invading fish to native amphibian hosts during the aquatic phases of their life cycle. By extension, we suggest that such novel/unknown parasites like *Gyrodactylus* spp. may be contributing to the global decline of amphibians.

Conclusions

The phylogenetic position of North American bullfrog parasite *G. jennyae* and two additional *Gyrodactylus* species demonstrate that they are not native to North America but instead likely originated from Asia, by invasive fish.

M. anguillicaudatus is a recent invader to several continents, and continues to spread into new/adjacent fresh waters. This Asian loach species carried *G. jennyae* from Asia to

North America and introduced it to bullfrog. We predict that *M. anguillicaudatus* has likely spread several other *Gyrodactylus* species or strains worldwide.

We support previously stated concerns (Paetow et al. 2013) that *M. anguillicaudatus* and its *Gyrodactylus* parasites may contribute to the global spread of chytrid fungi, as vectors via both legal and illegal trafficking routes.

Molecular phylogeography of *Gyrodactylus* (and *Misgurnus*) might well detect the routes of the insane trafficking of aquatic biota, and help to control it.

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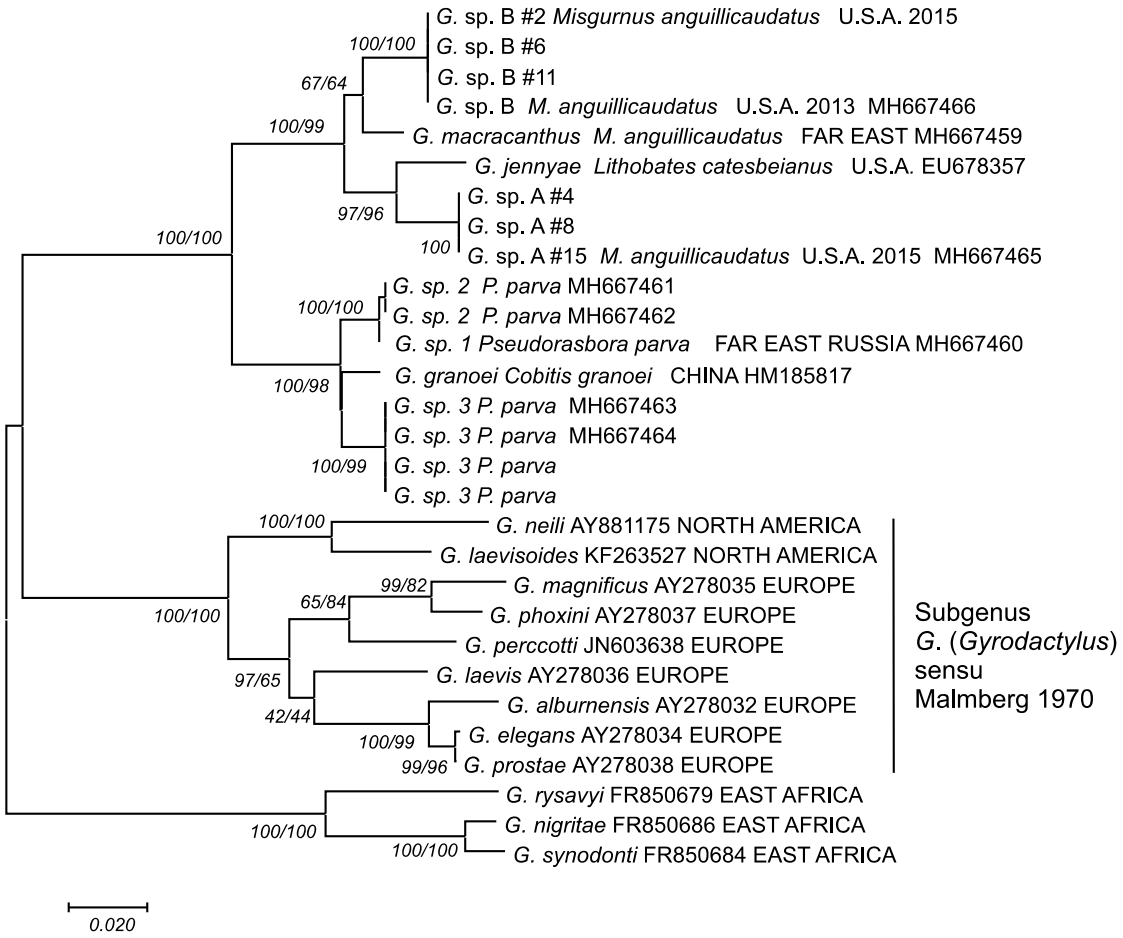
G. sp. B #2 Misgurnus anguillicaudatus U.S.A. 2015
G. sp. B #6
G. sp. B #11
G. sp. B M. anguillicaudatus U.S.A. 2013 MH667466
G. macracanthus M. anguillicaudatus FAR EAST MH667459
G. jennyae Lithobates catesbeianus U.S.A. EU678357
G. sp. A #4
G. sp. A #8
G. sp. A #15 M. anguillicaudatus U.S.A. 2015 MH667465
G. sp. 2 P. parva MH667461
G. sp. 2 P. parva MH667462
G. sp. 1 Pseudorasbora parva FAR EAST RUSSIA MH667460
G. granoei Cobitis granoei CHINA HM185817
G. sp. 3 P. parva MH667463
G. sp. 3 P. parva MH667464
G. sp. 3 P. parva
G. sp. 3 P. parva
G. neili AY881175 NORTH AMERICA
G. laevisoides KF263527 NORTH AMERICA
G. magnificus AY278035 EUROPE
G. phoxini AY278037 EUROPE
G. perccotti JN603638 EUROPE
G. laevis AY278036 EUROPE
G. alburnensis AY278032 EUROPE
G. elegans AY278034 EUROPE
G. prostae AY278038 EUROPE
G. rysavyi FR850679 EAST AFRICA
G. nigritae FR850686 EAST AFRICA
G. synodonti FR850684 EAST AFRICA
100/100
67/64
100/99
97/96
100
100/100
100/100
100/98
100/99
100/100
99/82
65/84
100/100
97/65
42/44
100/99
99/96
100/100
100/100
0.020
Subgenus
G. (Gyrodactylus)
sensu
Malmberg 1970

629

Figure. 1. The phylogenetic hypothesis of *G. jennyae* and the *Gyrodactylus* spp. on *Misgurnus anguillicaudatus* and *Pseudorasbora parva*, compared with subgenus *G.* (*Gyrodactylus)* sensu Malmberg (1970) and rooted with a sister clade of three species from East Africa (Přikrylová et al. 2012). Respective Bootstrap values (500 replicates) are indicated for both Neighbor-Joining and Maximum Likelihood. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 29 nucleotide sequences varying in length from 881-896 bp. All gaps were removed, leaving a total of 665 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

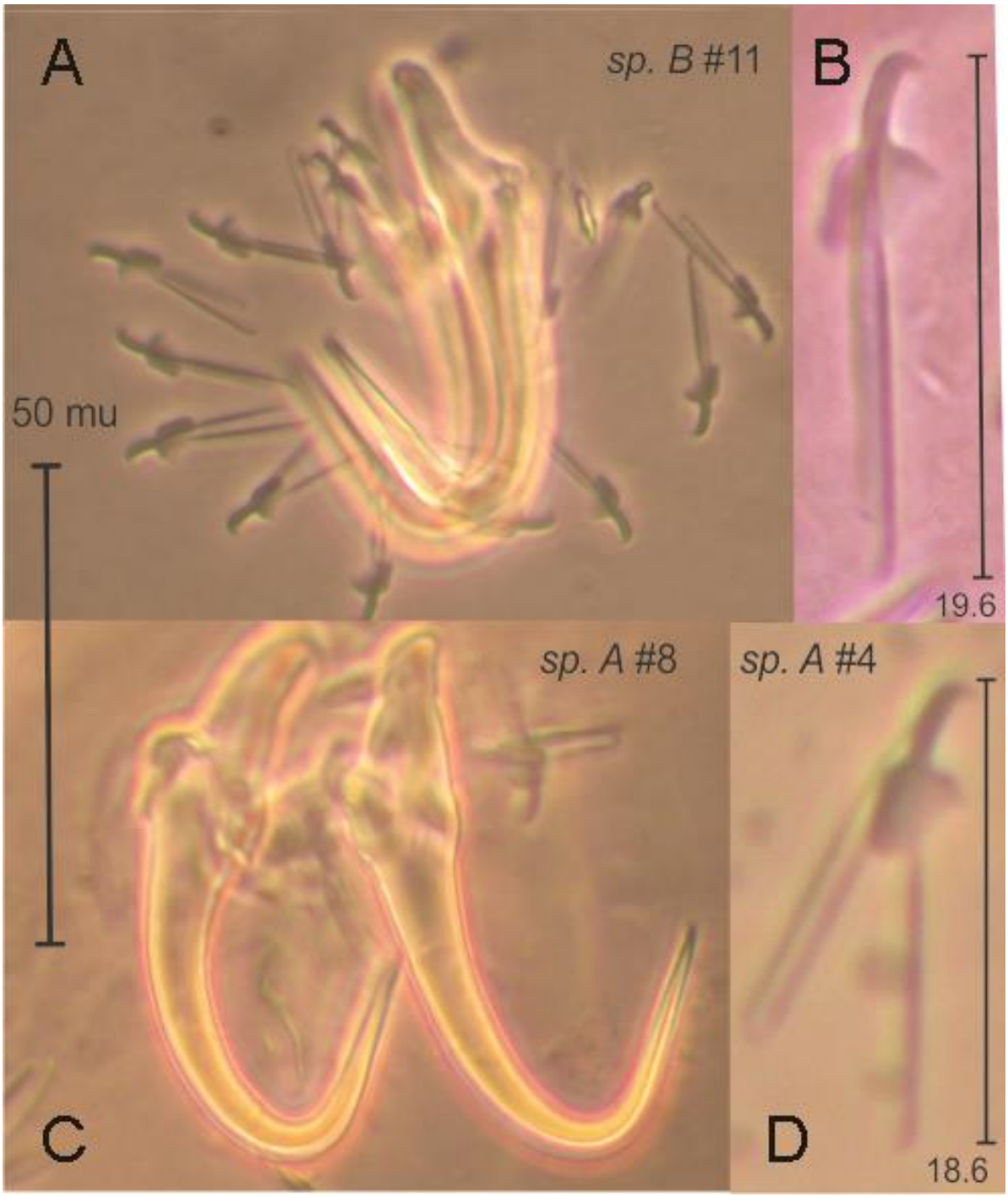

Figure. 2. Hamuli and marginal hooks of the *Gyrodactylus* sp. A (Figs. 2A,B; GenBank MH667465) and *G.* sp. B (Figs. 2C,D; GenBank MH667466) on *Misgurnus anguillicaudatus* in New York State, U.S.A. The hoof-like morphology of the base of marginal hooks is group-specific and is indistinguishable from *G. jennyae*, but separates the group clearly from the other frog parasites mentioned by Paetow et al. (2009).

Figure 1

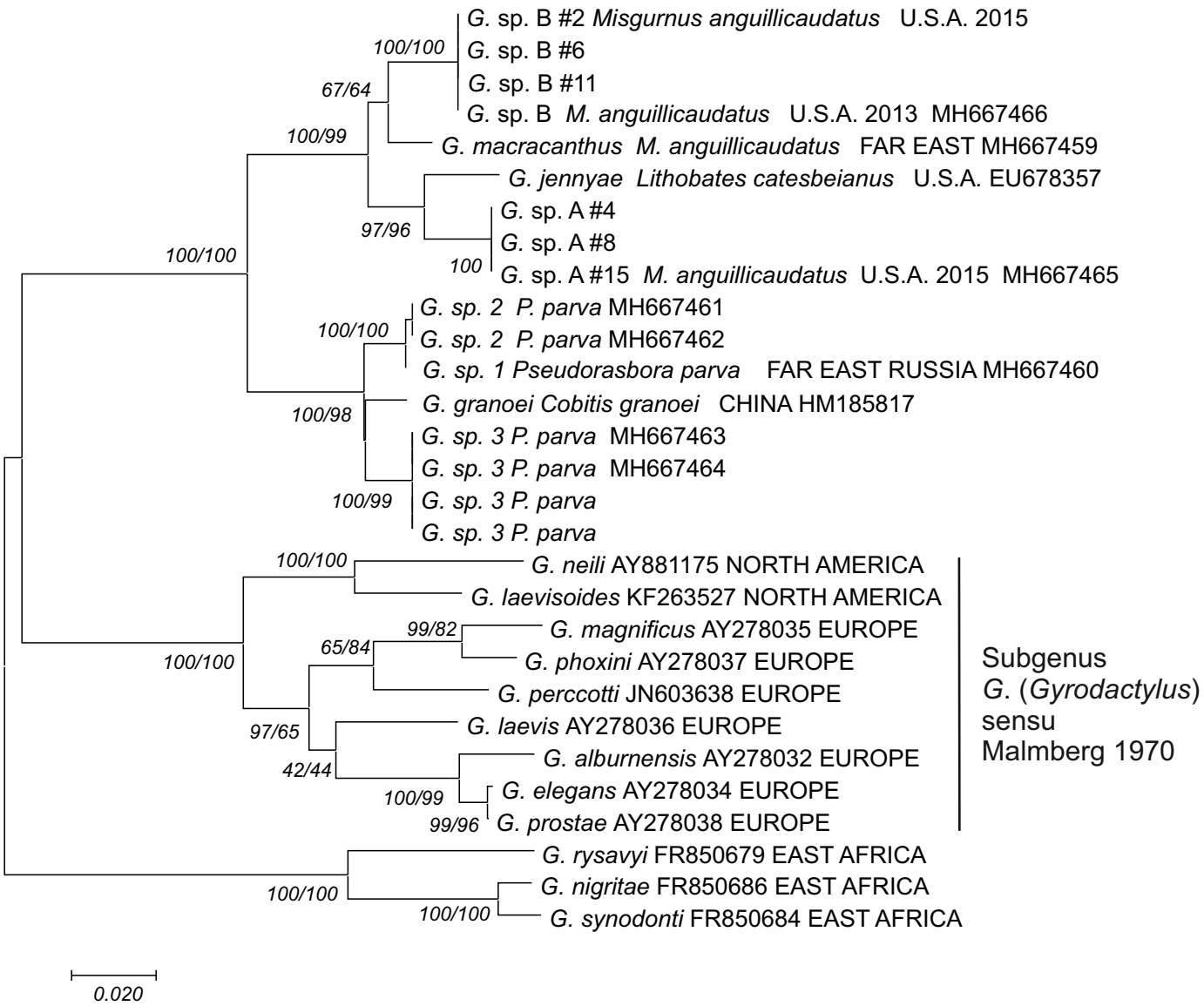

Figure 2

Click here to access/download;Figure;Fig 2.tif