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Speciation and host–parasite relationships in the parasite genus *Gyrodactylus* (Monogenea, Platyhelminthes) infecting gobies of the genus *Pomatoschistus* (Gobiidae, Teleostei)[☆]

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Abstract

Using species-level phylogenies, the speciation mode of *Gyrodactylus* species infecting a single host genus was evaluated. Eighteen *Gyrodactylus* species were collected from gobies of the genus *Pomatoschistus* and sympatric fish species across the distribution range of the hosts. The V4 region of the *ssrRNA* and the internal transcribed spacers encompassing the 5.8S rRNA gene were sequenced; by including published sequences a total of 30 species representing all subgenera were used in the data analyses. The molecular phylogeny did not support the morphological groupings into subgenera as based on the excretory system, suggesting that the genus needs systematic revisions. Paraphyly of the total *Gyrodactylus* fauna of the gobies indicates that at least two independent colonisation events were involved, giving rise to two separate groups, belonging to the subgenus *Mesonephrotus* and *Paranephrotus*, respectively. The most recent association probably originated from a host switching event from *Gyrodactylus arcuatus*, which parasitises three-spined stickleback, onto *Pomatoschistus* gobies. These species are highly host-specific and form a monophyletic group, two possible ‘signatures’ of co-speciation. Host specificity was lower in the second group. The colonising capacity of these species is illustrated by a host jump from gobiids to another fish order (Anguilliformes), supporting the hypothesis of a European origin of *Gyrodactylus anguillae* and its intercontinental introduction by the eel trade. Thus, allopatric speciation seems to be the dominant mode of speciation in this host–parasite system, with a possible case of sympatric speciation. © 2003 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

Keywords: Host specificity; Coevolution; Host switching; Host–parasite evolution; Internal transcribed spacers rDNA; Phylogeny; *Pomatoschistus*

1. Introduction

The relative contribution of the various modes of speciation can be assessed using species-level phylogenies with inclusion of the geographical distribution of sister taxa (Barraclough and Nee, 2001). However, in the case of free-living animals, the range of species can change rapidly, such that the observed range might not reflect the actual speciation mode. The niche of parasites is, by the nature of their life-style, relatively fixed, providing a more straightforward framework for studying speciation processes. Since parasites are dependent on their hosts,

speciation in the latter is likely to induce speciation in the parasite, resulting in mirror-image phylogenies referred to as Fahrenholz’s rule (Page, 1994; Page and Hafner, 1996; Poulin, 1998). However, parasites should not always be regarded as ‘passive’ members of the association; some taxa can also move independently from their hosts and actively switch hosts. Whereas co-speciation can be regarded as allopatric vicariant speciation, host switching can be regarded as peripatric or peripheral isolate speciation (Brooks and McLennan, 1993). A third phenomenon, sympatric speciation, is gaining recognition as an alternative speciation mode, operating under well-specified circumstances (Dieckmann and Doebeli, 1999; Via, 2001). Parasite groups belonging to the Monogenea meet many of these conditions (Poulin, 2002). Despite various interesting characteristics that render them an ideal study target, monogeneans have been rarely studied within this context.

[☆] Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers AY338429–AY338454 and AY339762–AY339776.

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The Monogenea is one of the largest groups of Platyhelminthes, characterised by high species diversity and high host specificity (Gusev, 1995; Kearns, 1994; Poulin, 1998). The direct life-cycle leads to auto-infection of the host, which means that they experience only one adaptive barrier in their life (Gusev, 1995), in contrast to other flatworm parasites that require one or more intermediate hosts. Members of the genus *Gyrodactylus* are hyperviviparous. Embryos develop within each other inside the mother's uterus and asexual reproduction alternates with sexual reproduction (Cable and Harris, 2002). This rapid reproduction in close relationship with the host, together with the high host specificity is thought to promote co-evolution between host and parasite (Humphrey-Smith, 1989; Page and Hafner, 1996). At first sight, the absence of a free-living larval stage might decrease the chance to encounter other host species, minimising the opportunities for host switching. However, it has been shown that gyrodactylids can survive for short periods independent of their host and a 'swimming behaviour' has been described (Cable et al., 2002). This 'active' dispersion capacity of *Gyrodactylus* in combination with the ability to produce a viable deme from only one pregnant individual might increase the chance for speciation by host switching. Brooks and McLennan (1993) state that auto-infection and the high level of host specificity might also enhance sympatric speciation. The succession of several generations on a single host specimen ensures the continuity of a population, but increases the chance of inbreeding. In spite of these life history traits known to promote sympatric speciation, limited evidence is available from the literature. Gusev (1995) presents numerous examples where congeneric doublets are likely to be the result of sympatric speciation, but without any molecular evidence. Molecular studies have been carried out on site-specific polystome monogeneans by Littlewood et al. (1997), but they did not find any evidence for speciation on the same host species. In conclusion, all speciation modes appear equally probable in this group, making *Gyrodactylus* an ideal candidate for speciation studies.

It is of special interest to focus on fine-scale parasite associations within a single host genus or within one group of sympatric host species. It is here that speciation takes place and all possible sister species and host transfer routes are expected to be included in the analysis. This study focuses on *Gyrodactylus* spp. parasitising gobies of the genus *Pomatoschistus* Gill 1864, which is the dominant gobiid genus of the Atlantic and Mediterranean coasts of Europe comprising about ten species (Miller, 1986). Until now, not much attention has been paid to their role as a host for *Gyrodactylus*. Four *Gyrodactylus* species are known to parasitise *Pomatoschistus* spp.: *Gyrodactylus longidactylus* Geets et al., 1998 (on the gills of *Pomatoschistus lozanoi*), *Gyrodactylus rugiensis* Gläser, 1974a (on the fins of *Pomatoschistus microps* and *Pomatoschistus minutus*), *Gyrodactylus micropsi* Gläser, 1974a (on the fins and gills of *P. microps* and *P. minutus*), *Gyrodactylus rugiensoides*

Huyse and Volckaert, 2002 (on the fins of *P. minutus*, *P. lozanoi* and *Pomatoschistus pictus*), and two unidentified species, one on *Pomatoschistus norvegicus* and *P. minutus* (Llewellyn et al. 1984) and one on *P. minutus* (Appleby, 1996). Furthermore, several other undescribed *Gyrodactylus* spp. are suspected to live on *Pomatoschistus* spp. (Geets, 1998). Therefore, we first made an inventory of the *Gyrodactylus* fauna parasitising the *Pomatoschistus* gobies and assessed their degree of host specificity (Huyse and Volckaert, 2002). Secondly, a robust phylogeny of both hosts and parasites is required. In this paper we focus on the parasites; interrelationships between the present *Gyrodactylus* species were inferred by using the V4 region of the ssrRNA and the complete ITS rDNA region. These markers consist of variable and conservative regions, which make them an ideal target to compare both closely as well as distantly related species (Hillis et al., 1996). This made it possible to test hypotheses on host–parasite evolution without the need for a host phylogeny: (1) *Gyrodactylus* spp. found on gobies of the genus *Pomatoschistus* are monophyletic; (2) congeners on the same host species or with the same niche are each other's closest relative; (3) *Gyrodactylus* spp. cluster according to their excretory system as defined by Malmberg (1970, 1998). The first scenario is expected under a mode of speciation through co-evolution. To test this, the fauna of sympatric non-*Pomatoschistus* species will be compared to that of *Pomatoschistus* spp. and all available sequences will be included in the phylogenetic analyses. The second hypothesis provides additional information on the mode of speciation within one host species, i.e. intra-host speciation, or speciation by host switching. Finally, the molecular phylogeny is used to evaluate the evolution of specific morphological traits as described by Malmberg (1970, 1998).

2. Material and methods

2.1. Collection of material, morphological determination and DNA extraction

Samples were collected along the coastlines of the English Channel and the North Sea up to Norway and in the Baltic Sea, during 1999–2002 (Table 1). In addition, *Gyrodactylus* spp. were sampled from the two-spotted goby *Gobiusculus flavescens* (Fabricius, 1779), European plaice, *Pleuronectes platessa* Linnaeus 1758, three-spined stickleback, *Gasterosteus aculeatus* Linnaeus 1758, nine-spined stickleback, *Pungitius pungitius* Linnaeus 1758, sea stickleback, *Spinachia spinachia* Linnaeus 1758 and the black goby, *Gobius niger* Linnaeus 1758. Fish were brought live to the laboratory and immediately screened for *Gyrodactylus*-infection using a stereo-microscope. Whenever possible, the attachment organ (opisthaptor) was separated from the body enabling simultaneous morphological and molecular analyses. The body was then placed in 5 µl of milli-Q water

Table 1
Collection sites, subgenus, respective hosts and location on the host of the *Gyrodactylus* species sequenced

Species	Subgenus	Host	Collection site	Country	Site on host	N
<i>Gyrodactylus</i> sp. 1 ^a	<i>Mesonephrotus</i>	<i>Pomatoschistus minutus</i>	Ostend, North Sea	Belgium	G/F	AF328866
<i>Gyrodactylus</i> sp. 1 ^a			Texel, Yerseke	The Netherlands	G/F	5
<i>Gyrodactylus</i> sp. 1 ^a			Trondheim	Norway	F	2
			Bergen	Norway	G	2
<i>Gyrodactylus</i> sp. 1 ^a	<i>Mesonephrotus</i>	<i>Pomatoschistus lozanoi</i>	North Sea	Belgium	G/F	2
<i>Gyrodactylus</i> sp. 1 ^a			Texel	The Netherlands	G/F	3
<i>Gyrodactylus</i> sp. 2 ^a	<i>Mesonephrotus</i>	<i>Pomatoschistus pictus</i>	Bergen	Norway	G/F	5
<i>Gyrodactylus</i> sp. 3	<i>Mesonephrotus</i>	<i>Pomatoschistus norvegicus</i>	Bergen	Norway	G	3
<i>Gyrodactylus</i> sp. ^b	<i>Mesonephrotus</i>	<i>Pomatoschistus microps</i>	Ostend, North Sea	Belgium	G	4
<i>Gyrodactylus</i> sp. ^b			Texel, Yerseke	The Netherlands	G	2
<i>Gyrodactylus</i> sp. ^b			Ambleteuse	France	G	2
<i>Gyrodactylus</i> sp. 4	<i>Mesonephrotus</i>	<i>Pomatoschistus microps</i>	Ostend, North Sea	Belgium	F	4
<i>Gyrodactylus</i> sp. 4			Texel, Yerseke	The Netherlands	F	2
<i>Gyrodactylus</i> sp. 4			Ambleteuse	France	F	2
<i>Gyrodactylus</i> sp. 5	<i>Mesonephrotus</i>	<i>Gobiusculus flavescens</i>	Trondheim	Norway	G/F	4
			Bergen	Norway	G	4
<i>Gyrodactylus</i> sp. 6	<i>Mesonephrotus</i>	<i>Gyrodactylus flavescens</i>	Trondheim	Norway	F	1
			Bergen	Norway	F	F
<i>Gyrodactylus rugiensis</i> ^c	<i>Paranephrotus</i>	<i>Pomatoschistus microps</i>	Ostend, North Sea	Belgium	F	AF328870
<i>Gyrodactylus rugiensis</i> ^c			Texel, Yerseke	The Netherlands	F	AF328870
			Èdeso, Stockholm	Sweden	F	2
			Ambleteuse	France	F	2
<i>Gyrodactylus rugiensis</i> ^c			Ostend, North Sea	Belgium	F	AJ427414
<i>Gyrodactylus rugiensoides</i> ^d	<i>Mesonephrotus</i>	<i>Pomatoschistus minutus</i>	Texel, Yerseke	The Netherlands	F	AJ427414
<i>Gyrodactylus rugiensoides</i> ^d			Bergen	Norway	F	AJ427414
<i>Gyrodactylus rugiensoides</i> ^d	<i>Mesonephrotus</i>	<i>Pomatoschistus lozanoi</i>	North Sea	Belgium	F	AJ427414
<i>Gyrodactylus rugiensoides</i> ^d			Texel	The Netherlands	F	AJ427414
<i>Gyrodactylus rugiensoides</i> ^d	<i>Mesonephrotus</i>	<i>Pomatoschistus pictus</i>	Bergen	Norway	F	AJ427414
<i>Gyrodactylus microps</i> ^c	<i>Mesonephrotus</i>	<i>Pomatoschistus microps</i>	Doel; North Sea	Belgium	G	AF328868
<i>Gyrodactylus</i> cf. <i>micropsi</i>	<i>Mesonephrotus</i>	<i>Pomatoschistus minutus</i>	Texel	The Netherlands	F	AJ427221
<i>Gyrodactylus</i> cf. <i>micropsi</i>	<i>Mesonephrotus</i>	<i>Pomatoschistus lozanoi</i>	Texel	The Netherlands	F	AJ427221
<i>Gyrodactylus</i> cf. <i>micropsi</i> 1	<i>Mesonephrotus</i>	<i>Pomatoschistus lozanoi</i>	Texel	The Netherlands	F	2
<i>Gyrodactylus</i> cf. <i>micropsi</i> 2	<i>Mesonephrotus</i>	<i>Pomatoschistus lozanoi</i>	Texel	The Netherlands	F	2
<i>Gyrodactylus</i> cf. <i>longidactylus</i>	<i>Mesonephrotus</i>	<i>Pomatoschistus norvegicus</i>	Bergen	Norway	G	5
<i>Gyrodactylus arcuatus</i> ^e	<i>Mesonephrotus</i>	<i>Gasterosteus aculeatus</i>	Doel	Belgium	?	AF328865
<i>Gyrodactylus arcuatus</i> ^e	<i>Mesonephrotus</i>	<i>Gyrodactylus aculeatus</i>	Èdeso, Stockholm	Sweden	F	1
<i>Gyrodactylus arcuatus</i> ^e	<i>Mesonephrotus</i>	<i>Pungitius pungitius</i>	Èdeso, Stockholm	Sweden	F	1
<i>Gyrodactylus</i> <i>pungitii</i> ^f	<i>Limnonephrotus</i>	<i>Pomatoschistus pungitius</i>	Èdeso, Stockholm	Sweden	F	1
<i>Gyrodactylus rarus</i> ^f	<i>Metanephrotus</i>	<i>Spinachia spinachia</i>	Trondheim	Norway	G	3
<i>Gyrodactylus flesi</i> ^c	<i>Paranephrotus</i>	<i>Pleuronectes platessa</i>	Trondheim	Norway	F	2
<i>Gyrodactylus flesi</i> ^c	<i>Paranephrotus</i>	<i>Pomatoschistus platessa</i>	Bergen	Norway	F	1
<i>Gyrodactylus niger</i> sp.	?	<i>Gobius niger</i>	North Sea	Belgium	G	3
<i>Gyrodactylus lotae</i>	<i>Paranephrotus</i>	<i>Lota lota</i>	Oulanka	Finland	G/F	AY061978
<i>Gyrodactylus alexgusevi</i>	<i>Paranephrotus</i>	<i>L. lota</i>	Oulu	Finland	F	AY061979
<i>Gyrodactylus truttae</i>	<i>Limnonephrotus</i>	<i>Salmo truttae</i>	Vlára river	Czech Republic	F	AJ132260
				Scotland		AJ407913
<i>Gyrodactylus salaris</i>	<i>Limnonephrotus</i>	<i>Salmo salar</i>	Fish farm	Finland	F	AF328871
						Z26942
<i>Gyrodactylus teuchis</i>	<i>Limnonephrotus</i>	<i>Oncorhynchus mykiss</i>	Brittany	France	F	AJ249349
						AJ249350
<i>Gyrodactylus gasterostei</i>	<i>Limnonephrotus</i>	<i>Gyrodactylus aculeatus</i>	Overpelt	Belgium	F	AF328867
<i>Gyrodactylus pungitii</i>	<i>Limnonephrotus</i>	<i>Pomatoschistus pungitius</i>	Overpelt	Belgium	F	AF328869
<i>Gyrodactylus branchicus</i>	<i>Metanephrotus</i>	<i>Gyrodactylus aculeatus</i>	Doel	Belgium	F	AF156669
<i>Gyrodactylus anguillae</i>	<i>Neonephrotus</i>	<i>Anguilla anguilla</i>		Spain/Australia	G	AB063294
<i>Gyrodactylus nipponensis</i>	?	<i>Anguilla japonica</i>	Lake Hamana	Japan	G	AB063295
<i>Gyrodactylus elegans</i>	<i>Gyrodactylus</i>	<i>Blicca bjoerkna</i>	Morava river	Czech Republic	F	AJ40792
						AJ407870

Sampling month/year are as follows: Belgium (Ostend, 8/1999; North Sea, 10/99 and 10/2001), The Netherlands (Texel, 06/99, 11/00; Yerseke: 11/99), France (09/99), Sweden (06/2001) and Norway (Trondheim, 06/2000; Bergen, 06/2000 and 05/2001). All extra *Gyrodactylus* sequences included in the analysis are shown with the respective GenBank accession number. N, number of specimens per species sequenced (ITS) or accession number (for the V4 region 2–3 specimens were sequenced). G, gills; F, fins; ?, the subgenus status or the site on host from the respective *Gyrodactylus* species is presently unknown from the literature. References to morphological descriptions:

^a Geets et al. (1999);

^b Geets (1998);

^c Gläser (1974a);

^d Huyse and Volckaert (2002);

^e Bychowski (1933);

^f Malmberg (1970).

and stored at -20°C . The opisthaptor was fixed in ammonium picrate glycerin as described by Malmberg (1970) to examine the haptor sclerites by phase contrast microscopy. DNA extractions were performed as described by Ziętara et al. (2002).

2.2. Amplification and sequencing of the ITS and the V4 region

Approximately 900–1200 bp of the rDNA complex, spanning the 3' end of the 18S subunit, the internal transcribed spacer 1 (ITS1), the 5.8S subunit, ITS2, and the 5' end of the 28S subunit were amplified for three to 15 specimens per species (Table 1). Amplification and sequencing were performed as described by Ziętara et al. (2002). A negative control was included in each PCR reaction. Sequences were verified by comparing each sequence with its complement, and in case of ambiguities, the sequencing reaction was repeated.

For the initial amplification of the V4 region (354 bp), the primers V4F and V4R designed by Cunningham et al. (1995) were used. Since the PCR products were not specific enough, new primers V4f2 (5'-GAGGGCAGTCTGGT-GCC-3') and V4r2 (5'-CAGGCTTCAAGGCCTGC-3') were designed, located 6 and 5 bp, respectively, inwards from the original primers. The amplification reactions consisted of 1 × polymerase chain reaction (PCR) buffer (Eurogentec, Seraing, Belgium), 1.75 mM MgCl₂ (Eurogentec), 200 μM of each dNTP (Amersham Biosciences, Sweden), 1 μM of each primer (Eurogentec), 2 μl lysate, 1 unit *Taq* polymerase (Eurogentec) and milli-Q water. The mixtures of 20 μl were layered with mineral oil, heated for 4 min at 97 °C and subjected to 35 cycles as follows: 95 °C for 1 min, 60 °C for 30 s and 72 °C for 30 s, followed by a final extension step at 72 °C for 7 min. The PCR products were visualised using ethidium bromide on a 1.2% agarose gel and purified by means of GFX columns according to the manufacturer's instructions (Amersham Pharmacia). The purified products were cloned following the manufacturer's instructions (TA cloning system, Invitrogen). The PCR products of the cloned products were purified by means of GFX columns and sequenced in both directions. Sequencing was done following the protocol of SequiTherm EXCEL II (Epicentre Technologies). The reaction products were separated on a LICOR 4200 system and visualised on a 6% Long Ranger gel (FMI BioProducts). For each species, 2–3 individual specimens were cloned and two clones per specimen were sequenced.

2.3. Sequence alignment

The ITS region shows substantial variation between *Gyrodactylus* spp. from different subgenera (Ziętara et al., 2002). Therefore sequences were aligned using the program SOAP 1.0 (Löytynoja and Milinkovitch, 2001). SOAP generates alternative CLUSTAL W alignments by using all

possible combinations of gap opening penalties, ranging from e.g. 4 to 10 and gap extension penalties ranging from e.g. 3 to 7. The program then identifies the 'unstable-hence-unreliable' positions by comparing the different alignments. Such ambiguous regions were then excluded and the file exported in NEXUS format. The same sites were re-included using PAUP* v. 4.01b (Swofford, 2001) to assess the impact of these unstable sites on our phylogeny. Exclusion of the unstable characters in the V4, 5.8S and ITS2 alignment resulted in a 675 bp fragment. An alternative evaluation of the alignment by means of dot plots implemented in the GeneWorks software (Intelligence, Oxford, UK) resulted in a very similar fragment of 690 bp. To compare relative speciation dates, the following sequences from GenBank were included: *Gyrodactylus truttae* (AJ132260, AJ407913), *Gyrodactylus salaris* (AF328871, Z26942) and *Gyrodactylus teuchis* (AJ249349, AJ249350). Finally, to analyse the evolutionary relationships between *Gyrodactylus* spp. collected from *Pomatoschistus* and *Gobiusculus* spp. and sympatric host species, and to study the evolution of specific morphological characters, the following GenBank sequences were added: *Gyrodactylus arcuatus* (AF328865), *Gyrodactylus branchicus* (AF156669), *Gyrodactylus gasterostei* (AF328867), *Gyrodactylus pungitii* (AF328869), *Gyrodactylus anguillae* (AB063294), *Gyrodactylus nipponensis* (AB063295), *Gyrodactylus lotae* (AY061978), *Gyrodactylus alexgusevi* (AY061979), *Gyrodactylus elegans* (AJ407920, AJ407870), *Gyrdicotylus* (AJ001843) and *Gyrodactyloides bychowskii* (AJ249348). Since the V4 region of those species was not available, this second dataset consisted of 5.8S and ITS2 sequences only. Again, SOAP was used to remove the unstable regions resulting in a 330 bp fragment.

2.4. Phylogenetic analyses

The V4, 5.8S and ITS sequences were treated as one dataset since the incongruence-length difference test (Farris et al., 1995) implemented in PAUP* provided no evidence for significant difference in the phylogenetic signal of both regions. Plotting transitions and transversions against divergence using DAMBE v4.0.75 (Xia and Xie, 2001) did not show saturation. *Gyrodactyloides bychowskii* was used as outgroup in the 5.8S and ITS2 dataset, but no sequence of the V4 region of this species was available. Therefore we implemented midpoint rooting for the V4-5.8S-ITS2 dataset. A likelihood-ratio test (LRT) showed that this tree was not significantly worse than the unrooted tree. First, a consensus tree was made from the topologies obtained by TREE-PUZZLE 5.0 (Schmidt et al., 2002), maximum parsimony (MP), maximum likelihood (ML) and neighbour-joining (NJ) using PAUP*. The consensus tree was used as input tree in the PAUP* command block from ModelTest 3.06 (Posada and Crandall, 1998). The parameters and likelihood scores were estimated upon

the consensus tree, and then the program uses the likelihood scores (LK) to select the model of DNA evolution that best fits the data. The parameters estimated under this best-fit model were entered in the ML search and nearest-neighbour-interchange branch swapping was performed. The respective parameters were optimised upon the tree through successive iteration. Trees were statistically tested by calculating *P* values for the ML tree. MP trees were inferred with the branch and bound algorithm. In these analyses gaps were treated successively as fifth base and as missing data, all sites were equally weighted and empirical transition:transversion (ti/tv) ratios were applied; 2:1 for the 5.8S and V4 region and 1:2 for ITS2. The minimum-evolution search was conducted (1000 replicates of tree-bisection reconnection branch swapping) from a matrix of ML genetic distances calculated under the optimised model. The base composition for all sequences was compared using a 5% χ^2 -test on the average composition (TREE-PUZZLE). The molecular-clock hypothesis was tested assuming the HKY model (Hasegawa et al., 1985) and γ -distributed rates across sites, with the likelihood ratio test for the clock hypothesis implemented in TREE-PUZZLE.

3. Results

3.1. Inventory of the *Gyrodactylus* fauna on gobies of the genus *Pomatoschistus*

In total, 71 complete ITS1-5.8S-ITS2 sequences of 18 species from six localities and 41 V4 sequences of 15 species were obtained (GenBank accession numbers AY338429–AY338454 and AY339762–AY339776). Table 1 lists the species with information on their subgenus status, their respective host and site on the host, geographic locality and the species included from GenBank. The 13 parasite species found on *Pomatoschistus* spp. clustered in two groups (A and B, see Fig. 1) differing about 24.8–28.7%, based on the V4-5.8S-partial ITS2 gamma-corrected distances. These groups are readily distinguished from each other since their ITS sequences differ by about 200 bp in length. None of these species were found on non-*Pomatoschistus* fish species examined in this study. Note that, although *G. flavescens* is placed in another genus by Fabricius, it is considered here a member of the genus *Pomatoschistus* as based on ITS1 rDNA, 12S and 16S mtDNA sequences (unpublished data). Within each group, genetic differentiation was much lower, ranging from 0.4 to 14% (uncorrected p-distances, complete ITS region).

Some of the species of group A have been described by Geets et al. (1999), but none of them have been named. They showed, by means of multivariate analyses on morphometric data of 17 anchor features, that each group could be separated by their host species. A combined morphometric and molecular sequencing analysis has been carried out to describe these species (in progress). They all

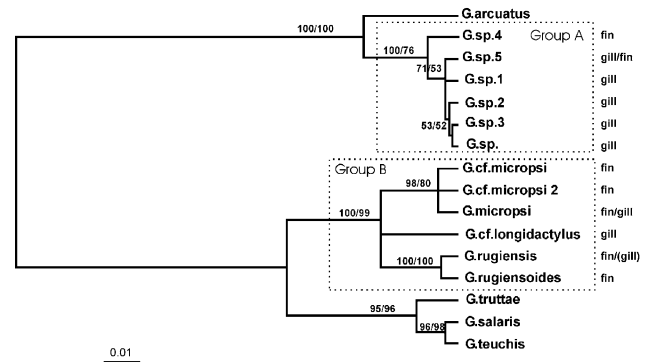


Fig. 1. Ultrametric tree constructed of sequences from the V4 ssrRNA region, 5.8S rRNA gene and partial ITS2 rDNA for 16 *Gyrodactylus* species. Bootstrap values are shown for the maximum parsimony/neighbour-joining analyses. *Gyrodactylus* spp. found on *Pomatoschistus* hosts separate into two groups (A and B), which are boxed with dotted lines; their site on the host is given.

belong to the subgenus *Mesonephrotus*. Each species was recorded from only one host species, except for *Gyrodactylus* sp. 1 that was found on both *P. minutus* and *P. lozanoi*. Group B contains three undescribed species/subspecies. It remains to be studied, by means of morphological and morphometric studies, whether the particular specimens found in this study represent actual species or not. One of them is only found on gills of *P. norvegicus*, hereafter referred to as *G. cf. longidactylus*, because of its morphological resemblance to *G. longidactylus* found on the gills of *P. lozanoi*. The other two are hereafter referred to as *G. cf. micropsi* 1 and *G. cf. micropsi* 2, respectively, in accordance with their morphological and genetical (ITS and V4 region) similarity to *G. micropsi*. They all belong to the subgenus *Paranephrotus*. More than one species of Group B was found on the same host, and some hosts shared the same species (see Table 1). Only *G. micropsi*, *G. rugiensis* and *G. cf. longidactylus* were recorded from a single host species. *Gobiusculus flavescens* was infected with *Gyrodactylus* sp. 5 and another species, of which only two specimens were found. The ITS1 region was identical to that of *Gyrodactylus* sp. 1 while the ITS2 region was identical to *Gyrodactylus* sp. 2. No mixing was possible since ITS1 and ITS2 were amplified at the same time, and sequence reactions were repeated twice. This species was excluded from the dataset since it would confound phylogenetic analyses.

A *Gyrodactylus*–*Pomatoschistus* association was found all over the distribution range of the host, but not all species were recorded at every location. *Pomatoschistus minutus* and *P. micropsi* have the widest distribution and were thus most frequently sampled. As a consequence, their *Gyrodactylus* fauna (*G. rugiensis*, *G. rugiensoides*, *Gyrodactylus* sp. and *Gyrodactylus* sp. 1) had the widest geographic record. Table 2 shows the geographic variation found in the ITS sequences expressed as pair-wise numbers of transitions, transversions and insertions/deletions. No variation was found in the V4 region. *Gyrodactylus arcuatus* sampled

Table 2

Intraspecific variation in the internal transcribed spacer rDNA region of *Gyrodactylus* spp. collected in the North Sea: by means of pairwise sequence comparison

<i>Gyrodactylus</i> species	Sequence comparison	Ti	Tv	Insertion/deletion events
<i>Gyrodactylus</i> sp. 1	Trondheim–Belgium	–	–	–
<i>Gyrodactylus rugiensis</i>	Belgium–Stockholm	1	–	2 (1 and 3 bp)
<i>Gyrodactylus rugiensis</i>	Trondheim–Belgium	–	–	–
<i>Gyrodactylus rarus</i>	Trondheim–Finland	2	–	–
<i>Gyrodactylus arcuatus</i>	Stockholm–Belgium	1	–	–
<i>Gyrodactylus pungitii</i>	Stockholm–Belgium	1	–	–
<i>Gyrodactylus pungitii</i>	Stockholm–Finland	–	–	–

Ti, transitions; Tv, transversions.

from *Gasterosteus aculeatus* and *P. pungitius* from Stockholm (Sweden) were identical and differed in one transition in the ITS2 region compared to *G. arcuatus* from Doel (GenBank, AF328865).

3.2. Phylogeny reconstruction

The 5.8S gene and the V4 region were least variable and p-distances between the gyrodactylids found on *Pomatoschistus* species ranged from 0–5.8% and 0–22.2%, respectively. The latter region showed four insertion/deletion events of one bp and one of three bp long. The ITS region was much more variable, a difference of 200 bp was found between Group A and B. Due to the large insertions/deletions, both groups could be easily separated by eye based on the alignment alone. The ITS p-distances within group A and B ranged from 0.4 to 14.2%. Whereas the dataset of Ziętara et al. (2002) suffered from deviating base composition (p-value: 37–99%) the V4-5.8S-ITS2 dataset created with SOAP had a base composition p-value of 70–92% and yielded 118 parsimony informative sites.

Modeltest selected the HKY + Γ_4 model (Hasegawa et al., 1985) with gamma shape parameter (α) = 0.3. After optimisation, the general reversible model (REV, Rodriguez et al., 1990) was selected (LRT $P < 0.001$), with rate matrix = (2.1 7.6 4.3 1.4 7.7) and $\alpha = 0.3$; ln L = -1902.75. The molecular clock was not rejected; Fig. 1 shows the clock tree of the V4-5.8S-ITS2 dataset (675 bp). The topology and bootstrap values are more or less in agreement with the trees generated by NJ and MP (118 parsimony informative sites; tree length: 914; consistency index (C.I.) = 0.81; retention index (R.I.) = 0.94); bootstrap values are shown in Fig. 1. Only the position of *G. cf. longidactylus* depended on the tree-building method used: NJ and ML grouped *G. cf. longidactylus* together with *G. micropsi*, *G. cf. micropsi* and *G. cf. micropsi* 1, while MP clustered it with *G. rugiensis* and *G. rugiensoides*. When the unstable alignment positions were re-included in the analysis or when gaps were treated as fifth character, the number of parsimony informative sites increased to 136 and 126, respectively, although this did not affect the topology; the bootstrap values varied only slightly.

The speciation events within both groups are relatively recent compared to those in the *Limnephrotus* group (Fig. 1). Note that only *G. salaris*, *G. teuchis* and *G. truttiae* are included since Bakke et al. (2002) argue that *G. salaris* speciated during the last ice age (see Section 4); a thorough phylogenetic analysis of *Limnephrotus* spp. has been carried out by Ziętara and Lumme (2002). Members of Group A have about the same relative age as *G. salaris*–*G. teuchis* while diversification among Group B appears more ancient. The analysis of the combined 5.8S and ITS2 region (330 bp, see Fig. 2) included all *Gyrodactylus* spp. found on the sympatric host species (Table 1); *Gyrodactyloides bychowskii* was used as outgroup. The likelihood ratio clock test showed a significant increase in the log-likelihood of the non-clock tree and TREE-PUZZLE showed that the base composition was not homogenous. In this case including or excluding ‘unstable alignment positions’ did affect phylogeny reconstruction, but only with respect to the clustering within each subgenus. The REV + Γ_4 model with gamma shape parameter = 0.7 was selected. Fig. 2 shows the NJ tree but an identical topology was obtained by TREE-PUZZLE and MP (213 parsimony informative sites when gaps were treated as a fifth character; C.I. = 0.62; R.I. = 0.85). The position of *G. flesi* and the unidentified species found on *G. niger* could not be resolved; both branched off at the base of the *Mesonephrotus* - *Metanephrotus* clade. None of the *Gyrodactylus* spp. found on the sympatric host species clustered in Group A while *G. anguillae* from European eel clustered within Group B.

3.3. Mapping morphological traits

Diagnostic morphological characters as defined by Malmberg (1970) were mapped onto the phylogenetic tree constructed from the partial 5.8S and ITS2 dataset (Fig. 2). *Gyrodactylus branchicus* infecting the gills of *Gasterosteus aculeatus* appeared to be very closely related to *G. rarus* found on *Spinachia spinachia* in Trondheim (ITS2, p-distance of 1.7%). They belong to the subgenus *Metanephrotus*. Both species clustered with *G. lotae* and *G. alexgusevi*, both belonging to *Paranephrotus*, forming a sister group to *Mesonephrotus* and *G. nipponensis*. *G. flesi* had a very distinct ITS sequence and clustered outside *Paranephrotus*,

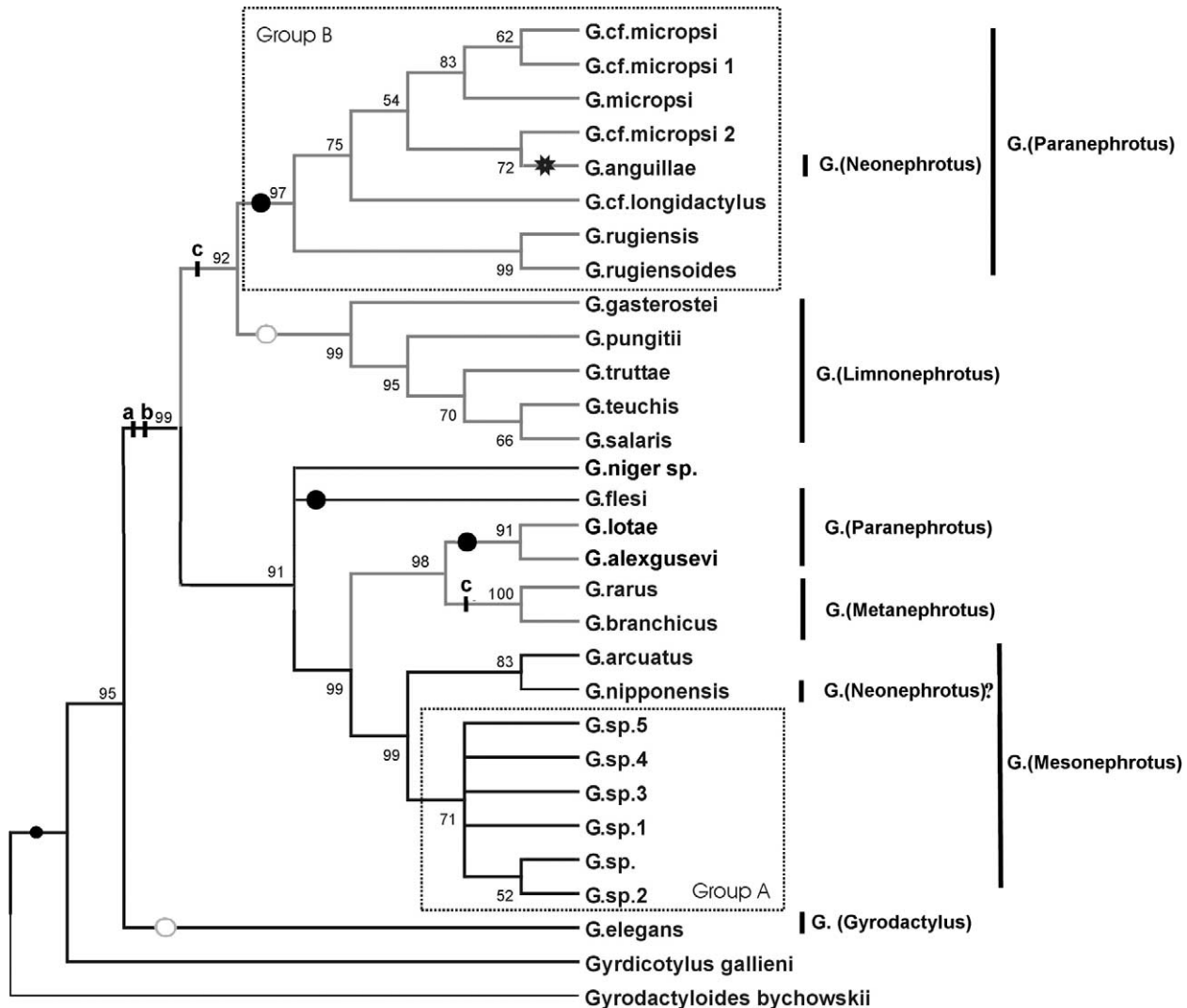


Fig. 2. Neighbour-Joining tree based on a 330 bp fragment of 5.8S rRNA and partial ITS2 rDNA sequences of representatives of all six *Gyrodactylus* subgenera (only transversions were taken into account). *Gyrodactylus* spp. found on *Pomatoschistus* hosts are boxed with dotted lines. Note that *Gyrodactylus anguillae* clusters within Group B although it infects European eel; all other *Gyrodactylus* spp. found on the sympatric fish hosts cluster outside Group A and B. Diagnostic features of the excretory system (Malmberg, 1970) are mapped on the tree. a: reduced number of flame bulbs; b: reduced number of lateral flame cells; c: no lateral flame cells. The size of the circle refers to the size of the bladders: small, large, absent (open circle) or constantly pumping (star). The subgenus status of *Gyrodactylus nipponensis* and *Gyrodactylus niger* sp. is not yet clear, as no information on the excretory system is available.

to which it is currently assigned. The remaining *Paranephrotus* species appeared as a sister group to the subgenus *Limnonephrotus*, which was monophyletic. *Gyrodactylus nipponensis* and *G. anguillae* from *Anguilla japonica* and *A. anguilla* respectively, were firmly joined with *Mesonephrotus* and *Paranephrotus* respectively. *Gyrodactylus elegans* branched off earlier than the other *Gyrodactylus* spp.; *Gyrdicotylus gallieni* clustered most basal.

4. Discussion

Fine-scale associations of the parasite *Gyrodactylus* within a single host genus were examined. Species-level phylogenies constructed from V4 region and complete ITS rDNA sequences were used to assess the contribution of

the different speciation modes. First, we made an inventory of the different parasite species infecting the gobies. Sampling has been extended over time and space ensuring that most possible sister species and host transfer routes were included in the analyses.

4.1. Inventory of the *Gyrodactylus* fauna on *Pomatoschistus* gobies

A total of 13 *Gyrodactylus* species were found on gobies of the genus *Pomatoschistus* and *Gobiusculus*. Only some of them have been described (Gläser, 1974a; Geets et al., 1999; Huyse and Volckaert, 2002); other species descriptions are in progress. The *Gyrodactylus* fauna could be separated into two groups; group A comprised species belonging to the subgenus *Mesonephrotus* while the species of Group B

belonged to the subgenus *Paranephrotus*. At first sight it seems that the site of infection is constrained by phylogeny: Group A is mainly found on gills, while Group B mostly infects fin and skin. However, *Gyrodactylus* sp. 4 of Group A was exclusively found on fins, while *G. cf. longidactylus* mainly infected gills, in contrast to the other members of Group B. Hence, the niche of *Gyrodactylus* spp. can switch on a relatively short evolutionary timescale. The pair-wise genetic distances within both groups were rather low (0.4–14%, p-distances from complete ITS region) compared to those reported in the literature (1.5–38.7% for ITS2, Kimura distances from Matejusova et al., 2001). However, our species were sampled from closely related hosts whereas in the latter study species were collected from different fish families. Thus, in this particular system it appears that closely related hosts are parasitised by closely related *Gyrodactylus* species (0.4–14%).

A *Gyrodactylus-Pomatoschistus* association was found all over the study area. As reported for other *Gyrodactylus* spp. (Ziętara et al., 2000, 2002; Matejusova et al., 2001), the geographic variation was very low: one or two point mutations in ITS2 and two instances of an insertion/deletion event of 1–3 bp in the ITS1 region. The V4 region did not show geographical variation.

As expected for gyrodactylids, true generalists are underrepresented in the present study. Most species were highly host-specific, although some were found on two or three *Pomatoschistus* species. None of the other fish species examined in this study or in other studies (Gläser, 1974b; Geets, 1998; Ziętara et al., 2000) were found to be infected with the present species, indicating phylogenetic host specificity towards gobies of the genus *Pomatoschistus*. It has been suggested that the frequency of co-speciation tends to be higher if host specificity is also high, since host-specific parasites are usually phylogenetically conservative in their host choice (Rohde, 1993). This can be evaluated by means of phylogeny reconstruction: in case of co-speciation; the parasites parasitising the *Pomatoschistus* gobies should form a monophyletic group.

4.2. Phylogeny reconstruction

The tree-like phylogenetic signal was very high in the V4-5.8S-partial ITS2 dataset comprising all species; the overall phylogeny was very robust and independent of the tree-building method. Phylogenetic relationships within subgenera were less clear. Split-decomposition analysis (SplitsTree v. 3-1, Huson, 1998) showed the presence of star- and net-like phylogenetic signals in the dataset (Huyse, unpublished data). Also, although Group B consists of very closely related species, the ITS and V4 regions were not behaving clock-like. This might point to the inadequacy of the ITS and V4 region to resolve interrelationships within the genus *Gyrodactylus* or it might be linked to the peculiar mode of reproduction these viviparous flatworms. This can

only be verified by sequencing more gene fragments and compare the obtained phylogenetic trees.

There was also one instance of shared ITS polymorphisms in two *Gyrodactylus* specimens found on *G. flavescens*, similar to what has been reported in a study on the coral genus *Alcyonium* (McFadden et al., 2001). According to the authors this can be explained by either a recent speciation event or hybridisation. As species of Group A apparently diverged very recently, the observed pattern might be the result of incomplete lineage sorting. However, recently sperm transfer has been observed between *G. arcuatus* and *G. gasterostei*, both parasitising three-spined stickleback (Cable, personal communication). Nothing is known yet about the possible offspring resulting from such pairings, but it does show that hybridisation might occur.

4.3. Are the parasite species infecting one host group monophyletic?

Paraphyly of the *Gyrodactylus* spp. infecting the gobies suggests that at least two independent colonisation events were involved. However, if groups A and B are monophyletic, co-evolution and co-speciation still might have played a role within these groups.

Group A is indeed monophyletic and each species infects only one host, except for *Gyrodactylus* sp. 1, which is found on *P. minutus* and *P. lozanoi*. A study of the *Gyrodactylus* fauna of sympatric fish species and an additional screening of GenBank showed that the most closely related species were *G. arcuatus* and *G. nipponensis*. Morphologically, *G. arcuatus* is remarkably similar to the present species (Geets et al., 1999) but genetically they differ by 8.2% (ITS2, uncorrected p-distance). Thus, it is possible that a host switching event from *G. arcuatus* of the three-spined stickleback onto the various goby species might lie at the origin of Group A. It is not unlikely that euryhaline *Pomatoschistus* gobies and stickleback shared the same refugium during the Pleistocene (e.g. in the Bay of Biscay or the Atlantic drainages of the Western Iberian Peninsula, Garcia-Marin et al., 1999). The close relationship between *G. nipponensis*, infecting the gills of Japanese eel *A. japonica*, and the *Mesonephrotus* species included herein, was not suspected. Based on the similar hook morphology of *G. nipponensis* and *G. anguillae*, Ernst et al. (2000) stated that *G. nipponensis* might belong to *G. anguillae*-species group. The present phylogenetic analyses suggest that it belongs to *Mesonephrotus*. This may indicate another relatively recent host switching event, between stickleback/gobies and eel. It also supports the idea that *G. nipponensis* has been introduced into Japan as a result of the eel trade (Hayward et al., 2001), with a possible origin in Europe.

Group B is not monophyletic: *G. anguillae*, infecting European eel *Anguilla anguilla*, clustered firmly within this group. Based on the 5.8S and ITS2 dataset it appeared

most closely related to *G. cf. micropsi* 2, pointing to a relatively recent host switching event. The direction of the host transfer is more likely to be from *Pomatoschistus* to *Anguilla* since (1) there are more *Gyrodactylus* sister species on *Pomatoschistus* than on *Anguilla*, and (2) according to Malmberg (1970), the occurrence of a species with a specialised excretory system like *G. anguillae* on a primitive fish like *A. anguilla* points to a secondary infestation. When comparing sclerite morphology, there are similarities in the shape of the anchors, ventral bar and marginal hook, although the sclerites of *G. micropsi* are smaller. Since *G. anguillae* appeared to be a relatively recently evolved species, it supports the hypothesis for the intercontinental introduction of this species by the eel trade (Hayward et al., 2001). The close relationship with the *Gyrodactylus* fauna of the *Pomatoschistus* gobies furthermore supports the author's hypothesis that *G. anguillae* originated in Europe. In order to obtain a complete picture of other possible host transfer routes, it would be of interest to obtain sequences of the *Gyrodactylus* fauna of the Gadidae (all members of the subgenus *Mesonephrotus*) and the species infecting Cottidae, Pleuronectidae and Zoarcidae (all members of the subgenus *Paranephrotus*).

4.4. Are congeners on the same host species each other's closest relative?

Co-existence of congeneric parasites on the same host species might be an indication of sympatric speciation by site shift (Gusev, 1995). For example, *P. microps* harbours two very closely related species: *Gyrodactylus* sp. is exclusively found on the gills and *Gyrodactylus* sp. 4 on the fins. However, *Gyrodactylus* sp. is more closely related to *Gyrodactylus* sp. 1, 2 and 3 found on the gills of *P. minutus*, *P. lozanoi*, *P. pictus* and *P. norvegicus*, respectively. Hence parasites of different hosts are more closely related to each other than parasites are on the same host. This indicates an allopatric mode of species formation. Such a scenario can be explained by (1) strict co-speciation with the host, (2) host switching followed by speciation or (3) a combination of both scenarios. Several statistical methods are available to test for co-speciation (Page, 1994; Huelsenbeck et al., 2000; Legendre et al., 2002), but most of them require a robust and resolved phylogeny for both hosts and parasites, preferably constructed from molecular data. This allows a comparison of evolutionary rates in both groups, facilitating the differentiation between co-speciation and host switching (Page and Hafner, 1996). Until now, only morphology and allozyme based goby phylogenies are available (Wallis and Beardmore, 1984). Although a molecular phylogeny is in preparation (Huyse et al., unpublished data), the speciation modes in *Gyrodactylus* spp. are here evaluated by means of the parasite phylogeny and the ecological background of the hosts.

According to Bakke et al. (2002) host switching in gyrodactylids has been facilitated by the mixing of

fish strains following glaciation during the Pleistocene. *G. salaris* infecting *Salmo salar* is thought to have diverged within the North Sea ice lake during the last ice age. Based on the ultrametric tree (Fig. 1), the speciation events in Group A seem to have almost the same evolutionary age as the speciation of *G. salaris* and *G. teuchis*. It is possible that Pleistocene conditions promoted host switching between the various goby species. However, the results of Wallis and Beardmore (1984) suggest that the Pleistocene was very important in the genealogical history of the *P. minutus* complex (*P. minutus*, *P. lozanoi* and *P. norvegicus*). In that case, those host and parasite lineages might have co-speciated, triggered by the Pleistocene ice ages.

By analogy with the literature on plant feeding insects (Craig et al., 2001; Emelianov et al., 2001; Via, 2001), *Gyrodactylus* spp. of Group A could also be regarded as 'host races'. Since the *Pomatoschistus* species are very abundant and occur in sympatry, they might belong to the same cruising range of actively dispersing gyrodactylids. As such, speciation by host switching could be regarded as sympatric speciation. However, in the present study we adopted the definition of sympatric speciation of Brooks and McLennan (1993), implying speciation on the same host species (intra-host speciation).

Such an example of intra-host speciation might be found in Group B: *G. cf. micropsi* and *G. cf. micropsi* 1 are each other's closest relatives and were found on the same host species. Hyperviviparity results in a very short generation time, allowing *Gyrodactylus* to evolve faster than its host. Also, during automictic parthenogenesis, inbreeding might create different 'strains' of *Gyrodactylus* spp. found on the same host (Brooks and McLennan, 1993). The other species belonging to Group B represent host-associated species complexes (Huyse and Volckaert, 2002): the two sister species *G. rugiensis* and *G. rugiensoides* are found on *P. microps*, and on *P. pictus*, *P. lozanoi* and *P. minutus*, respectively; *G. micropsi* and *G. cf. micropsi* occur on *P. microps*, and on *P. lozanoi* and *P. minutus*, respectively. This suggests that co-speciation or speciation by host switching shaped the observed pattern. If the genetic divergence between the host-associated species complex is comparable to the divergence between the respective hosts, co-speciation is favoured.

4.5. Does the molecular phylogeny reflect the morphological groupings?

On the basis of six main types of protonephridial systems, Malmberg (1970, 1998) subdivided *Gyrodactylus* into six subgenera: *Gyrodactylus*, *Mesonephrotus*, *Metanephrotus*, *Paranephrotus*, *Neonephrotus* and *Limnonephrotus*. A complex excretory system (subgenus *Gyrodactylus*) is considered as primitive, while the simplest systems (subgenus *Limnonephrotus*) are regarded as more advanced. As such, the excretory system of *Mesonephrotus* may have given rise to the system of *Metanephrotus* by a reduction of the lateral

flame cells. This excretory system may have developed into that of *Neonephrotus* through the excretory bladders specialising for a constantly pumping function. It is suggested that it has also given rise to the subgenus *Limnonephrotus* through reduction of the excretory bladders. The absence of excretory bladders is thought to be a limnic adaptation since this character is shared with the freshwater subgenus *Gyrodactylus*, while large bladders found in *Paranephrotus* might originally have been an adaptation to salt water. This subgenus probably developed from *Mesonephrotus* by a total reduction of the lateral flames and an enlargement of the excretory bladders (Malmberg, 1970). The system of the closely related genus *Gyrdicotylus* is in accordance with that in *Mesonephrotus*. It has however, a lower number of lateral flames and a higher number of flame bulbs (Malmberg, 1998). The gyrodactylid genus *Gyrodactyloides* has no published record on its excretory system but according to Malmberg (personal communication) it seems to have small bladders indicating a protonephridial system of either *Mesonephrotus* or *Metanephrotus* type.

All subgenera were included in the present molecular analysis. The phylogeny confirms that a small excretory bladder is likely to be a symplesiomorphic character state in *Gyrodactylus*. The evolution of big bladders apparently happened more than once while the modification into constantly pumping excretory bladders happened relatively recently. Excretory bladders disappeared at least twice: in *Gyrodactylus* and in *Limnonephrotus*. A complex excretory system characterised by many flame bulbs and lateral flame cells is confirmed to be primitive, with a decrease in number along the lineage leading from *Gyrodactylus* to the other subgenera. A further simplification of the excretory system by the loss of lateral flames evolved twice: in the lineage leading to *Metanephrotus* and the lineage leading to *Limnonephrotus* and *Paranephrotus*.

Gyrodactylus flesi branched off at the base of the *Mesonephrotus* and *Metanephrotus* clade while *G. lotae* and *G. alexgusevi* clustered strongly with the *Metanephrotus* species. This implies that either *Paranephrotus* is paraphyletic or these species do not belong to this subgenus. As suggested by Ziętara et al. (2002), the 5.8S rDNA sequence could be used as a tool for identifying *Gyrodactylus* subgenera. Taking this approach, *G. flesi*, *G. lotae*, *G. alexgusevi* and *G. niger* sp. would belong to *Metanephrotus*, *G. nipponis* to *Mesonephrotus* and *G. anguillae* to *Paranephrotus*, invalidating *Neonephrotus* as a distinct subgenus.

4.6. Conclusions

Gobies of the genus *Pomatoschistus* were colonised by at least two independent evolutionary lineages of *Gyrodactylus*, belonging to the subgenera *Mesonephrotus* and *Paranephrotus*. Most likely, the first group (A) evolved from a host switching event of *G. arcuatus* from the three-spined stickleback. If it occurred before

the speciation of the gobies, the host–parasite association might have evolved through co-speciation. In case of a more recent host-switching event, the present pattern might be the result of successive host switching between the extant goby hosts. Paraphyly of Group B shows that host switching even to other fish orders (*A. anguilla*) occurred as well. Numerous host-switches crossing the host family barrier have also been described for *Limnonephrotus* species (Ziętara and Lumme, 2002). The origin of clade B is still unknown, as no closely related species were available. The inclusion of other *Paranephrotus* species parasitising Cottidae and Zoarcidae is advisable.

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