CrossMark

Ancient and modern genome shuffling: Reticulate mito-nuclear phylogeny of four related allopatric species of *Gyrodactylus* von Nordmann, 1832 (Monogenea: Gyrodactylidae), ectoparasites on the Eurasian minnow *Phoxinus phoxinus* (L.) (Cyprinidae)

Jaakko Lumme · Marek S. Ziętara · Dar'ya Lebedeva

Abstract Phylogenetic analyses including four allopatric species of *Gyrodactylus* von Nordmann, 1832 on the Eurasian minnow *Phoxinus phoxinus* (L.) (Cyprinidae) revealed incongruence between the nuclear ITS1-5.8S-ITS2 and mitochondrial *cox*1 phylogenies due to ancient hybridisation. *Gyrodactylus pannonicus* Molnár, 1968 was sampled close to its type-locality, the upper reaches of River Tisza, tributary of Danube in the Black Sea Basin. Faunistic search detected three new related species with maximum composite likelihood distances in *cox*1 between 16.8–23.2% (tentatively 1.3 to 1.8 My of divergence). *Gyrodactylus albolacustris* n. sp. recorded in the White Sea Basin, eastern Baltic Basin and Mongolia was close to *G. pannonicus* in the nuclear ITS (divergence of 0.9%), but diverged in *cox*1 by 19.8%. The Mongolian isolate of *G. albolacustris* n. sp. diverged from the European isolates in *cox*1 by 8.9%, suggesting 0.7 My of isolation. The two other new species differed from *G. pannonicus* by >4% in ITS and some large indels in ITS1, and by >20% in *cox*1. *Gyrodactylus danastriae* n. sp. was found in River Strwiąż, a tributary of the River Dniester (Black Sea Basin) and was characterised by smaller size of anchors and by 29–41 bp dimorphic insertion in ITS1. *Gyrodactylus botnicus* n. sp. is considered endemic in the Baltic Basin, but was also found in the White Sea Basin as a postglacial immigrant, where it had hybridised with *G. albolacustris* n. sp. in spite of the high divergence in ITS (3.9%) and *cox*1 (22%). The discordant nuclear and mitochondrial phylogenies revealed an ancient mitochondrial introgression: *G. albolacustris* n. sp. was derived from a hybridisation combining proto-pannonicus ITS with proto-danastriae mitochondria, perhaps 1.3 My ago. The postglacial hybridisation of *G. albolacustris* n. sp. (as the donor of mtDNA^{alb} and ITS^{alb}) and *G. botnicus* n. sp. (donor of the ITS^{bot}) offered a model of shuffling of the genomic components: the process of the homogenisation and stabilisation of nuclear ITS (concerted evolution) and the lineage sorting has hardly begun.

J. Lumme
Department of Biology, University of Oulu, 90014 Oulu, Finland

M. S. Ziętara
Department of Molecular Evolution, University of Gdańsk, Wita Stwosza St., 59, 80-308 Gdańsk, Poland

D. Lebedeva (✉)
Institute of Biology of Karelian Research Center, Pushkinskaya St., 11, Petrozavodsk, Russian Federation 185910
e-mail: daryal78@gmail.com

Introduction

Among monogenean flatworms of the genus *Gyrodactylus* von Nordmann, 1832 (Gyrodactylidae van

Beneden & Hesse, 1864), the number of Linnaean species names is still well below half thousand, while the most conservative estimate of the number of species was 20,000 a few years ago (Bakke et al., 2002). From the taxonomist's point of view, the task of naming the remaining species seems hopeless, but checking of the existing names is equally demanding. Simply, there are not enough professional parasitologists for such a work, in any part of the world.

The use of molecular methods for solving taxonomic questions seems to be unavoidable. Most recently, Hahn et al. (2015) analysed two salmonid parasite species, i.e. *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel & Vigneuille, 1999 and *G. truttae* Gläser, 1974 utilising both ribosomal transcribed spacer ITS2 and mitochondrial *cox*1 sequences, covering widely the distribution area of both species on salmonid hosts. Wide sampling of *G. truttae* revealed very limited genetic variation, but *G. teuchis* was divided into five clades, mostly associated with the divergence of the main host *Salmo trutta* L. in the major drainage systems in southern and central Europe. The mitochondrial variation between the allopatric clades of *G. teuchis* was deeper (max. *c.*10%) than that found in *G. lucii* Kulakovskaya, 1952 (*c.*7%) or in *G. salaris* Malmberg, 1957 / *G. thymalli* Žitňan, 1960 (max. *c.*3%). An exception was the "alien" mitochondrial DNA in *G. salaris* found on rainbow trout farm strains in Macedonia and Poland, differing by *c.*19% (Ziętara et al., 2010). In all these species, the rDNA variation in ITS1 and ITS2 was very limited, supporting the inclusion of the deviant strains as geographical variants of the species.

The Eurasian minnow *Phoxinus phoxinus* (L.) (Cyprinidae) is hosting the most diverse fauna of *Gyrodactylus* described on any fish species in the Palaearctic region, emphasis on "described" (Harris et al., 2004; Bakke et al., 2007). The number of species of *Gyrodactylus* recorded on *P. phoxinus* in the latest enumeration was 17 (Harris et al., 2004) and as far as we know, there are no new species added to the list. Therefore, these species might be a suitable "assemblage" to study the ecology and evolution in a host-parasite system, without the complications caused by host switching (Ziętara & Lumme, 2002). The first step in such approach must be the α-taxonomy: the names of the species in the "assemblage".

The taxon or group of taxa in focus here was originally described on *P. phoxinus* as *Gyrodactylus wageneri aphyae* Malmberg, 1957, in the island Nämdö in Stockholm archipelago (59.18°N, 18.69°E, decimal degrees). Later, Malmberg (1964) raised *G. w. aphyae* to species rank, updating the convention of naming the "difficult" *wageneri* group members as subspecies. Further, he split the species and accepted only specimens from the freshwater localities Aneboda (Växsjö) and Jämtland into *G. aphyae*, and left several other samples nameless, including those in Nämdö (Malmberg, 1970). Ergens (1975) revisited the "*aphyae*-problem" and concluded that the complex contained an acceptable *G. aphyae* and another species, for which the name *G. pannonicus* Molnár, 1968 was available.

Ergens (1975) presented a large collection of morphometric data and drawings to study the problem of geographical and seasonal size variation in *G. aphyae*. His collection contained specimens from the Czech Republic, Slovakia, Hungary, Montenegro and Russia, including the Kola Peninsula. We shall analyse the "*aphyae-gasterostei* problem" later. From the point of view of the present study, it is a problem that the samples of *G. pannonicus* measured seasonally (figure 3 in Ergens, 1975), all came from the small brook Rokytka in Prague (roughly 50.09°N, 14.53°E), draining *via* Vltava (Moldau) and Labe (Elbe) to the North Sea, and thus are not from the watershed of the type-locality of *G. pannonicus*.

Our molecular survey of minnow parasites was conducted in four sea basins, the White and Baltic Sea basins in Fennoscandia; the Black Sea Basin in the Carpathian mountains in Poland and Slovakia; and the Arctic Ocean Basin at Lake Baikal in Mongolia. In addition to several other recognisable and described or new species of *Gyrodactylus*, this geographical survey revealed a cluster of four cryptic species closely resembling *G. pannonicus*, described by Molnár (1968) in River Tisza in Hungary. We recorded and tagged this species by molecular markers in Slovakia, in the watershed near the type-locality. Here we introduce three new sibling species: *G. botnicus* n. sp., *G. albolacustris* n. sp. and *G. danastriae* n. sp., on the basis of DNA characteristics, i.e. by barcoding by nuclear and mitochondrial DNA sequences. We also describe the morphology and standard measurements of the haptors of the species, but we want to warn that morphology cannot be used alone to distinguish these species: they are sibling and cryptic species in the strict meaning of the terms.

There are differences in the drawings, but ranges of variation have not been assessed yet.

However, the most interesting observation in this phylogenetic analysis was the mito-nuclear incongruence, as indication and evidence of the reticulate phylogenetic history of the four related taxa (e.g. Funk & Omland, 2003). The first hybridisation leading to an ancient mitochondrial introgression has occurred in the phylogeny of *G. albolacustris* n. sp. between >1 and 0.6 million years ago. The second analogous case is postglacial: *G. botnicus* n. sp. and *G. albolacustris* n. sp. have hybridised and the progeny was found as still segregating, offering a model demonstration of the genetic process leading to reticulate phylogeny.

Materials and methods

Fish and parasite samples

Eurasian minnows *Phoxinus phoxinus* (L.) were sampled by a hand net or electrofishing in different localities in waters in the Baltic, White Sea and Black Sea basins, and in Mongolia (Table 1; Supplementary Fig. S1). Fish were immediately euthanised and stored in 96% ethanol. Parasites were picked from the skin or fins of the fish later in laboratory, under a stereo microscope.

DNA extraction, amplification and sequencing of the mitochondrial cox1 gene

DNA was isolated by digesting single parasite specimens in 10 µl of lysis buffer (1× PCR buffer, 0.45% (v/v) Tween 20, 0.45% (v/v) NP 40 and 60 µg/ml proteinase K). Tubes 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 remaining impurities in the lysate did not interfere with the PCR process.

The analysis of mitochondrial DNA was conducted as described earlier (Meinilä et al., 2002; Ziętara et al., 2006; Huyse et al., 2007). The 20 µl PCR reaction consisted of 1× PCR buffer, 0.2 mM dNTPs, 2 mM MgCl₂, 1 µM of each primer, 0.5 U of Fermentas Taq DNA polymerase and 2 µl of DNA template. The gene *cox*1 was amplified either as single or as two overlapping fragments. The complete *cox*1 gene fragment was amplified using primers Trp2F (5′-TTT TAG ACG ATT TGT TTT CA-3′)

and Thr2R (5′-ATA GAT TGC TTG GTA TTA CA-3′). The 5′-end fragment of the shorter overlapping fragments was amplified with Trp2F and with either RCox7 (5′-GTA GGT ACA GCG ATT ATC AT-3′; *G. pannonicus*, *G. albolacustris* n. sp. or *G. danastriae* n. sp.) or RCox9 (5′-GTA GGT ACT GCA ATA ATC AT-3′; *G. botnicus* n. sp.) primers. The 3′-end was amplified with FCox7 (5′-TTT TCA ATA GGT ATG GAC GT-3′) and Thr2R. The PCR program was run as follows: 94°C for 3 min, 38 cycles (94°C for 30 s, 48°C for 1 min and 72°C for 1 min 50 s), 72°C for 7 min, and final cooling to 4°C. The same primers were used for sequencing.

Amplification and sequencing of the ITS of species and hybrids

The entire ITS region of the ribosomal DNA array (spanning ITS1-5.8S-ITS2 and flanking terminal fragments of the 18S and 28S rRNA genes) was amplified with primers ITS1F (5′-GTT TCC GTA GGT GAA CCT-3′) (Rokicka et al., 2007) and ITS2R (5′-GGT AAT CAC GCT TGA ATC-3′) (Ziętara et al., 2000). The PCR reaction contained 2 µl of lysate, 1× PCR buffer, 2 mM MgCl₂, 1 µM of each primer, 200 µM of each dNTP and 0.4 unit of *Taq* polymerase (Fermentas, Vilnius, Lithuania) in a final volume of 20 µl. Amplification mixtures were heated for 3 min at 95°C, then subjected to 37 cycles (94°C, 48°C and 72°C for 1 min each), heated for 7 min at 72°C and cooled down to 4°C. The amplified fragments were purified from the agarose gel and sequenced directly with two additional primers ITS1R (5′-ATT TGC GTT CGA GAG ACC G-3′) and ITS2F (5′-TGG TGG ATC ACT CGG CTC A-3′) as described earlier (Ziętara & Lumme, 2003). The forward allele-specific primers for amplifying the two separate alleles from the heterozygous *G. albolacustris* n. sp. × *G. botnicus* n. sp. hybrid worms were ITS1tF (5′-GTG TTG TCA TTG CCT AAA AT-3′) for ITS[alb] and ITS1aF (5′-GTG TTG TCA TTG CCT AAA AA-3′) for ITS[bot] . They were designed to start as near of the 5′-end of the sequence as possible.

Data analysis

The phylogenetic trees and genetic distance estimates were based on the complete *cox*1 (1,545 bp) gene and on specified fragments of the ITS1-5.8S rDNA-ITS2. In the latter, the hypervariable segment of ITS1 (Supplementary Figs. S2 and S3) was removed from

Table 1 Sampling sites and GenBank accession numbers of the parasites, all on *Phoxinus phoxinus* (L.)

	Locality, date*	n	ITS rDNA	mtDNA *cox*1
***Gyrodactylus pannonicus* Molnár, 1968 (Tisza, Danube)**				
ITS genotype (gnt)1–cox1 haplotype (hp)2	1 Black Sea Basin	1	HM192915	HM488962
ITS gnt2–cox1 hp3	1	1	HM192916	HM488963
ITS gnt1/2–cox1 hp1	1	1	HM192917	HM488961
ITS gnt1–cox1 hp1	2 Black Sea Basin	1	HM192918	HM488964
ITS gnt2–cox1 hp4	2	1	HM192919	HM488965
ITS gnt2–cox1 hp5	2	1	HM192919	HM488966
ITS gnt2–cox1 hp8	2	1	HM192919	HM488969
ITS gnt2–cox1 hp9	2	1	HM192919	HM488970
ITS gnt3–cox1 hp8	2	1	HM192920	HM488969
ITS gnt4–cox1 hp6 (n = 5)	2	5	HM192921	HM488967
ITS gnt4–cox1 hp7	2	1	HM192921	HM488968
***Gyrodactylus danastriae* n. sp. (Black Sea Basin, Dniester)**				
ITS missing –cox1 hp1	3 Black Sea Basin	1	–	HM488971
ITS T-minus–cox1 hp2	3	2	HM192925	HM488972
ITS T-plus–cox1 hp2	3		HM192926	HM488972
ITS del-minus–cox1 hp3	3	1	HM192927	HM488973
ITS del-plus–cox1 hp3	3		HM192928	HM488973
ITS del-minus–cox1 hp4	3	1	HM192927	HM488974
ITS del-plus–cox1 hp4	3		HM192928	HM488974
***Gyrodactylus botnicus* n. sp.**				
ITS bot/bot	4 Baltic Basin	1	AF484542	–
ITS bot/bot	5 Baltic Basin	1	EU727178	–
ITS bot/bot	6 Baltic Basin	1	EU727177	–
ITS bot/bot–cox1 bot1	7 Baltic Basin	1	EU554413	EU554422
ITS bot/bot–cox1 bot3	7	4	EU554413	HM488975
ITS bot/bot–cox1 bot4	7	2	EU554413	HM488976
ITS bot/bot–cox1 bot5	7	1	EU554413	HM488977
ITS bot/bot–cox1 bot6	7	1	EU554413	HM488978
ITS bot/bot–cox1 bot7	7	1	EU554413	HM488979
ITS bot/bot–cox1 bot8	7	1	EU554413	HM488990
ITS bot/bot–cox1 bot2	8 White Sea Basin	3	EU554416	EU554421
***Gyrodactylus albolacustris* n. sp.**				
ITS alb/alb–cox1 alb1	9 Baltic Basin	3	EU554412	EU554409
ITS alb/alb–cox1 alb2	9	1	EU554412	EU554410
ITS alb/alb–cox1 alb3	9	1	EU554412	EU554411
ITS alb/alb Alburnoides bipunctatus	9	1	KU365754	
ITS alb/alb–cox1 alb6	10 White Sea Basin	4	HM192924	HM488981
ITS alb/alb–cox1 alb7	10	2	HM192924	HM488982
ITS alb/alb–cox1 alb8	10	1	HM192924	HM488983
ITS alb/alb–cox1 alb9	10	1	HM192924	HM488984
ITS alb/alb–cox1 alb10	10	1	HM192924	HM488985
ITS alb/alb	11 Baltic Basin	1	HM192922	–
ITS alb/alb	11	1	HM192923	–
ITS alb/alb–cox1 albM	12 Lake Baikal Basin	1	KU365755	KU365756

Table 1 continued

	Locality, date*	n	ITS rDNA	mtDNA *cox*1
***Gyrodactylus albolacustris* × *Gyrodactylus botnicus* hybrid population**				
*ITS bot/bot–cox*1 *alb5*	13a White Sea Basin	3 + 3	EU554414 (*bot*)	EU554407
		1	EU554415 (*bot*)	
*ITS alb/bot–cox*1 *alb4*	13a	5 + 1	EU678640 (*alb*)	EU554408
			EU678641 (*bot*)	
*ITS alb/bot–cox*1 *alb5*	13b	1 + 2	EU678640 (*alb*)	EU554407
			EU678641 (*bot*)	
*ITS bot/bot/alb⁺–cox*1 *alb5*	13b	1	–	EU540407
*ITS alb/alb/bot⁺–cox*1 *alb5*		7	–	EU540407
*ITS bot/bot–cox*1 *alb5*	13c	1	EU554417 (*bot*)	EU554419
*ITS alb/bot–cox*1 *alb4*	13c	2	EU678642 (*alb*)	EU554418
			EU678643 (*bot*)	
*ITS alb/bot–cox*1 *alb5*	8	1	EU678644 (*alb*)	EU540420
			EU678645 (*bot*)	

*Localities and dates of sampling: 1, River Topl'a near Rokytov, Slovakia (30.vii.2007; 49.3133N, 21.1932E); 2, River Ondava near Nižný Mirošov, Slovakia (30.vii.2007; 49.3515N, 21.4635E); 3, River Strwiąż near Brzegi Dolne, Poland (31.vii.2007; 49.4544N, 22.6393E); 4, Gulf of Bothnia, Oulu, Finland (6.vii.2000; 65.0333N, 25.4117E); 5, Gulf of Bothnia, Hailuoto, Finland (30.viii.2000; 65.0391N, 24.5556E); 6, River Kiiminkijoki, Oulu, Finland (21.viii.2000; 65.1488N, 25.6629E); 7, Gulf of Bothnia, Varjakka, Finland (11.ix.2005; 64.9213N, 25.1014E); 8, River Oulankajoki, Pikkuköngäs, Finland (31.viii.2005; 66.3695N, 29.3352E); 9, River Vidlitsa, Lake Ladoga, Russia (15.vii.2006; 61.1966N, 32.4213E); 10, River Strel'na, Kola Pensinsula, Russia (4.viii.2008; 66.0967N, 38.5248E); 11, River Bol'shaya Uya, Onego Lake, Russia (21.vii.2005; 61.6028N, 34.6603E); 12, River Tuul, Ulaanbaatar, Mongolia (28.viii.2012; 47.8863N, 106.9336E); 13a, Hangaspuro, Oulanka, Finland (20.vi.2007; 66.3406N, 29.3297E); 13b, Hangaspuro, Oulanka, Finland (28.vii.2008); 13c, Merenoja, Oulanka, Finland (29.viii.2005; 66.3507N, 29.3493E)

⁺ Specimens showing low proportion of "wrong" ITS

the distance calculations which therefore represent minimum values. Sequence alignments were made by ClustalW or Muscle implemented in MEGA6 (Tamura et al., 2013), leading to the same result. The phylogenetic trees were constructed by the Neighbor Joining method based on Kimura's two parameter distance (K2P for ITS), as implemented in the MEGA6 program package. Distances for the *cox*1 gene were calculated by the maximum composite likelihood method, correcting the large distances (reaching saturation) upwards. The divergence time estimates were based on the 13% divergence per million years, estimated for *G. salaris* (see Kuusela et al., 2007). We may give the timing also for the 5.1% suggested by Hahn et al. (2015), but all the calibrations are quite weak (Supplementary Fig. S4).

Morphological descriptions

Worms investigated morphologically were cut in two parts. The opisthaptor was prepared as a microscopy slide in ammonium picrate-glycerin (Malmberg, 1970),

and the rest of the body was used for molecular analysis. Isolated haptors were partially digested by Proteinase K in a final concentration of 60 μg/ml prior to their preservation in ammonium picrate-glycerin. Measurements of haptoral hard parts were taken with a microscope and digital camera (Nikon Optiphot - 2) and estimated by the interactive measuring system IMT iSolution Lite (Ver.7.4, IMT iSolution Inc.). The holotype of each species was drawn and several paratypes measured; all measurements are in micrometres.

Systematic analysis of species resembling *G. pannonicus* by ribosomal ITS and mtDNA

The minnow parasites were first placed into phylogenetic-systematic context by constructing a phylogenetic hypothesis for *wageneri* group of the subgenus *Gyrodactylus* (*Limnonephrotus*) based on 495 nucleotides (nt) of ITS2 alignment (Supplementary Fig. S5). The clade of the "*pannonicus* cluster" was monophyletic (93% bootstrap support). Within
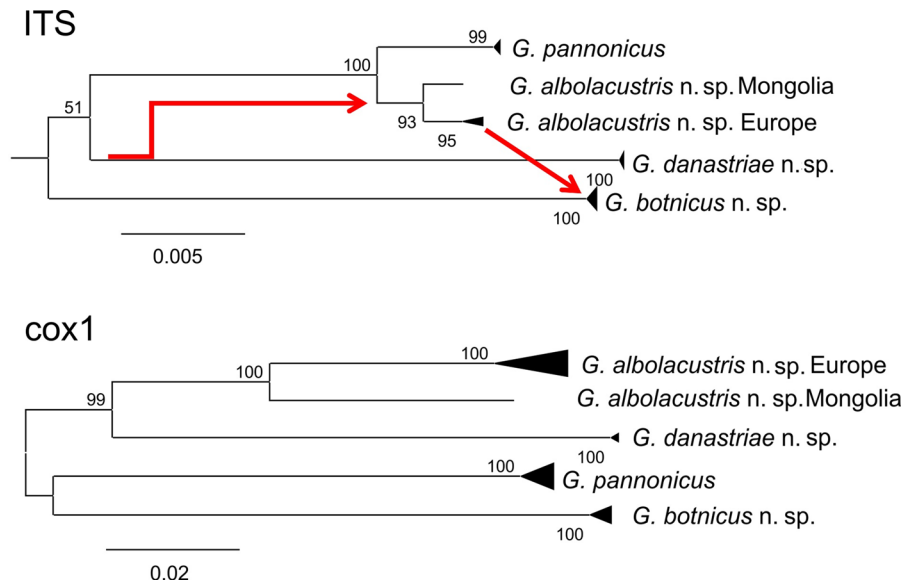
Fig. 1 Compressed phylogenetic hypotheses based on ITS and *cox*1 sequences. The elbow arrow shows the positions of the mitochondrial introgression from pro-danastriae to *G. pannonicus* Molnar, 1968, leading to the *G. albolacustris* n. sp. The straight arrow shows the modern hybridisation between *G. botnicus* n. sp. and *G. albolacustris* n. sp.

the *pannonicus* cluster, the analysed phylogenetic tree was based on 814 nt positions in the ITS1-5.8S-ITS2 alignment using pairwise deletion option (Fig. 1; Supplementary Fig. S6). The species discussed here formed a monophyletic cluster within the *wageneri* species group, with three deeply separated main clades (99–100% bootstrap support), and the third clade further split into two (91–99% support).

In the *pannonicus* cluster, most of the interspecific variation of ITS1 was in a segment closed with the conservative stem motif 5′-AAAAGUA … UACUUUU-3′ (Supplementary Figs. S2 and S3). The length of this segment was 226 nt in *G. albolacustris* n. sp. and *G. pannonicus*, 227 nt in *G. botnicus* n. sp. and much longer, 268 or 256 nt in *G. danastriae* n. sp. (Supplementary Fig. S2). While the three first named species had the ITS1 of equal length, the content was changed. The Mfold-predicted (Zuker, 2003) A, B and C types for the secondary structure of the hypervariable segment. A hairpin stem structure depicted in Supplementary Figs. S2 and S3 was the only structurally conserved motif within this hypervariable segment. Transforming this kind of qualitative and taxonomically definitive insertion/deletion variation into distance estimates is difficult and the mechanisms leading to indels remain unexplained.

As a complementation of the ITS analysis, we produced a mitochondrial phylogenetic hypothesis of the subgenus (Supplementary Fig. S7) and the species group (Fig. 1, Supplementary Fig. S6). The trees were based on an alignment of the complete *cox*1 gene sequence (1,545 nt, 515 amino acids. The "*pannonicus* cluster" appeared as a perfectly monophyletic clade (92% bootstrap support) and separated for four nearly equidistant clades (Supplementary Fig. S7).

Ancient genome shuffling *via* hybridisation

The incongruence of the ITS and *cox*1 trees was serious, with respect of the topology and distances as well (Fig. 1 and Supplementary Fig. S6, Table 2, graphical illustration in Supplementary Fig. S8). This can be explained by an ancient mitochondrial introgression *via* hybridisation (kinked arrow in Fig. 1) of a member of the ancestral strain from the Dniester Basin (to be *G. danastriae* n. sp.) with a member of the lineage from the Danube Basin (leading to *G. pannonicus*). This sidekick hybridisation event was the origin of the lineage leading to *G. albolacustris* n. sp., which has the ITS quite similar to *G. pannonicus* (0.8–0.9%), but the *cox*1 gene more related to *G. danastriae* n. sp. (16.8%) than to *G. pannonicus* (19.8%). This introgression has occurred prior to the divergence of the Mongolian and north-east European

Table 2 ITS distances (Kimura 2-parameter model, above diagonal) and *cox*1 distances (Maximum Composite Likelihood method, MCL, below diagonal) for the species studied

		1	2	3	4	5
1	*G. albolacustris* n. sp.	–	0.004	0.0393	0.0381	0.0089
2	*G. albolacustris* n. sp. (Mongolia)	0.089	–	0.0385	0.0372	0.0081
3	*G. botnicus* n. sp.	0.218	0.219	–	0.0486	0.0419
4	*G. danastriae* n. sp.	0.168	0.172	0.232	–	0.0389
5	*G. pannonicus* Molnár, 1968	0.198	0.181	0.202	0.219	–

lineages of *G. albolacustris* n. sp. which differ by 8.9% in the mtDNA.

Contemporary genome shuffling: *G. albolacustris* n. sp. × *G. botnicus* n. sp. hybrid population

The postglacial faunal transfer from the Baltic Basin to the White Sea Basin occurred in the modern Iijoki tributary system in Finland. Lake Kitka was originally connected to the Baltic Basin in the Ancylus lake phase (Heikkinen & Kurimo, 1977; Koutaniemi, 1999). The aquatic fauna, e.g. brown trout (Huusko et al., 1990) or *Gyrodactylus salaris* on grayling (Meinilä et al., 2004) arrived to the lake from the Baltic. About 8,400 years ago, the isostatic land uplift tilted Lake Kitka to drain east *via* River Koutajoki system to the White Sea (Heikkinen & Kurimo, 1977; Koutaniemi, 1999). Since then, the faunal components of the primordial Lake Kitka had a free access downstream. Upstream dispersal was prevented by a nine meter waterfall called Jyrävä. How far and how rapidly the eastward dispersal occurred has not been yet studied.

Gyrodactylus botnicus n. sp. had evolved in isolation, until the latest postglacial period, when it succeeded to cross the line less than 8,400 years ago and mate with *G. albolacustris* n. sp. in the White Sea Basin (straight arrow in Fig. 1). The hybrid population (heterozygous ITS) was found in the creek Merenoja in 2001 (66.3507N, 29.3493E, 209 metres above sea level, masl). Next samplings were conducted half a km upstream, in a creek called Hangaspuro.

In Hangaspuro (66.3406N, 29.3297E, 227 masl) in years 2007 and 2008, homozygous and heterozygous combinations of ITS specific for *G. albolacustris* n. sp. and *G. botnicus* n. sp. were found in the sample analysed by rapid PCR-RFLP: seven specimens of

ITS$^{alb/alb}$, nine ITS$^{alb/bot}$ and eight ITS$^{bot/bot}$. All these specimens carried the mitochondrial DNA of *G. albolacustris* n. sp., also those with homozygous-looking *G. botnicus* n. sp. ITS$^{bot/bot}$. Proper *G. botnicus* n. sp. (including the proper *cox*1) was also observed in the River Oulanka, downstream of the creek.

The two allelic forms of ITS representing *G. albolacustris* n. sp. and *G. botnicus* n. sp. in the heterozygous specimens were amplified and sequenced by utilising allele-specific primers. The ITS1 sequences in the Supplementary Fig. S2 clearly show that direct sequencing of the hybrids should produce unreadable mess (as it was first observed in 2001 by us). The results suggest segregation after the primary hybridisation, in addition to the fact that the *cox*1 gene in Hangaspuro parasites was always from *G. albolacustris* n. sp.

The genotypes detected as homozygous by the PCR-RFLP were sequenced directly using universal ITS primers. Seven specimens of apparent ITS$^{alb/alb}$ homozygotes on the basis of PCR-RFLP were supposed to represent the maternal parent of the hybrids. However, they always showed low intensity ambiguous nucleotide characteristics to the ITS^{bot} allele, when sequenced by using the general *Gyrodactylus* primers amplifying both alleles. The mean ratio of high/low peak heights in the ABI phenograms was 1: 0.27 (*G. albolacustris* n. sp.: *G. botnicus* n. sp. allele). Because the sequences were made from individuals, the aberration from 1:1 ratio indicates recombination within the repeated rDNA cassette. The recombination is modelled in Supplementary Fig. S9.

In contrast, the spontaneous ITS$^{bot/bot}$ homozygotes showed no 'contaminant' ambiguities except in a single specimen, which showed a similar variation

as described above. However, these ITS$^{bot/bot}$ homozygotes carried the *G. albolacustris* n. sp. *cox*1 gene and thus necessarily were recovered from hybrid heterozygotes either *via* mating of F$_1$ × F$_1$ or, perhaps *via* backcrossing F$_1$ × *G. botnicus* n. sp. male. Pure *G. botnicus* n. sp. was not found just in this creek, but it was observed downstream in the Oulanka River (66.3695N, 29.3352E, 155 masl).

The GenBank accession numbers of the ITS (n = 22) and *cox*1 (n = 22) sequenced from the various hybrid combinations are given in Table 1.

Family Gyrodactylidae van Beneden & Hesse, 1864
Genus *Gyrodactylus* von Nordmann, 1832
Subgenus *Gyrodactylus* (*Limnonephrotus*) Malmberg, 1970
Species group *wageneri sensu* Ziętara & Lumme (2002)

Gyrodactylus albolacustris n. sp.

Type-host: *Phoxinus phoxinus* (L.) (Cypriniformes: Cyprinidae), Eurasian minnow.
Other host: *Alburnoides bipunctatus* (Bloch) (Cypriniformes: Cyprinidae), Schneider.
Type-locality: River Vidlitsa draining to Lake Ladoga, Baltic Basin, Russia (61.1966N, 32.4213E).
Other localities: *P. phoxinus*: River Bol'shaya Uya draining to Lake Onega, Russia (61.6028N, 34.6603E); River Strel'na, south coast of Kola Peninsula, White Sea Basin, Russia (66.0967N, 38.5248E); Kuusamo, White Sea Basin, Finland, (66.3406N, 29.3297E), only ITS and *cox*1 DNA found in hybrids of *G. albolacustris* n. sp. with *G. botnicus* n. sp.; River Tuul near Ulaanbaatar, Mongolia (47.8863N, 106.9336E), in the Lake Baikal watershed, draining to the Arctic Ocean *via* River Yenisey. *A. bipunctatus*: River Vidlitsa draining to Lake Ladoga, Baltic Sea Basin, Russia (61.1966N, 32.4213E).
Site on host: Skin and fins.
Type-material: Type-specimens (the holotype and four paratypes): slides of the haptors are deposited in the Finnish National History Museum in Helsinki University, under accession numbers MZH118057 (holotype) and MZH118058–MZH118061 (paratypes) (all collected on 15.vi.2006). The Mongolian specimens are deposited as MZH118085–MZH 118086 (paratypes) (collected on 28.viii.2012).

Representative DNA sequences: GenBank accession numbers for ITS (n = 18) and *cox*1 (n = 16) sequences are given in Table 1.
Etymology: The name *albolacustris* refers to the mythic region of *Lacvs Albvs* in the *Carta Marina* (1539) of Olavus Magnus, a Swedish cleric and historian. Because the eastern regions of the map were mainly based on stories of fur traders, it is not clear whether the name refers to the Lake Onega or to the White Sea, and therefore, the name is suitable to a parasite living in both basins.

Description (Figs. 2.1, 2.2, 2.6)

[Based on 7 specimens: 5 European (from the type-locality, River Vidlitsa) and 2 Mongolian specimens, see Table 3 for metrical data.] Haptor ovate. Anchor 39–52 long with fold; anchor root 13–19 long, tends to bend ventrally; anchor shaft pronounced, 33–40 long. Ventral bar 4–9 × 18–26, with short processes and medium triangular membrane, 9–10 long. Dorsal bar delicate, 1–4 long, 14–16 wide. Marginal hook 25–30 long, with robust marginal hook sickle, 4–6 long; marginal hook shaft leaning; marginal hook point extending toe and pointing backwards; filament loop long extending almost to middle of handle; transition of shaft to point not distinct.

DNA characteristics

The length of the nuclear ribosomal ITS1-5.8S-ITS2 is 1,218 nt (EU554412, KU365754, HM192922–HM19 2924). The Mongolian sequence is one nt shorter (KU365755), due to a variation G5 or G4 in sites 236–240 within the hypervariable segment of ITS1 (Supplementary Fig. S2). The hypervariable segment is 226 nt long. Its predicted secondary RNA structure is with three clear hairpins and few unpaired segments (Supplementary Fig. S3). The sequences deviate from those of *G. pannonicus* by ten single nt changes (0.81%). There are no long indels observed. Mitochondrial *cox*1 sequences (EU554409–EU554411, HM488981–HM488985) are deeply divergent from the other species in the *pannonicus* cluster (Fig. 1, Supplementary Figs. S6, S7, S8). Three amino acid substitutions between local isolates are found (Val > Ile at sites 419 and 460, Leu > Phe 459; Supplementary Fig. S10). The Mongolian *cox*1 sequence (KU365756) differs from the European sequences by 8.9% (Supplementary Fig. S8).
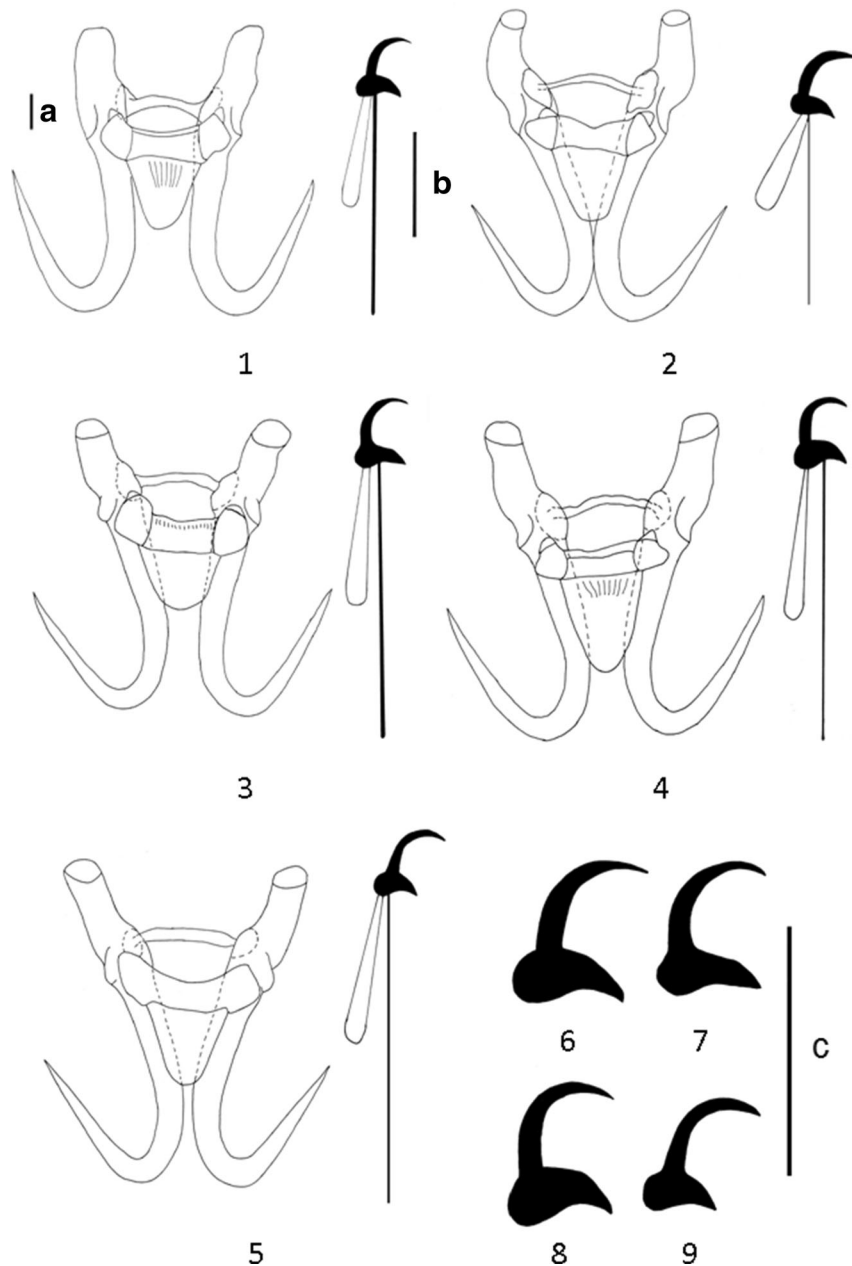
Fig. 2 Morphology of haptoral parts of *Gyrodactylus* spp. 1, *G. albolacustris* n. sp. from Europe (River Vidlitsa, holotype); 2, *G. albolacustris* n. sp. from Mongolia (River Tuul, paratype); 3, *G. botnicus* n. sp. from the Baltic Basin (brackish water, Varjakka, holotype); 4, *G. danastriae* n. sp. from south-eastern Poland (River Strwiąż, holotype); 5, *G. pannonicus* from Slovakia (River Ondava); 6–9, Marginal hook sickles: 6, *G. albolacustris* n. sp.; 7, *G. botnicus* n. sp.; 8, *G. danastriae* n. sp.; 9, *G. pannonicus*. *Scalebars*: a (anchors), 10 µm; b (marginal hooks), 10 µm; c (marginal hook sickles), 10 µm

Remarks

The general haptor morphology of *G. albolacustris* n. sp. is in accordance with *wageneri* group *sensu* Malmberg ([1970](#)) as follows: folded anchors; ventral bar with short processes; marginal hook shape typical for *wageneri* group, with rounded heel and pointed toe; sickle point extends sickle toe which

Table 3 Measurements (range in μm) of haptoral hard parts of *Gyrodactylus* spp. from *Phoxinus phoxinus*. The relative length of each part (in % in parentheses) was calculated as percentage of the total length of anchor/marginal hook, using the maximum values

Species	*G. pannonicus* Molnár, 1968			*G. danastriae* n. sp.	*G. albolacustris* n. sp.		*G. botnicus* n. sp.	*G. aphyae* Malmberg, 1970		
Reference	Molnár (1968)	Ergens (1975)	Present study (River Topl'a, Ondava)	Present study (River Strwiąż)	Present study (River Vidlitsa)	Present study (River Tuul)	Present study (Oulu)	Ergens (1975)	Present study (River Topl'a)	Present study (River Tuul)
	(n = 23)	(n = 90)	(n = 7)	(n = 5)	(n = 5)	(n = 2)	(n = 5)	(n = 120)	(n = 7)	(n = 7)
Anchor										
Total length	45–47	45–54	45–49	45–48	39–52	47–48	49–57	51–69	53–60	54–63
Point length	32–34 (72%)	33–38 (70%)	22–25 (51%)	23–26 (54%)	23–35 (67%)	25–27 (56%)	36–43 (75%)	37–51 (74%)	25–31 (52%)	27–32 (51%)
Shaft length	24–26 (55%)	23–27 (50%)	33–36 (73%)	33–35 (73%)	33–40 (77%)	35–35 (73%)	24–28 (49%)	25–34 (49%)	41–45 (75%)	44–47 (75%)
Root length	12–16 (34%)	13–18 (33%)	12–17 (35%)	12–15 (31%)	13–19 (37%)	13–14 (29%)	12–17 (39%)	14–22 (32%)	15–18 (30%)	13–20 (32%)
Ventral bar										
Width	21–22	13–18	15–20	17–19	18–26	18–19	18–23	22–32	21–25	22–28
Length	5–7	5–6	4–5	5–7	4–9	4–7	4–8	5–9	5–10	5–9
Membrane length	8–10	9–13	9–11	10–12	9–10	9–10	8–9	12–18	12–18	12–20
Dorsal bar										
Width	19–20	13–20	12–17	12–16	14–16	14–14	13–14	16–24	18–27	16–28
Length	1–2	1–2	1–2	1–2	1–4	2–2	1–2	1–3	2–3	2–3
Marginal hook										
Total length	25–27 (57%)	25–29 (54%)	24–29 (59%)	24–25 (52%)	25–30 (58%)	27–30 (63%)	21–30 (53%)	28–41 (59%)	29–31 (52%)	31–34 (54%)
Sickle length	3–5	5–6	5–6	4–6	4–6	4–5	4–5	5–6	5–6	5–6

makes distal part of sickle wider than proximal part. Further, sickle point is not pointing more upwards like in *G. gracilihamatus* Malmberg, 1964, the outgroup (see figure 1A in Ziętara & Lumme, 2002 and figure 2 in Ziętara & Lumme, 2004) of *wageneri* group *sensu* Ziętara & Lumme (2002). *Gyrodactylus albolacustris* n. sp. possesses 5.8S rDNA identical with 5.8S rDNA of *G. aphyae* Malmberg, 1970 (GenBank AF484527). Sequence of 5.8S rDNA is the main DNA character of *wageneri* group *sensu* Ziętara & Lumme (2002).

The size range of *G. albolacustris* n. sp. is wider than in the other species. Specimens from Mongolia were smaller than those collected in Europe. Still, the size of *G. albolacustris* n. sp. hardly overlaps with the smallest values for the range of *G. aphyae* (Table 3). The relative point length in *G. albolacustris* n. sp. is 67% which is clearly more than in *G. danastriae* n. sp. (54%) and *G. pannonicus* (51%), as well as in *G. aphyae* samples measured by us from Topl'a, Slovakia (52%) and Tuul, Mongolia (51%), but the values given by Ergens (1975) indicate relatively longer anchor point (74%) in of *G. aphyae*. The marginal hook sickle of *G. albolacustris* n. sp. is the most prominent among the three new species, but less robust than that in *G. aphyae,* and differs from the latter by leaning shaft and then less prominent transition from the shaft to the point. The only constant difference from *G. aphyae* is the tendency of bending the roots of anchors ventrally.

The two specimens from Mongolia did not differ in shape from those collected in Europe, their dimensions fall within the lower range specified in Table 3. All the measurements overlap with the other three species in the *pannonicus* cluster, so they are of limited use to distinguish the species.

Many reports of *G. pannonicus* in north-eastern Europe may actually concern *G. albolacustris* n. sp., at least within the postglacially colonised triangle Ladoga - Kola Peninsula - Mongolia.

Gyrodactylus albolacustris n. sp. was found on *P. phoxinus* as sympatric (often also on the same host specimens) with *G. botnicus* n. sp., *G. macronychus* Malmberg, 1957, *G. aphyae*, *G. jussii* Ziętara & Lumme, 2003, *G. phoxini* Malmberg, 1957, *G. magnificus* Malmberg, 1957 and *G. laevis* Malmberg, 1957 (unpublished, molecularly confirmed data of the authors, Supplementary Fig. S11).

Gyrodactylus botnicus n. sp.

Type-host: *Phoxinus phoxinus* (L.) (Cypriniformes: Cyprinidae), Eurasian minnow.
Type-locality: Varjakka, Finland, brackish water (64.9213N, 25.1014E).
Other localities: *P. phoxinus*: Oulu, Finland, brackish water Baltic Basin (65.0333N, 25.4117E); Marjaniemi in Hailuoto, Finland, brackish water Baltic Basin (65.0333N, 25.4117E); Kiiminkijoki, Oulu, Finland, freshwater Baltic Basin (65.1488N 25.6629E); River Oulanka, Kuusamo, Finland, freshwater White Sea Basin (66.3695N, 29.3352E).
Site on host: Skin and fins.
Type-material: Type-specimens (the holotype and four paratypes): slides of the haptors are deposited in the Finnish National History Museum in Helsinki University, under accession numbers MZH118063 (holotype) and MZH118064–MZH118067 (paratypes) (all collected on 11.ix.2005).
Representative DNA sequences: GenBank accession numbers for ITS (n = 17) and *cox*1 (n = 14) sequences are given in Table 1.
Etymology: The name *botnicus* is derived from the Latin name *Mare Botnicvm*, referring to the Bothnian Bay of the Baltic Sea between Sweden and Finland in the old map of Olavus Magnus.

Description (Figs. 2.3, 2.7)

[Based on 5 specimens from the type-locality, Varjakka, Finland, brackish water, see Table 3 for metrical data.] Haptor ovate. Anchor 49–57 long with fold; anchor root 12–17 long, tends to bend ventrally; anchor shaft shorter, 24–28 long. Ventral bar 4–8 × 18–23, with short processes and medium triangular membrane less pointed, 8–9 long. Dorsal bar delicate, 1–2 long, 13–14 wide. Marginal hook 21–30 long, with prominent marginal hook sickle, 4–5 long; marginal hook shaft more strait; marginal hook point extending toe and pointing less backwards; filament loop long extending almost to middle of handle; transition of shaft to point not distinct.

DNA characteristics

The length of nuclear ribosomal ITS1-5.8S-ITS2 is 1,214 nt (AF484542, EU554413, EU554416,

EU727177). It is most basal when the *pannonicus* cluster is embedded in *wageneri* group phylogeny (Supplementary Fig. S5). The hypervariable segment is 227 nt long. Its predicted secondary RNA structure is with five tight hairpins and many unpaired segments (Supplementary Figs. S2, S3). The sequence deviates from *G. pannonicus* by numerous single nucleotide changes (4.2%) in well-aligned segments. There are longer indels in the hypervariable segment. Mitochondrial *cox*1 sequence (EU554421–EU554422, HM488975–HM488990) is deeply divergent from the other species in the *pannonicus* cluster (Supplementary Figs. S6, S7, S8).

Remarks

The general haptor morphology of *G. botnicus* n. sp. is in accordance with *wageneri* group *sensu* Malmberg (1970) (see the remarks in the first paragraph for *G. albolacustris* n. sp.). *Gyrodactylus botnicus* n. sp. possesses 5.8S rDNA identical with *G. aphyae* Malmberg, 1970 (GenBank AF484527). Sequence of 5.8S rDNA is the main DNA character of *wageneri* group *sensu* Ziętara & Lumme (2002).

Gyrodactylus botnicus n. sp. is the largest of the new species described here, but the samples are from northern part of the Baltic Sea Basin, thus perhaps influenced by the colder waters. The species *G. danastriae* n. sp. and *G. pannonicus* hardly overlap with *G. botnicus* n. sp. in the total length of the anchor. Also the anchor point is relatively longer (75%). The sickle point of *G. botnicus* n. sp. marginal hook less extended its sickle toe. It has a very thin dorsal bar and a ventral bar with small processes. Ventral bar membrane is rounded.

Specimens from Oulu and Oulanka were originally reported as *G. pannonicus* (Ziętara & Lumme, 2002) but it was confirmed in this study as misidentification, and the GenBank entries were corrected. Blazek et al. (2008) most probably were experimenting with *G. botnicus* n. sp. in Finnish inland lakes, instead of *G. pannonicus*, but the species name has no relevance with respect of their results: in a multiple-choice experiment, *pannonicus*-like parasite remained on *P. phoxinus*.

The species has been found on *P. phoxinus* together with *G. aphyae*, *G. macronychus*, *G. phoxini*, and *G. laevis*.

Gyrodactylus danastriae n. sp.

Type-host: *Phoxinus phoxinus* (L.) (Cypriniformes: Cyprinidae), Eurasian minnow.
Type-locality: River Strwiąż near Brzegi Dolne draining to River Dniester, Black Sea Basin, south-easter corner of Poland in Carpathian Mountains (49.4544N, 22.6393E).
Site on host: Skin and fins.
Type-material: Type-specimens (holotype and four paratypes): slides of haptors are deposited in the Finnish National History Museum in Helsinki University, specimen numbers MZH118051 (holotype) and MZH118052–MZH118055 (paratypes) (all collected on 31.vii.2007).
Representative DNA sequences: GenBank accession numbers for ITS (n = 5) and *cox*1 (n = 5) sequences are given in Table 1.
Etymology: The name *danastriae* is from an ancient Latin literature name *Danastris* of the river Dniester.

Description (Figs. 2.4, 2.8)

[Based on 5 specimens from the type-locality, River Strwiąż near Brzegi Dolne.]. Haptor ovate. Anchor 45–48 long with fold; anchor root 12–15 long, tends to bend ventrally; anchor shaft 33–35 long. Ventral bar 5–7 × 17–19, with short processes and longer triangular membrane prominently pointed, 10–12 long. Dorsal bar delicate, 1–2 long, 12–16 wide. Marginal hook 21–25 long, with prominent marginal hook sickle, 4–5 long; marginal hook shaft more strait; marginal hook point less extending toe and pointing downwards; filament loop long extends almost to middle of handle; transition of shaft to point more distinct.

DNA characteristics

The length of nuclear ribosomal ITS1-5.8S-ITS2 is either 1,258 (HM192925–HM19292) or 1,246 nt (HM192928). The hypervariable segment is either 268 or 256 nt long. Intraspecific variation is due to 12 nucleotides long heterozygous indel GAGAGTG-TAAGA, situated in tandem with more GAGA repeats. Predicted secondary RNA structure is with five tight hairpins and many unpaired segments. The sequence deviates from *G. pannonicus* by many

single nucleotide changes (3.9%) in well-aligned segments. There are several long indels observed in the hypervariable segment (Supplementary Figs. S2, S3). Mitochondrial *cox*1 sequence (HM488971–HM488974) is deeply divergent from the other species in the *pannonicus* cluster, least from European *G. albolacustris* n. sp. (16.8%; Supplementary Fig. S8). A single unique amino acid substitution Leu > Met is present in site 442 (Supplementary Fig. S10.).

Remarks

The general haptor morphology of *G. danastrae* n. sp. is in accordance with *wageneri* group *sensu* Malmberg ([1970](#)) (see the remarks in the first paragraph for *G. albolacustris* n. sp.). *Gyrodactylus danastriae* n. sp. possesses 5.8S rDNA identical with 5.8S rDNA of *G. aphyae* Malmberg, 1970 (GenBank AF484527). Sequence of 5.8S rDNA is the main DNA character of *wageneri* group *sensu* Ziętara & Lumme ([2002](#)). The anchor in *Gyrodactylys danastriae* n. sp. is slightly smaller than in the other two new species and the relative point length is only 54%, almost as small as in *G. pannonicus* in our measured material. In spite of all effort, measuring the haptors is not exact science, and the dimensions vary seasonally (Ergens, [1975](#)). The marginal hook sickle is clearly more curved than in other species and resembles more *G. aphyae* sickle but is less massive. Sickle point is almost not extending sickle toe. The dorsal bar is very thin and the ventral bar is with small processes. Ventral bar membrane is longer and more oval in comparison with the three other species.

The species was found in River Strwiąż with *G. aphyae* and an undescribed species related to *G. aphyae*.

Gyrodactylus pannonicus Molnár, 1968

Type-host: *Phoxinus phoxinus* (L.) (Cypriniformes: Cyprinidae), Eurasian minnow.
Other hosts: *Barbus barbus* (L.) (Cypriniformes: Cyprinidae), barbel (Molnár, [1968](#)).
Localities: Present study only. River Ondava draining to Danube, Black See Basin, Slovakia (49.3513N, 21.4635E); River Topl'a draining to Danube, Black Sea Basin, Slovakia (49.3133N, 21.1932E).
Site on host: Skin and fins.

Voucher material: Specimens collected from the same watershed as the holotype: slides of the haptors are deposited in the Finnish National History Museum in Helsinki University, under accession numbers MZH118040–MZH118047 (River Ondava, near Nižný Mirošov) and MZH118048–MZH118049 (River Topl'a, near Rokytov) in Slovakia (all collected on 30.vii.2007).
Representative DNA sequences: GenBank accession numbers of ITS (n = 15) and mitochondrial *cox*1 (n = 15) sequences are given in Table [1](#).

Description (Figs. [2.5](#), [2.9](#))

[Based on 10 specimens: 8 from River Ondava and 2 from River Topl'a.] Haptor ovate. Anchor 45–48 long with fold; anchor root 12–17 long, tends to bend ventrally; anchor shaft 33–36 long. Ventral bar 4–5 × 15–20, with short processes and triangular membrane prominently pointed, 9–11 long. Dorsal bar delicate 1–2 long, 12–17 wide. Marginal hook 24–27 long, with delicate marginal hook sickle, 5–6 long; marginal hook shaft leaning prominently; marginal hook point visibly extending toe and pointing backwards; filament loop long extends almost to middle of handle; transition of shaft to point not distinct.

DNA characteristics

The length of nuclear ribosomal ITS1-5.8S-ITS2 is 1,217 nt (HM192915–HM192921). The hypervariable segment is 226 nt long. It is similar to *G. albolacustris* n. sp.. The predicted secondary RNA structure of the hypervariable segment is with three tight hairpins and few unpaired nucleotides. Both isolates from River Ondava and River Topl'a share A/R/G heterozygosity (Supplementary Figs. S2, S3). Mitochondrial *cox*1 sequence (HM488961–HM488970) are deeply divergent from the other species in the *pannonicus* cluster, least divergent from Mongolian *G. albolacustris* n. sp. (18.1%; Supplementary Fig. S8). A unique amino acid substitution Ala > Val in site 134 in isolate from River Ondava is observed (Supplementary Fig. S10.).

Remarks

The general haptor morphology of *G. pannonicus* is in accordance with *wageneri* group *sensu* Malmberg

([1970](#)) (see the remarks in the first paragraph for *G. albolacustris* n. sp.). *Gyrodactylus pannonicus* possesses 5.8S rDNA identical with *G. aphyae* Malmberg, 1970 (GenBank AF484527). Sequence of 5.8S rDNA is the main DNA character of *wageneri* group *sensu* Ziętara & Lumme (2002). Parasites from authentic description of Molnár ([1968](#)) did not differ in size and shape from those found and investigated in the Rivers Ondava and Topl'a. All sizes vary within the limits specified in Table [3](#). The maximum values of all diagnostic characters of *G. pannonicus* measured in this study are either shorter or equal to minimum metrical values given by Gusev et al. ([1985](#)) for *G. aphyae*.

The Slovakian Rivers Ondava and Topl'a are connected upwaters of the tributary system Tisza to Danube to the Black Sea, i.e. from the tributary system of the type-locality of *G. pannonicus*, and therefore we feel safe to suggest that the DNA sequences reported here represent the genuine *G. pannonicus* Molnár, 1968. We suggest that it is the best way of tagging with DNA markers already described *Gyrodactylus* species. The topotype specimens should be collected from the same watershed as the type-material, although not necessarily at the same time (Winston, [1999](#)).

Because the specimens described and measured as *G. pannonicus* and compared with *G. aphyae* by Ergens ([1975](#)) were from the brook Rokytka in Prague, North Sea Basin, it is possible in the present context that they do represent some other, more western lineage than the genuine *G. pannonicus* Molnár, 1968.

Gyrodactylus pannonicus was reported by Ziętara & Lumme (2002) from the Bothnian Bay area, but because the ITS sequence (AF484542) was provided, we here confirm it as a misidentification, and the GenBank entry was corrected to represent *G. botnicus* n. sp. The experiments done by Blazek et al. ([2008](#)) in Finland most probably were made using *G. botnicus* n. sp. and not *G. pannonicus*. The species name *G. pannonicus* has been reported in many regions from the Baltic Basin to Mongolia, e.g. by Rumyantsev & Ieshko ([1997](#)), Gusev et al. ([1985](#)), Pugachev ([2002](#)), and Pugachev et al. ([2009](#)). Perhaps Ergens & Dulmaa ([1967](#)) described parasites of this cluster also as *Gyrodactylus* sp. from skin of minnow from River Kerlen (Mongolia). Specimens from River Kolyma (Pugachev, [1983](#)) clearly represent larger

parasites, and the molecular comparison might be very interesting.

This list of the wide use of the name *G. pannonicus* in wide geographical areas is in contrast to our results, and demonstrates that even the best specialists have not observed anything suspicious among the samples by measuring and comparing the parasites visually, in distant localities.

In River Ondava, *G. pannonicus* was found on minnows with *G. aphyae*. In River Dunajec on the Baltic side of the watershed, *G. pannonicus* was not found among twenty one *G. aphyae* specimens.

Discussion

The convention of using the internal transcribed spacers of the tandem repeats of nuclear ribosomal gene (ITS rDNA) for species identification of *Gyrodactylus* was initiated in the mid-nineties (Cunningham et al., [1995a](#), [b](#); Cunnigham, [1997](#)).

The ITS of most species of the genus can be amplified by a set of optimised primers, not only among salmonid parasites (Cable et al., [1999](#); Cunningham et al., [2001](#)) but widely in parasites found from many fish species in European freshwaters (Matějusová et al., [2001](#), [2003](#); Ziętara & Lumme, [2002](#), [2003](#), [2004](#)), as well as among subgenera, species groups and species in European marine environments (Huyse & Volckaert, [2002](#), [2005](#); Huyse et al., [2003](#), [2015](#); Huyse & Malmberg, [2004](#)), in North American coasts (e.g. Gilmore et al., [2012](#)), in the Caribbean (Xavier et al., [2015](#)), in the Far East Asia (Hayward et al., [2001](#)), in Amazonas (Boeger et al., [2003](#)), Antarctica (Rokicka et al., [2009](#)), Chile (Ziętara et al., [2012](#)) and Africa (Vaughan et al., [2010](#); Přikrylová et al., [2012](#)).

The ITS marker serves perfectly the purposes of a taxonomist. The most important rationale confirmed later is that ITS is almost invariable in conspecific samples, and *homozygous* and *homogeneous* in all the specimens studied (Ziętara et al., [2000](#)). It has been suggested that a genetic distance of 1% between homozygous ITS sequences might justify species separation within the genus (Ziętara & Lumme, [2003](#); Huyse et al., [2004](#)). This suggestion was based on observations that this small genetic differentiation was detected between sympatric parasites on different hosts, which were originally described as separate

species on the basis of morphology by one of the best specialists (Malmberg, 1970). The lack of heterozygous ITS types *a posteriori* justified the separation of species (Ziętara & Lumme, 2003). Considering the material in this study, the genetic distance 0.81% of the ITS of *G. albolacustris* n. sp. and *G. pannonicus* just remains below the 1% level we have ourselves suggested to tell apart species (Ziętara & Lumme, 2003). However, the 19.8% difference in the mtDNA supports the decision made.

The rarity of heterogeneity in the rDNA is the evidence that the mechanisms of concerted evolution (e.g. Polanco et al., 1998, 2000) homogenise the radical changes in ITS observed between species of *Gyrodactylus*. Even among the closely related *wageneri* species group, the length of ITS1 is variable, indicating that insertions and deletions are frequent in the evolutionary time scale (Ziętara & Lumme, 2002). Indels were also separating the species studied in this paper, and 11 bp indel was found even segregating in the population of *G. danastriae* n. sp. (Supplementary Fig. S2).

A clear disadvantage of the ITS is that it is not well suitable for molecular clock approach. Especially the ITS1 seems not to be evolving *via* point nucleotide substitutions, but rather as large blocks of insertions and/or deletions. In the Supplementary Fig. S4, some tentative transformations of the genetic distances to divergence timing are given. For the ITS, Huyse & Volckaert (2005) suggested divergence rate of 0.7%/My when studying the *rugiensis* species group. For mitochondrial DNA divergence rate within the subgenus *Limnonephrotus*, 13%/My was suggested by Kuusela et al. (2007) and 5.1%/My by Hahn et al. (2015). According to the phylogenetic hypothesis provided here, the comparisons between ITS and *cox*1 of *G. danastriae* n. sp., *G. botnicus* n. sp. and *G. pannonicus* distances are without shuffling effects, and the 5.1%/My seems to be more in concert with the ITS suggestion. However, both calibrations are quite unreliable.

A very good advantage of the discontinuous variation of the ITS is that it is easy to construct single step DNA-based identification tools for faunistic comparisons (e.g. Rokicka et al., 2007; Supplementary Fig. S11). From experience we claim that species identification by PCR-RFLP method is easier, faster, applicable to a technician, and more accurate than morphological inspection, also avoiding seasonal variation. Therefore, species description based mainly on DNA characteristics, or at least supported by DNA is clearly preferable (Ziętara & Lumme, 2003).

As demonstrated in the results of this study, the gene trees may depart, and the species trees are another phenomenon (Vanhove et al., 2013). In some animal groups, nuclear and mitochondrial evolutionary trajectories depart by continuous or repeated hybridisations (Linnen & Farrell, 2007, 2008; Toews & Brelsford, 2012). In the *pannonicus* cluster, two hybridisations were demonstrated, one ancient and the other contemporary. Without analysing all four lineages by nuclear and mitochondrial marker, the ancient hybridisation had remained undetected.

Some contemporary cases of hybridisation between divergent lineages of *Gyrodactylus* have been reported in fish farms, on introduced host species *Oncorhynchus mykiss* Walbaum, 1972 (see Rokicka et al., 2007; Kuusela et al., 2008; Ziętara et al., 2010; Ieshko et al., 2015). Hybridisation might instantly lead to higher degree of heterozygosity than a transient change, and it should therefore be very obvious and easily detectable by molecular analysis. In the above fish farm cases, the hybrid clones were permanently heterozygous and showed no segregation. As a hermaphroditic parthenogenic flatworm, a single *Gyrodactylus* hybrid is capable to found a viable deme. Evidently, the ancient and modern hybrids observed here among the minnow parasites have been sexual and the heterozygosity and variation in the ribosomal gene cassette has been or is going to be eliminated *via* Mendelian segregation and/or by the process of concerted evolution.

This specific taxonomic and systematic case will contribute significantly to the understanding of the evolution and speciation process among *Gyrodactylus*. Adaptive radiation *via* host-switching has been postulated as an explanation of the high species number in this genus, but the genetic processes associated with host-switching remain elusive (Ziętara & Lumme, 2002). In the present case, the evolving parasites remained on minnow and experienced allopatric mode of divergence, but the hybridisation might be the mechanism to obtain genetic variation needed for adaptation to new hosts as well.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national and international guidelines for the use of animals were followed.

References

Bakke, T. A., Cable, J., & Harris, P. D. (2007). The biology of gyrodactylid monogeneans: The "Russian-Doll killers". *Advances in Parasitology, 64,* 161–377.

Bakke, T. A., Harris, P. D., & Cable, J. (2002). Host specificity dynamics: observations on gyrodactylid monogeneans. *International Journal for Parasitology, 32,* 281–308.

Blazek, R. D., Bagge, A., & Valtonen, E. T. (2008). Monogenean assemblages and the apparent transmission capability of monogeneans between related fish species: an experimental study. *Parasitology Research, 102,* 1359–1366.

Boeger, W. A., Kritsky, D. C., & Pie, M. R. (2003). Context of diversification of the viviparous Gyrodactylidae (Platyhelminthes, Monogenoidea). *Zoologica Scripta, 32,* 437–448.

Cable, J., Harris, P. D., Tinsley, R. C., & Lazarus, C. M. (1999). Phylogenetic analysis of *Gyrodactylus* spp. (Platyhelminthes, Monogenea) using ribosomal DNA sequences. *Canadian Journal of Zoology, 77,* 1439–1449.

Cunningham, C. O. (1997). Species variation within the internal transcribed spacer (ITS) region of *Gyrodactylus* (Monogenea: Gyrodactylidae) ribosomal RNA genes. *Journal of Parasitology, 83,* 215–219.

Cunningham, C. O., McGillivray, D. M., MacKenzie, K., & Melvin, W. T. (1995a). Discrimination between *Gyrodactylus salaris*, *G. derjavini* and *G. truttae* (Platyhelminthes: Monogenea) using restriction fragment length polymorphisms and an oligonucleotide probe within the small subunit ribosomal RNA gene. *Parasitology, 111,* 87–94.

Cunningham, C. O., McGillivray, D. M., MacKenzie, K., & Melvin, W. T. (1995b). Identification of *Gyrodactylus* (Monogenea) species parasitizing salmonid fish using DNA probes. *Journal of Fish Diseases, 18,* 539–544.

Cunningham, C. O., Mo, T. A., Collins, C. M., Buchmann, K., Thiery, R., Blanc, G., & Lautraite, A. (2001). Redescription of *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel, & Vigneuille, 1999 (Monogenea, Gyrodactylidae); a species identified by ribosomal RNA sequence. *Systematic Parasitology, 48,* 141–150.

Ergens, R. (1975). Contribution to the knowledge of the species *Gyrodactylus aphyae* Malmberg, 1957 and *G. pannonicus* Molnár, 1968 (Monogenoidea: Gyrodactylidae). *Věstník Československé Společnosti Zoologické, 39,* 1–8.

Ergens, R., & Dulmaa, A. (1967). Monogenoidea from genus *Phoxinus* from Mongolia. *Folia Parasitologica, 14,* 321–333.

Funk, D. J., & Omland, K. E. (2003). Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Evolution, Ecology and Systematics, 34,* 397–423.

Gilmore, S. R., Cone, D. K., Lowe, G., King, S. K., Jones, S. R. M., & Abbott, C. L. (2012). Molecular phylogeny of *Gyrodactylus* (Monogenea) parasitizing fishes in fresh water, estuarine, and marine habitats in Canada. *Canadian Journal of Zoology, 90,* 776–786.

Gusev, A. V., Dubinina, M. N., Pugachev, O. N., Raykova, E. V., Khotenovskiy, I. A., & Ergens, R. (1985). [*Key to the parasites of freshwater fishes of the USSR fauna. Vol 2. Parasitic metazoans. Part 1.*] Leningrad: Nauka (In Russian).

Hahn, C., Weiss, S. J., Stojanowski, S., & Bachman, L. (2015). Co-speciation of the ectoparasite *Gyrodactylus teuchis* (Monogenea, Platyhelminthes) and its salmonid hosts. *PLoS One, 10,* e0127340.

Harris, P. D., Shinn, A. P., Cable, J., & Bakke, T. A. (2004). Nominal species of the genus *Gyrodactylus* von Nordmann 1832 (Monogenea: Gyrodactylidae), with a list of principal host species. *Systematic Parasitology, 59,* 1–27.

Hayward, C. J., Iwashita, M., Ogawa, K., & Ernst, I. (2001). New evidence that *Gyrodactylus anguillae* (Monogenea) is another invading pest of anguillid eels. *Biological Invasions, 3,* 417–424.

Heikkinen, O., & Kurimo, H. (1977). The post-glacial history of Kitkajärvi, North-Eastern Finland, as indicated by trend-surface analysis and radiocarbon dating. *Fennia, 153,* 1–32.

Huusko, A., van der Meer, O., & Koljonen, M.-L. (1990). Life history patterns and genetic differences in brown trout (*Salmo trutta* L.) in the Koutajoki river system. *Polskie Archiwum Hydrobiologii, 37,* 63–77.

Huyse, T., Audenaert, V., & Volckaert, F. A. M. (2003). Speciation and host-parasite relationships in the parasite genus *Gyrodactylus* (Monogenea, Platyhelminthes) infecting gobies of the genus *Pomatoschistus* (Gobiidae, Teleostei). *International Journal for Parasitology, 33,* 1679–1689.

Huyse, T., & Malmberg, G. (2004). Molecular and morphological comparisons between *Gyrodactylus ostendicus* n. sp. (Monogenea: Gyrodactylidae) on *Pomatoschistus microps* (Krøyer) and *G. harengi* Malmberg, 1957 on *Clupea harengus membras* L. *Systematic Parasitology, 58,* 105–113.

Huyse, T., Malmberg, G., & Volckaert, F. A. M. (2004). Four new species of *Gyrodactylus* von Nordmann, 1832 (Monogenea, Gyrodactylidae) on gobiid fishes: combined DNA and morphological analyses. *Systematic Parasitology, 59,* 103–120.

Huyse, T., Plaisance, L., Webster, B. L., Mo, T. A., Bakke, T. A., Bachmann, L., & Littlewood, D. T. J. (2007). The mitochondrial genome of *Gyrodactylus salaris* (Platyhelminthes: Monogenea), a pathogen of Atlantic salmon (*Salmo salar*). *Parasitology, 134,* 739–747.

Huyse, T., Vanhove, M. P. M., Mombaerts, M., Volckaert, F. A. M., & Verreycken, H. (2015). Parasite introduction with an invasive goby in Belgium: double trouble? *Parasitology Research, 114,* 789–793.

Huyse, T., & Volckaert, F. A. M. (2002). Identification of a host-associated species complex using molecular and morphometric analysis, with the description of *Gyrodactylus rugiensoides* n. sp. (Gyrodactylidae, Monogenea). *International Journal for Parasitology, 32,* 907–919.

Huyse, T., & Volckaert, F. A. M. (2005). Comparing host and parasite phylogenies: *Gyrodactylus* flatworms jumping from goby to goby. *Systematic Biology, 54,* 710–718.

Ieshko, E., Lebedeva, D., & Lumme, J. (2015). A new *Gyrodactylus* strain on brown trout (*Salmo trutta*) in Jänisjärvi, Russian Karelia, and a literature revision of salmonid parasites of the genus *Gyrodactylus* in North-Western Russia and adjacent areas. *Acta Parasitologica, 60,* 75–84.

Koutaniemi, L. (1999). Physical characteristics and palaeogeography of the Oulanka-Paanajärvi region of the Finnish-Karelian border. *Fennia, 177,* 2–9.

Kuusela, J., Ziętara, M. S., & Lumme, J. (2007). Hybrid origin of Baltic salmon-specific parasite *Gyrodactylus salaris*: a model for speciation by host switch for hemiclonal organisms. *Molecular Ecology, 16,* 5234–5245.

Kuusela, J., Ziętara, M. S., & Lumme, J. (2008). Description of three new European cryptic species of *Gyrodactylus* Nordmann, 1832 supported by nuclear and mitochondrial phylogenetic characterization. *Acta Parasitologica, 53,* 120–126.

Linnen, C. R., & Farrell, B. D. (2007). Mitonuclear discordance is caused by rampant mitochondrial introgression in *Neodiprion* (Hymenoptera: Diprionidae) sawflies. *Evolution, 61,* 1417–1438.

Linnen, C. R., & Farrell, B. D. (2008). Phylogenetic analysis of nuclear and mitochondrial genes reveals evolutionary relationships and mitochondrial introgression in the sertifer species group of the genus *Neodiprion* (Hymenoptera: Diprionidae). *Molecular and Phylogenetic Evolution, 48,* 240–257.

Malmberg, G. (1957). [On the occurrence of *Gyrodactylus* on Swedish fishes.] *Skrifter Utgivna av Södra Sveriges Fiskeriförening Årsskrift*, 1956, 19–76 (in Swedish).

Malmberg, G. (1964). Taxonomical and ecological problems in *Gyrodactylus*. In: Ergens, R. & Ryšavý, B. (Eds) *Parasitic worms and aquatic conditions. Proceedings of a symposium held in Prague from October 29 to November 2, 1962*. Prague: Publishing House of the Chechoslovak Academy of Sciences, pp. 203–230.

Malmberg, G. (1970). The excretory systems and the marginal hooks as a basis for the systematics of *Gyrodactylus* (Trematoda, Monogenea). *Arkiv för Zoologi, 23,* 1–237.

Matějusová, I., Gelnar, M., McBeath, A. J. A., Collins, C. M., & Cunningham, C. O. (2001). Molecular markers for gyrodactylids (Gyrodactylidae: Monogenea) from five fish families (Teleostei). *International Journal of Parasitology, 31,* 738–745.

Matějusová, I., Gelnar, M., Verneau, O., Cunningham, C. O., & Littlewood, D. T. J. (2003). Molecular phylogenetic analysis of the genus *Gyrodactylus* (Platyhelminthes: Monogenea) inferred from rDNA ITS region: subgenera versus species groups. *Parasitology, 127,* 603–611.

Meinilä, M., Kuusela, J., Ziętara, M., & Lumme, J. (2002). Primers for amplifying 820 bp highly polymorphic mitochondrial COI gene of *Gyrodactylus salaris*. *Hereditas, 137,* 72–74.

Meinilä, M., Kuusela, J., Ziętara, M., & Lumme, J. (2004). Initial steps of speciation by geographic isolation and host switch in salmonid pathogen *Gyrodactylus salaris* (Monogenea: Gyrodactylidae). *International Journal for Parasitology, 34,* 515–525.

Molnár, K. (1968). Beitrage zur Kenntnis der Fischparasiten Ungarn. 3.Weitere Monogenoiden-Arten aus Fischen. *Acta Veterinaria Hungarica, 18,* 295–311.

Polanco, C., Gonzalez, A. I., de la Fuente, D., & Dover, G. A. (1998). Multigene family of ribosomal DNA in *Drosophila melanogaster* reveals contrasting patterns of homogenization for IGS and ITS spacer regions: a possible mechanism to resolve this paradox. *Genetics, 149,* 243–256.

Polanco, C., Gonzalez, A. I., & Dover, G. A. (2000). Patterns of variation in the intergenic spacers of ribosomal DNA in *Drosophila melanogaster* support a model for genetic exchanges during X-Y pairing. *Genetics, 155,* 1221–1229.

Přikrylová, I., Blažek, R., & Vanhove, M. P. (2012). An overview of the *Gyrodactylus* (Monogenea: Gyrodactylidae) species parasitizing African catfishes, and their morphological and molecular diversity. *Parasitology Research, 110,* 1185–1200.

Pugachev, O. N. (1983). Monogenea of freshwater fishes of the North-East. In O. N. Bauer (Ed.), *Studies on morphology and faunistics of parasitic worms*. Leningrad: Nauka, pp. 22–34 (in Russian).

Pugachev, O. N. (2002). [Checklist of the freshwater fish parasites of the Northern Asia (Cnidaria, Monogenoidea, Cestoda).] *Proceedings of the Zoological Institute: Saint-Petersburg, 297,* 1–248 (In Russian).

Pugachev, O. N., Gerasev, P. I., Gussev, A. V., Ergens, R., & Khotenowsky, I. (2009). *Guide to Monogenoidea of freshwater fish of Palaearctic and Amur Regions*. Milan: Ledizione-Ledi Publishing, 564 pp.

Rokicka, M., Lumme, J., & Ziętara, M. S. (2007). Identification of *Gyrodactylus* ectoparasites in Polish salmonid farms by PCR-RFLP of the nuclear ITS segment of ribosomal DNA (Monogenea, Gyrodactylidae). *Acta Parasitologica, 52,* 185–195.

Rokicka, M., Lumme, J., & Ziętara, M. S. (2009). Two new Antarctic *Gyrodactylus* species (Monogenoidea): description and phylogenetic characterization. *Journal of Parasitology, 95,* 1112–1119.

Rumyantsev, E. A., & Ieshko, E. L. (1997). *Parasites of fish in waterbodies of Karelia*. Petrozavodsk: Russian Academy of Sciences, Karelian Research Center, 120 pp.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30,* 2725–2729.

Toews, D. P. L., & Brelsford, A. (2012). The biogeography of mitochondrial and nuclear discordance in animals. *Molecular Ecology, 21,* 3907–3930.

Vanhove, M. P. M., Tessens, B., Schoelink, C., Jondelius, U., Littlewood, D. T. J., Artois, T., & Huyse, T. (2013). Problematic barcoding in flatworms: A case-study on monogeneans and rhabdocoles (Platyhelminthes). *Zoo-Keys, 365,* 355–379.

Vaughan, D. B., Christison, K. W., Hansen, H., & Shinn, A. P. (2010). *Gyrodactylus eyipayipi* sp. n. (Monogenea: Gyrodactylidae) from *Syngnathus acus* (Syngnathidae) from South Africa. *Folia Parasitologica, 57,* 11–15.

Winston, J. E. (1999). *Describing species. Practical taxonomic procedure for biologists*. New York: Columbia University Press.

Xavier, R., Faria, P. J., Paladini, G., van Oosterhout, C., Johnson, M., & Cable, J. (2015). Evidence for cryptic speciation in directly transmitted gyrodactylid parasites of Trinidadian guppies. *PLoS ONE, 10,* e0117096.

Ziętara, M. S., Arndt, A., Geets, A., Hellemans, B., & Volckaert, F. A. M. (2000). The nuclear rDNA region of *Gyrodactylus arcuatus* and *G. branchicus* (Monogenea: Gyrodactylidae*). Journal of Parasitology, 86,* 1368–1373.

Ziętara, M. S., Kuusela, J., & Lumme, J. (2006). Escape from an evolutionary dead end: a triploid clone of *Gyrodactylus salaris* is able to revert to sex and switch host (Platyhelminthes, Monogenea, Gyrodactylidae). *Hereditas, 143,* 86–92.

Ziętara, M. S., Lebedeva, D., Muñoz, G., & Lumme, J. (2012). A monogenean fish parasite, *Gyrodactylus chileani* n. sp., belonging to a novel marine species lineage found in the South-Eastern Pacific and the Mediterranean and North Seas. *Systematic Parasitology, 83,* 159–167.

Ziętara, M. S., & Lumme, J. (2002). Speciation by host switch and adaptive radiation in a fish parasite genus *Gyrodactylus* (Monogenea: Gyrodactylidae). *Evolution, 56,* 2445–2458.

Ziętara, M. S., & Lumme, J. (2003). The crossroads of molecular, typological and biological species concept: two new species of *Gyrodactylus* Nordmann, 1832 (Monogenea, Gyrodactylidae). *Systematic Parasitology, 55,* 39–52.

Ziętara, M. S., & Lumme, J. (2004). Comparison of molecular phylogeny and morphological systematics in fish parasite genus *Gyrodactylus* Nordmann, 1832 (Monogenea, Gyrodactylidae). *Zoologica Poloniae, 49,* 5–28.

Ziętara, M. S., Rokicka, M., Stojanovski, S., & Lumme, J. (2010). Introgression of distant mitochondria into the genome of *Gyrodactylus salaris*: Nuclear and mitochondrial markers are necessary to identify parasite strains. *Acta Parasitologica, 55,* 20–28.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research, 31,* 3406–3415.