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VERSITA

Introgression of distant mitochondria into the genome of *Gyrodactylus salaris*: Nuclear and mitochondrial markers are necessary to identify parasite strains

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Abstract

Novel combinations of mitochondrial DNA (CO1) and internal transcribed spacers of nuclear ribosomal DNA (ITS) were detected among *Gyrodactylus* parasites on brown trout (*Salmo trutta* L.), rainbow trout (*Oncorhynchus mykiss* (Walbaum)), and Ohrid trout (*Salmo letnica* (Karaman)) from salmonid farms in Poland and Macedonia. Some clones differed from standard ITS only by ≤ 4 nucleotides, but they belonged to a mtDNA clade that differed from the Northern European lineages of *G. salaris* by $d_{MCL} = 0.266 \pm 0.108$ (maximum composite likelihood distance). The divergence of $d_{MCL} = 0.013 \pm 0.005$ within the alien mtDNA clade suggested introgression from an unknown maternal ancestor into the *G. salaris* Malmberg genome 137 to 57 kyr ago (or, less probably, repeated introgression). A comparable modern hybrid was detected based on permanently heterozygous ITS (28 bp/1264 = 2.2%) in a clone that is widespread throughout Finnish rainbow trout farms. This was a F_1 hybrid of maternal *G. pomeraniae* Kuusela, Ziętara et Lumme (on roach, *Rutilus rutilus* (L.)) and *G. lavareti* Malmberg (on whitefish, *Coregonus lavaretus* (L.)). The mtDNA of the parental species differed by $d_{MCL} = 0.290 \pm 0.130$. The observations emphasize that both nuclear and maternally-inherited DNA markers are necessary to characterize sexually-produced lineages or clones of *Gyrodactylus*. The hybridization events demonstrated predict incongruence of mitochondrial vs. nuclear gene trees, i.e., reticulate evolution in species trees.

Keywords

Hybrid speciation, ITS of nrDNA, mitochondrial phylogeny, barcoding

Introduction

The infamous monogenean parasite of Atlantic salmon (*Salmo salar* L.), *Gyrodactylus salaris*, is neither a homogeneous panmictic species, nor a uniform clone. Sequencing of the mitochondrial DNA (mtDNA) has revealed an unexpected scale of diversity and geographical structure in the natural Baltic range on Atlantic salmon, *Salmo salar* L. (Kuusela *et al*. 2009), as well as on different host species like rainbow trout, *Oncorhynchus mykiss* (W.) and Arctic charr, *Salvelinus alpinus* (L.) (Meinilä *et al*. 2002, 2004; Hansen *et al*. 2003, 2006, 2007a; Robertsen *et al*. 2007). The mtDNA offers high resolution for separating numerous host-specific and geographically separated strains of salmon parasites and those on grayling, *Thymallus thymallus* (L.) (which are also called *G. thymalli* Žitňan). However, the mitochondrial marker alone is not enough for a detailed evolutionary analysis of the aspects of host specificity and pathogenity, as discussed in the comprehensive review by Hansen *et al*. (2007b). The hybrid origin of the main mtDNA clade parasitizing Baltic salmon was solved by adding a variable nuclear DNA marker to the toolbox (Kuusela *et al*. 2007, 2009). The mitochondrially separate clade associated in several localities with rainbow trout, Arctic charr, and salmon, and specified as the RBT clone by Ziętara *et al*. (2006), or clade **F** by Hansen *et al*. (2007b), remains of unknown origin. Three cases of transfer of this mtDNA clade to salmon populations have been explained as the consequences of sexual recombination, again, using nuclear markers (Ziętara *et al*. 2006, 2008a).

The ITS region of ribosomal DNA (ITS) is a very good taxonomic marker for *Gyrodactylus* in general, but within the *G. salaris* (including *G. thymalli*) cluster, it is nearly monomor-

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phic and fails to identify divergent lineages (Cunningham 1997, Meinilä *et al*. 2004, Hansen *et al*. 2007b). Single nucleotide ITS polymorphism (SNP) was found in *G. salaris* on Arctic charr in Norway associated with the mitochondria of the **F** clade (Robertsen *et al*. 2007), while another was found on rainbow trout in Denmark (Jørgensen *et al*. 2007, Kania *et al*. 2007). Considering this uniformity, the Danish strain ***Gx*** characterized by *three* heterogeneous nucleotides in the ITS deserved specific attention (Lindenstrøm *et al*. 2003). The clone raised in the laboratory from a single worm preferred rainbow trout as a host, suggesting that it was not directly descended from salmon (Lindenstrøm *et al*. 2003). Recently, we identified the same unusual ITS sequence of *G. salaris* in a molecular survey of Polish salmonid farms (Rokicka *et al*. 2007).

The aim of the current study was to further characterize the *G. salaris* strains carrying the ITS variants by analyzing their maternally inherited mitochondrial DNA. The observed variation was compared with the molecular variation of *G. salaris* and some other *Gyrodactylus* species parasitizing rainbow trout, including a clone of the F_1 species hybrid reported recently (Kuusela *et al*. 2008). The results contribute to the general understanding of the reproductive biology and evolution of *Gyrodactylus* spp. Most importantly, the results also have practical consequences with respect to the idea of molecular recognition of parasite species and strains, which is also referred to as 'barcoding'.

Materials and methods

Parasite collection

The *Gyrodactylus* specimens investigated here are listed in Table I. They were sampled from brown trout (*Salmo trutta* L.) and rainbow trout (*Oncorhynchus mykiss* (Walbaum)) in May and July in 2006 at eight fish farms in northern and southern Poland along tributaries of the Vistula River, and on independent rivers near the Pomeranian coast, all of which are located within the Baltic Sea basin (Rokicka *et al*. 2007). The Macedonian parasites were collected from rainbow trout and Ohrid trout (*Salmo letnica* (Karaman)) from a fish farm located along the Vardar River, Aegean Sea basin, in July 2005. All the fish farms were relatively small, mostly self-sustained salmonid farms with natural water flow through the ponds.

The origin of the outgroup species was as follows: *G. truttae* Gläser from rainbow trout in a farm in northern Poland (2002); *G. derjavini* Mikailov from rainbow trout in a farm in Iran (2005); *G. derjavinoides* Malmberg, Collins, Cunningham et Jalali from brown trout in Älvkarleby and Mörrumsån in Sweden (2001) and from rainbow trout in a farm in Poland (2006); *G. lucii* from northern pike (*Esox lucius* L.) was collected in various rivers in northwestern Russia (2006), northern Finland (2004 and 2007), and northeastern Poland (2004). The sampling of the F_1 hybrid of *G. pomeraniae* × *G. lavareti* in Finland was described by Kuusela *et al*. (2008). A single sample from grayling (*Thymallus thymallus* (L.)) was also added.

The fish were inspected for *Gyrodactylus* infection using a low-power stereomicroscope. Infected fins were clipped and stored in 70–90% EtOH.

Molecular methods

For all PCR procedures, single EtOH-preserved worms were picked from the fins and digested in 10 µl of solution containing 1 × Fermentas PCR-buffer, 0.45% Tween 20, 0.45% Igepal, 0.6 µg Proteinase K. Each worm was spun to the bottom in 200 µl Eppendorf vials and incubated at 65°C for 25 min, the Proteinase K was denatured at 94°C for 10 minutes. The process ended at 4°C.

The overall ITS region of the nuclear ribosomal DNA (rDNA) spanning from the 3'end of 18S rDNA (15 bp) through ITS1, 5.8S rDNA (157 bp), and ITS2 to the 5'end of 28S rDNA (9 bp) was amplified by primers *ITS1F* 5'-GTT TCC GTA GGT GAA CCT and *ITS2R* 5'-GGT AAT CAC GCT TGA ATC as in Ziętara *et al*. (2000). Two internal primers were used for sequencing the ITS-*ITS1R* (5'-ATT TGC GTT CGA GAG ACC G-3') and *ITS2F* (TGG TGG ATC ACT CGG CTC A-3'). The new ITS sequences were deposited in GenBank under the following accession numbers: *G. derjavinoides* EF445940–EF445941, EU304810; *G. lucii* EU304811–EU304816; *G. salaris* EF524573–EF524574 and EF117888.

Mitochondrial DNA encompassing the CO1 gene was amplified as two overlapping fragments. A major ~1600 bp fragment was amplified as in Ziętara *et al*. (2006) by *FCox6* (5'-TTG GAT CAT AAG CGC ATY GGT AT-3') and *16SR* (5'-CAT TTA ATC ATG ATG CAA AAG G-3') primers. The complementary 5'end of the CO1 gene was amplified as follows: *G. derjavini*, *G. derjavinoides*, and the alien mitochondria of *G. salaris* by *Trp1F* (5'-ATA TAG ACG ATT TGT TTT CA-3') and *RCox4* (5'-AGA AG GTG AAG CGA AAA CA-3'); *G. lucii* by *Trp1F* and *RCox6* (5'-AAA TGC TGG AAT AAC ACT GG-3'); *G. truttae* by *Trp4F* (5'-AAT AAG ACG ATT TGT TTT CA-3'); *RCox7* (5'-GTA GGT ACA GCG ATT ATC AT-3'). Additional internal primers used for sequencing the mtDNA were as follows: *G. derjavini – FCox7* (5'-TTT TCA ATA GGT ATG GAC GT-3'), *LA1* (5'-TAA TAG GGG GGT TTG GTA A-3'), and *RCox2* (5'-TAG AGA ACA TGG CGA ACA CC-3'); *G. derjavinoides – FCox7* and *RCox7* (5'-GTA GGT ACA GCG ATT ATC AT-3'); *G. lucii – FCox7*, *LA1*, *RCox7*; *G. salaris* and *G. truttae – FCox7*.

The overall mitochondrial fragment covers the 3' end of the transfer RNA gene of tryptophan (30–32 bp, $tRNA^{trp}$), the complete CO1 gene (cox1, 1548 bp including the stop codon), the complete transfer RNA gene of threonine (75 bp, $tRNA^{thr}$), and the flanking 5' fragment of the16S ribosomal RNA gene (31 bp, 16S rRNA) (for the gene order, see Huyse *et al*. 2007). To shorten the amplification step to a single amplicon, the following sets of primers could be used: *G. derjavini – Trp1F* and *Thr11R* (5'-AAA GGT TAC TTG GTA TTA CA-3'); *G. der-*

Table I. Localities and accession numbers of new DNA sequences presented in this paper

***Gyrodactylus* species**	**Host**	**Locality**	**Collection date**	**ITS ac. No.**	**No.**	**COI ac. No.**	**No.**
G. derjavini Mikailov, 1975	*Oncorhynchus mykiss* (W.)	Iran, fish farm	Oct. 2005	DQ323402[a]	2	EF524575	2
G. derjavinoides Malmberg *et al.,* 2007	*Salmo letnica* (K.)	Macedonia, fish farm	July 2005	EU304810	1	EU304827	1
	Salmo trutta L.	Poland, fish farm	May 2006	EF445939[a]	2	EF446758	2
		River Dalälven, S 60°34′N, 17°26′E	Nov. 2001	EF445941	4	EF446761	4
		River Mörrum, S 56°11′N, 14°45′E	Nov. 2001	EF445940	1	EF446759	1
				EF445940	3	EF446760	3
G. lucii Kulakovskaya, 1952	*Esox lucius* L.	River Einojoki, RUS 61°20′N, 32°09′E	July 2006	EU304811	2	EU304817	2
		River Jaziewianka, Jaziewo, PL 53°40′N, 22°54′E	Aug. 2004	DQ993187[b]	1	EU304824	1
		River Hiitolanjoki, RUS 61°10′N, 29°51′	July 2006	EU304812	2	EU304818	2
		Lake Kuohkimajärvi, FIN 69°03′N, 20°33′E	Sept. 2004	EF113105[b]	5	EU304819	5
		River Kutujoki, FIN 71°66′N, 34°90′E	May 2007	EU304815	3	EU304822	3
		River Syskynjoki, RUS 61°42′N, 31°28′E	July 2006	EU304814	2	EU304820	2
		River Tuloksa, RUS 60°70′N, 32°35′E	July 2006	EU304813	2	EU304821	2
	Rutilus rutilus (L.)	River Kutujoki, FIN 71°66′N, 34°90′E	May 2007	EU304816	2	EU304823	2
G. salaris Malmberg, 1957	*Oncorhynchus mykiss* (W.)	Macedonia, fish farm	July 2005	EF524573	1	EF524577	2
				EF524574	2		
		Poland, fish farm	July 2006	EF464676[a]	8	EF524576	8
				EF464677[a]	2	EF524578	2
	Thymallus thymallus (L.)	Krunnit Islands, FIN 65°25′N, 25°00′E	1999	EF117888	1	AF540894[c]	1
G. truttae Gläser, 1974	*Salmo trutta* L.	Poland, fish farm	Mar. 2002	EF464681[a]	1	EU304826	1

FIN – Finland, PL – Poland, RUS –Russia, S – Sweden. [a]Rokicka *et al*. 2007; [b]Ziętara *et al.* 2008b; [c]Meinilä *et al.* 2004.

javinoides – Trp6F (5'-TAA TAG ACG ATT TGT TTT CA-3') and *Thr12R* (5'-GTA AGT ATC TTG GTA TTA CA-3'); *G. lucii – Trp6F* and *Thr10R* (5'-TGA AGT RTC TTG GTA TTA CA-3'); *G. salaris* alien CO1 gene – *Trp1F* and *Thr8R* (5'-ACA GAT TGC TTG GTA TTA CA-3'); *G. truttae – Trp4F* (5'-AAT AAG ACG ATT TGT TTT CA-3') and *Thr9R* (5'-ATA GGT TTC TTG GTG TTA CA-3'). The new sequences were deposited in GenBank under the following accession numbers: *G. derjavini* EF524575; *G. derjavinoides* EF446758–EF446761, EU304827; *G. lucii* EU304817–EU304824; *G. truttae* EU304826; *G. salaris* EF524576–EF524578.

The PCR reactions were prepared by adding 2 µl of a digested worm to 18 µl of PCR solution: 1 x Fermentas PCR-buffer, 0.2 mM dNTP, 2 mM $MgCl_2$, 1 µM of each primer, and 0.5 U Fermentas DNA polymerase. The PCR program for ITS was 3 min at 95°C and 37 cycles for 40 sec at 94°C, 30 sec at 48°C, 1 min at 72°C, final elongation was 7 min at 72°C, and the process ended at 4°C. The PCR program for the CO1 gene was 3 min at 95°C and 37 cycles for 30 sec at 94°C, 1 min at 50°C, 1 or 1.5 min at 72°C, final elongation was 7 min at 72°C, and the process ended at 4°C. The DNA fragments were run in 1% agarose gel, cut out, and purified by precipitation in ethanol. The DNA pellet was diluted in 20 µl sterile water and kept at –20°C.

Sequencing was based on the Big Dye Terminator Cycle Sequencing kit protocol and an ABI 3730 DNA Analyzer.

In this study, a fragment of 1545 nucleotides (515 amino acids) was used in the mitochondrial phylogenetic analysis. The phylogenetic tree (Fig. 1) was constructed with the Neighbor Joining method based on Maximum Composite Likelihood distance (d_{MCL}), as implemented in the MEGA4 software package (Tamura *et al*. 2007). The bootstrap test was conducted with 1000 repeats. Some informative genetic distances were also expressed as the number of nucleotide or amino acid differences, or as Kimura's two-parameter distances (d_{K2P}).

Results

There were two cases of distant hybridization in the parasite material analyzed in the current study. One was found based on introgressed alien mtDNA among strains carrying *nearly* standard ITS sequences of *G. salaris*, which indicated that hybridization was followed by backcrossing. The introgressed mtDNA was from an unknown species. The other case was modern: a non-segregating F_1 hybrid clone was found in rainbow trout farms, and the ITS and mtDNA sequences of both parental species were known. These cases were compared

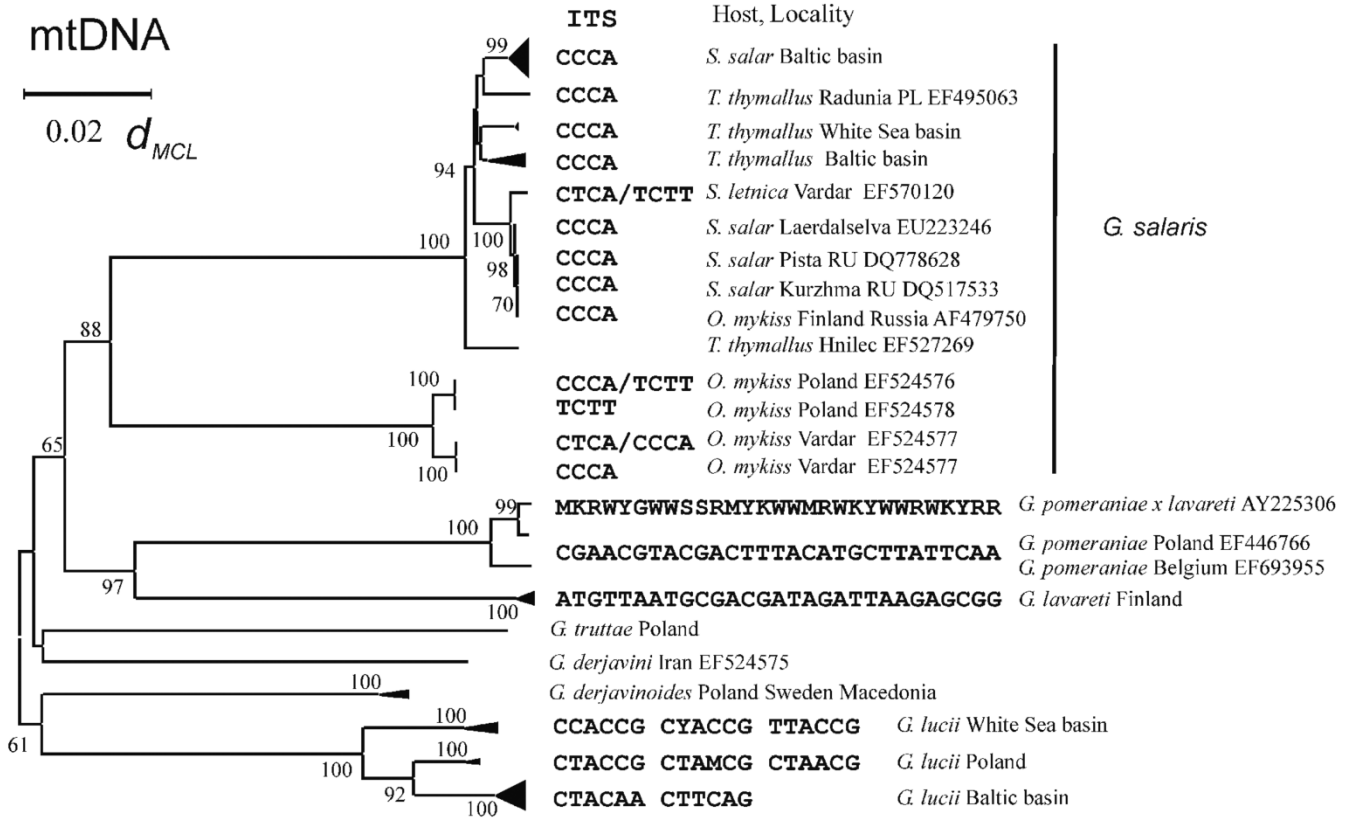

Fig 1. Mitochondrial phylogenetic hypothesis and the variable sites of ITS in each haplotype. Some *G. salaris* clades which contain large number of haplotypes were compressed. The tree is based on 76 complete mitochondrial CO1 sequences (1545 bp), Neighbor Joining, 1000 repeats for bootstrap, maximum composite likelihood distance (MEGA4, Tamura *et al*. 2007)

with some other species in order to establish the scale of the evolutionary events observed.

Mitochondrial phylogeny of the taxa involved in hybridizations

The variant *G. salaris* clone was first observed in Polish farms as small differences in ITS (see below), but the most interesting character of the clones was the alien mitochondria identified in this study from Polish and Macedonian salmonid farms. The mitochondrial phylogeny is presented in Figure 1. To characterize the distance between the introduced mtDNA and the standard haplotypes of *G. salaris,* we included new sequences, which are mostly unpublished, from the salmonid parasites *G. derjavini*, *G. derjavinoides*, *G. truttae*, and *G. lavareti*, all from the wageneri group. Testing the mtDNA of these species was also an exclusive maternity test. The mtDNA sequence of *G. teuchis*, which is also a salmonid farm parasite in Poland, is unknown.

In Figure 1, two distant mtDNA clades under the species name *G. salaris* are connected by the ITS and exhibit only four variable sites. Even if these variants were the reason to analyze the strains (see below), the difference in ITS is very small compared to the large divergence in mtDNA. Estimated over the complete CO1 coding sequence (1545 bp, 515 amino acids), the novel mtDNAs of the Polish farm types ITS^{YCYW} and ITS^{TCTT} differed from the Finnish rainbow trout specific RBT clone of *G. salaris* by 261 nucleotide substitutions and eight amino acid differences ($d_{MCL} = 0.266 \pm 0.108$; $d_{K2P} = 0.187 \pm 0.012$). The CO1 sequences were deposited as EF524576 and EF524578, respectively.

The species hybrid *G. pomeraniae* × *lavareti* found on rainbow trout (*Oncorhynchus mykiss*) combining parasites from roach (*Rutilus rutilus*) and whitefish (*Coregonus lavaretus*), as well as the parents, were included in the mitochondrial tree as another example of a distant hybrid of *Gyrodactylus*. This hybrid is found only on rainbow trout, and it is non-segregating (= asexual) F_1 clone. The ITS alleles of the parental species differ by 19 single nucleotide substitutions, which were heterozygous in the hybrid (Kuusela *et al*. 2008). With respect to the mtDNA, the paternal *G. lavareti* and the hybrid clone differ by 217 bp along the 1545 bp of CO1, and by 22 amino acid substitutions. The distance estimates are $d_{MCL} = 0.2901 \pm 0.1298$ and $d_{K2P} = 0.2030 \pm 0.0113$, indicating that the purifying selection on mtDNA has been less intense in this species pair than among the *G. salaris* variants.

To illustrate the degree of comparable intraspecific geographical variation in the mtDNA, another wageneri group species, i.e. *G. lucii* sampled in the northern European area, including the Baltic Sea and White Sea basins, was included in the phylogenetic tree. The maximum divergence found between Polish and White Sea specimens of *G. lucii* mtDNAs was $d_{MCL} = 0.0851 \pm 0.0298$ and $d_{K2P} = 0.0782 \pm 0.0078$. There were 112 nucleotide and 9 amino acid substitutions.

A simple comparison of the ratio of nucleotide substitutions/amino acid substitutions shows that in the history of *G. salaris* variants, the purifying selection was much stronger ($N_{bp}/N_{aa} = 32.6$) than in the divergence of *G. lavareti* vs. *G. pomeraniae* (12.3) or among geographical isolates of *G. lucii* (12.4).

Comparison of the nuclear marker ITS with mtDNA phylogeny

The internal transcribed spacer region of rDNA was sequenced in all the species, strains, and specimens presented in the mitochondrial phylogenetic tree in Figure 1. Based on previous experience, ITS corresponds well to the morphological delineation of the *Gyrodactylus* species. It has even been suggested that divergence of ITS at about 1% could be used as a pragmatic indicator of conspecificity of sister species (Ziętara and Lumme 2003), because ITS sequences are nearly always homozygous, even when they differ very little between host-specific parasites living in the same environment. The lack of heterozygotes is evidence of sexual isolation (Huyse *et al*. 2004).

In the present study, all of the *Gyrodactylus* parasites collected in the Polish farms were originally identified to species using PCR-RFLP of the ITS fragment cut by the enzyme *Hinc* II (Rokicka *et al*. 2007). The cutting sequence of *Hinc* II is GTYRAC, which was expected to hit the heterogeneous ITS 276C/T of the Danish strain described by Lindenstrøm *et al*. (2003) at site 5'-G<u>GTTAA**Y**</u>CATGGCA-3'. Site 276 was indeed observed as heterozygous in several specimens (C/T = Y, N = 35), but also as homozygous with respect to the variant allele (T/T, N = 2). Two homozygous specimens were found at separate farms (Rokicka *et al*. 2007).

Next, the whole ITS segment was sequenced directly from eight heterozygous and both homozygous specimens (deposited as EF464676 and EF464677, respectively), and the diagnostic C/T and T/T nucleotides were confirmed at site 276. Further, the other two variable sites reported in Denmark were either heterozygous or homozygous: 911C/T (in two specimens T/T) and 1090A/T (T/T). Since another variable nucleotide of ITS is presented in the current study (572C/T, Table II), the 'phenotypes' are referred to as ITS^{YCYW} and ITS^{TCTT}, while the standard phenotype and 'allele' of *G. salaris* is referred to as ITS^{CCCA}, the site numbers are 276, 572, 911, and 1090 (Table II, Fig. 1). The site numbering follows the sequence of Lindenstrøm *et al*. (2003, GenBank accession number AJ515912).

The specimens of both heterozygous and homozygous Polish variant clones ITS^{YCYW} and ITS^{TCTT} carried a nearly identical mitochondrial CO1 sequence (Fig. 1); however, this deviated widely from the sequences recorded so far for *G. salaris*. The ITS^{TCTT} homozygotes had a single heteroplasmic Y in the mtDNA, which indicated that the two specimens represented the same clone in two farms.

The *G. salaris* strains collected from a Macedonian fish farm were analyzed during the same exploratory phase of the study, and these proved to be the most interesting. The specimens from rainbow trout had mtDNA that was closely related to the Polish farm strains. The mtDNA diverged by 19 nucleotide substitutions (mtDNA: EF524577). However, one

Table II. Variable sites in ITS1 and ITS2 of *Gyrodactylus salaris* strains

Author	Host	Accession number	Site numbering according to AJ515912 (Lindenstrøm *et al*. 2003)								
			102*	**276**	299	422	**572**	**911**	988	**1090**	1099
Cunningham *et al*. 2000	*S. salar*	Z72477	R	**C**	G	T	**C**	**C**	G	**A**	G
Ziętara *et al*. 2002	*O. mykiss*	AF328871	A	**C**	G	T	**C**	**C**	G	**A**	G
Ziętara and Lumme 2002	*T. thymallus*	AF484544	A	**C**	G	C	**C**	**C**	G	**A**	G
Lindenstrøm *et al*. 2003	*O. mykiss*	AJ515912	R	**Y**	G	T	**C**	**Y**	G	**W**	G
Jørgensen *et al*. 2007	*O. mykiss*	DQ823390	A	**C**	G	T	**C**	**C**	G	**A**	A
Robertsen *et al*. 2007	*S. alpinus*	DQ898302	A	**C**	G	T	**C**	**C**	G	**A**	A
Hansen *et al*. 2007a	*T. thymallus*	DQ916137	A	**C**	T	T	**C**	**C**	G	**A**	G
Hansen *et al*. 2007a	*T. thymallus*	DQ916138	A	**C**	G	T	**C**	**C**	A	**A**	G
Kuusela *et al*. 2007	*S. salar*	EF117877	A	**C**	G	T	**C**	**C**	G	**A**	G
Kuusela *et al*. 2007	*T. thymallus*	EF117888	A	**C**	G	T	**C**	**C**	G	**A**	G
Kuusela *et al*. 2007	*S. letnica*	EF570119	A	**Y**	G	T	**Y**	**Y**	G	**W**	G
Rokicka *et al*. 2007	*O. mykiss*	EF464676	A	**Y**	G	T	**C**	**Y**	G	**W**	G
Rokicka *et al*. 2007	*O. mykiss*	EF464677	A	**T**	G	T	**C**	**T**	G	**T**	G
This paper Macedonia	*O. mykiss*	EF524573	A	**C**	G	T	**C**	**C**	G	**A**	G
This paper Macedonia	*O. mykiss*	EF524574	A	**C**	G	T	**Y**	**C**	G	**A**	G

The four diagnostic nucleotides considered in this paper (e.g., Fig. 1) are marked as bold. *G/A variation was only observed in one laboratory. Because it was screened from Fennoscandian isolates of *G. salaris* and not confirmed in other laboratories having samples from the same localities, it is stated to be a technical error.

specimen carried the standard ITS^{CCCA} of 'normal' *G. salaris* (ITS: EF524573), and two of the three had a unique additional Y ambiguity not found in any known *G. salaris* strains, T/C in site 572: ITS^{CYCA} (EF524574, Table II).

A parasite clone was found in the Macedonian farm stock of Ohrid trout (*Salmo letnica*) that had heterozygous ITS^{YYYW}; thus, it was a combination of the alleles found in Poland (ITS^{TCTT}) and Macedonia (ITS^{CTCA}) (EF570119). Surprisingly, it did not carry the alien mtDNA found in two other Macedonian *G. salaris* strains, but the mtDNA sequence (EF570120) was next to the standard mtDNA sequence of the rainbow trout-specific *G. salaris* RBT clone (Ziętara *et al*. 2006), from which it differed by ten nucleotides. These ten nucleotide differences were most surprising, because this mitochondrial clade has been extremely conservative so far on many hosts and in many localities. There are published sequences of *G. salaris* specimens on salmon in Lærdalselven, Norway (1 nucleotide difference EU223246, Ziętara *et al*. 2008a); on salmon in Lake Kuito, Russian Karelia (Ziętara *et al*. 2006); on rainbow trout in several countries (invariable RBT clone in Finland, Ziętara *et al*. 2006); and on Arctic charr in Norway (shorter sequence, Robertsen *et al*. 2007).

On the scale of the mitochondrial phylogenetic tree (Fig. 1), the graphic visualization shows that the heterozygous *G. salaris* ITS^{YCYW} and the homozygous ITS^{TCTT} clones in Poland and the Macedonian ITS^{CCCA} and ITS^{CYCA} all belong to a maternal lineage that originated as a species hybrid, but because of backcrossing, at present it contains rather typical *G. salaris* ITS alleles. The unknown maternal ancestor probably also carries a widely divergent ITS that deserves a full species rank if it is ever found.

Discussion

The introgression of mitochondrial DNA between species is not uncommon (Funk and Omland 2003). It leads to the incongruence of mitochondrial and nuclear gene trees, and uncertainty with so-called species trees (Kunz 2002). If a species has a history of introgression, the mitochondrial tree shows polyphyly or paraphyly (Funk and Omland 2003). However, the degree of genetic divergence of the mtDNA sequences that were demonstrated to be introgressed in two cases in the current study is extraordinary.

In the case of the *G. pomeraniae* × *lavareti* hybrid, the future of the heterologous genetic components remains open. If the hybrid backcross to males of *G. lavareti*, the mtDNA will be introgressed into *G. lavareti*. If it backcrosses to the roach parasite, males or females of *G. pomeraniae*, the hybridization episode will be swept away, and will only be detected by the collection of heterologous nuclear markers. It is also possible that only the F_1 hybrid of *G. pomeraniae* × *lavareti* maintains the host recognition system needed to stay on rainbow trout, and the segregating offspring is just lost, without being able to orient to roach or whitefish. Further, it is possible that the F_1 is not

sexually fertile at all. The segregation of ITS was not detected in a large sample from many farms (Kuusela *et al*. 2008).

In the parasite strains treated under the name *G. salaris* in Figure 1, mitochondrial introgression was completed by mating the hybrid female to *G. salaris*. The starting point of this study was to recover the *G. salaris* clone in Poland marked by the same three diagnostic nucleotides described in Denmark (Lindenstrøm *et al*. 2003). The detection of the homozygous ITS^{TCTT} demonstrated that ITS^{YCYW} was a *heterozygous* hybrid, not a lineage slowly crossing the heterogeneous phase between two homogeneous rDNA stages by means of concerted evolution (Polanco *et al*. 2000). The hybrid character of the heterozygote was confirmed by the observation that the clone carried distantly-related alien mtDNA. It was previously demonstrated that the cattle parasite *Schistosoma bovis* and the human parasite *S. haematobium* could naturally hybridize in the Senegal River system. Two hybrid lines were found, an F_1 generation hybrid of *S. haematobium* × *S. bovis* and a backcross, or F_2 generation, of the *S. bovis* × *S. haematobium* hybrid (Huyse *et al*. 2009).

In the current study, several single nucleotide substitutions in ITS, which were sometimes observed in a few specimens, are significant. All DNA sequencing was direct, thus excluding the PCR artefacts. All singletons were confirmed from independent samples, and most of the reported singletons appeared in other localities as well. It should be underscored that these conclusions are not biased by the small number of specimens observed. The molecular markers are qualitative: seeing three variant nucleotides in the same positions in the ITS sequence is absolute evidence of the common descent of the haplotypes (alleles) involved, and similarly, the mitochondrial sequences differing by twenty nucleotides are related, but definitely not identical.

Age of the mitochondrial introgression into G. salaris?

The mitochondrial sequences of *G. salaris* ITS^{CCCA} and ITS^{CYCA} genotypes from Macedonia were closely related but not identical to the Polish farm strains (19 nucleotide substitutions, $d_{MCL} = 0.0126 \pm 0.0052$). If it is justly assumed that distant hybridization is rare, this Polish-Macedonian divergence in the mitochondrial sequence suggests that the alien mtDNA was introduced into the *G. salaris* genome once, but a long time ago. A divergence rate of up to 13% per million years has been suggested for this segment of mtDNA in *G. salaris* (Kuusela *et al*. 2007). Using this value, the hybridization occurred about one hundred thousand years ago (137–57 kyr). There were enough geological disturbances in continental Europe within this time interval that would have permitted salmonids and parasites to meet in moving watersheds on the ice margin. In this case, the host of hybridization was definitely not rainbow trout, which was introduced to Europe only some 150 years ago (Fausch 2007). An alternative scenario that the two alien mitochondrial haplotypes were introduced into the *G. salaris* background twice is less probable since the ITS difference between the Polish and Macedonian strains is so small. However, the case cannot be closed definitively before more salmonid populations from the central and southeastern parts of Europe are screened, or before more nuclear DNA markers are developed.

Two of the Macedonian *G. salaris* specimens had a unique additional Y ambiguity in ITS site 572, indicating sexual reproduction with an unknown male of *G. salaris*. The parasite on Ohrid trout also carried this Macedonian extra T, and was thus heterozygous at site 572 of ITS, as paired with the Polish ITS^{TCTT}. This shuffling might well be modern, i.e., generated in fish farms. Observations show that there might be *G. salaris* strains carrying as yet unidentified combinations of mtDNA among European salmonid populations in the wild or in the fish farming network.

The unknown maternal ancestor of the alien mtDNA clade of *G. salaris* described here deserves a species rank and name, when it is found (Kunz 2002). A candidate name is available among salmonid parasites – *G. bohemicus* Ergens, which was described from rainbow trout farms in the Czech Republic and Slovakia (Ergens 1983). Unfortunately, this species was not recorded in two comprehensive molecular surveys of *Gyrodactylus* in the Czech Republic (Matějusová *et al*. 2001, 2003). Therefore, *G. bohemicus* can be any of the new salmonid parasite forms separated after the application of molecular methods, e.g., *G. teuchis* (Cunningham *et al*. 2001), or it might even be of the lineage with alien mtDNA itself, as discussed by Lindenstrøm *et al*. (2003). Seven different molecular strains representing the species *G. salaris*, *G. truttae*, *G. derjavinoides*, and *G. teuchis* were found in Polish farms (Rokicka *et al*. 2007). With the exception of *G. teuchis*, they were all excluded from this maternity case. However, it would be of interest to extend the search for the donor of the alien maternal mitochondria to other host fish species; however, as Ziętara and Lumme (2002) demonstrated, host-switching is common in this genus, which means that the ancestor could be one of many other species. Thus, the quest to find it would be like searching for a needle in a haystack.

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