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Identification of a host-associated species complex using molecular and morphometric analyses, with the description of *Gyrodactylus rugiensoides* n. sp. (Gyrodactylidae, Monogenea)☆

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Abstract

Gyrodactylus rugiensis was originally described as a parasite occurring on the marine gobies *Pomatoschistus minutus* and *Pomatoschistus microps*. In our preliminary survey this species was also frequently found on *Pomatoschistus pictus* and *Pomatoschistus lozanoi*. Subsequent molecular analysis of the internal transcribed spacers rDNA region revealed that this parasite actually represents a complex of two apparently cryptic species, one restricted to *P. microps* and the other shared by *P. minutus, P. lozanoi* and *P. pictus*. Morphometric analyses were conducted on 17 features of the opisthaptoral hard parts of specimens collected from all four host species. Standard discriminant analysis showed a clear separation of both genotypes by significant differences in marginal hook and ventral bar features. Statistical classifiers (linear discriminant analysis and nearest neighbours) resulted in an estimated misclassification rate of 4.7 and 3.1%, respectively. Based on molecular, morphological and statistical analyses a new species, *Gyrodactylus rugiensoides* is described. This species seems to display a lower host-specificity than generally observed for *Gyrodactylus* species as it infects three sympatric host species.However, seasonal and host-dependent morphometric variation is shown for *G. rugiensoides* collected on *P. pictus*. Host-switching and gene flow might be important factors preventing speciation on closely related and sympatric host species. The presence of host associated species complexes in this *Gyrodactylus-Pomatoschistus* system is also confirmed by the presence of two host-dependent genotypes within *G. micropsi* found on *P. minutus* and *P. lozanoi*, and *P. microps*, respectively. By comparing host and parasite phylogeny, phylogenetic and ecological factors influencing host-specificity are discussed.

Keywords: *Gyrodactylus rugiensoides* n. sp; Internal transcribed spacers rDNA; Host-parasite relationships; Host-specificity; Morphometrics; *Pomatoschistus*

1. Introduction

Since the introduction of molecular tools into taxonomy, systematics and phylogeny, many species descriptions are re-evaluated, new species are described while some are assigned new taxonomic positions (e.g. Burridge and White, 2000; Jousson et al., 2000; Lazoski et al., 2001; Desdevises, 2001). In classical morphological analysis, cryptic speciation may lead to an underestimation of the number of species while phenotypic plasticity may induce the reverse effect. In parasitic organisms, the morphological identification can be furthermore obliterated by convergent evolution (Price, 1980). In this study we assess the validity of molecular markers, comparative morphometric analysis and statistical classifiers in discriminating closely related *Gyrodactylus* species.

Gyrodactylus is a species-rich genus of monogenean ectoparasites, mostly found on fish. Anatomical conservatism as a result of viviparity and progenesis has led to a reduced number of useful taxonomic characteristics (Cable et al., 1999). A morphological identification method has been developed by Malmberg (1970) based on the hard parts of the posterior attachment organ. Marginal hook features appeared crucial for discrimination of very closely related species, but the discrimination of some taxa, including the pathogenic *Gyrodactylus salaris,* remained problematic. Shinn et al. (1996) used univariate and multivariate analyses on morphometric data of the opisthaptoral sclerites to address this problem, but an unambiguous separation did not seem feasible. More recently, Kay et al. (1999) constructed a classification system with the use of statistical classifiers. According to these authors identification of *G. salaris* is possible from measurements of the marginal hook

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alone when based on scanning electron microscopy. However, when using light microscopy based images, the total complement of sclerites is required. Cunningham et al. (1995) introduced molecular markers, namely the rDNA region with the V4 region and the internal transcribed spacers (ITS), as a new tool for species identification. By using RFLP and DNA probe hybridisation a relatively rapid screening for potential pathogenic *G. salaris* specimens was possible. However, recently it has become clear that these molecular tools are not always as straightforward as generally accepted. DNA probe hybridisation to the amplified V4 region misidentified *Gyrodactylus teuchis* samples as *G. salaris*. Direct sequencing remains the most reliable method for *Gyrodactylus* identification to date (Cunningham et al., 2001).

Here we use as a model *Gyrodactylus* specimens living on gobies of the genus *Pomatoschistus* Gill, 1864. They are among the most abundant fish species along the Eastern Atlantic and Mediterranean coasts of Europe, playing an important role in the marine ecosystem as predator of meiobenthos and prey for economically important fish species (Wallis and Beardmore, 1984; Miller, 1986). The genus forms an interesting complex of species showing various degrees of relatedness and niche overlap. The species belonging to the *Pomatoschistus minutus* complex, namely *P. minutus, Pomatoschistus lozanoi* and *Pomatoschistus norvegicus*, speciated only recently and hybrids of the former two species have been reported (Fonds, 1973; Wallis and Beardmore, 1984). The question arises to which degree these relationships, as well as their biological characteristics, are reflected in their *Gyrodactylus* fauna. However, until now, not much attention has been paid to their role as a host for *Gyrodactylus*. Geets et al. (1998) described *Gyrodactylus longidactylus* on the gills of *P. lozanoi*. The only other species descriptions are made by Gläser (1974a): *Gyrodactylus rugiensis* and *Gyrodactylus micropsi* occurring on the common goby *Pomatoschistus microps* and the sand goby *P. minutus*. In 1998, Geets (Host-parasite interactions between sympatric *Pomatoschistus* species (Gobiidae, Teleostei) and their helminth parasites: ecological and phylogenetic aspects. Doctoraatsthesis, Katholieke Universiteit Leuven) reported one specimen of *G. rugiensis* on the skin of *P. lozanoi*. In our parasitological survey we found *P. lozanoi* and *P. pictus* highly infected with *G. rugiensis*-like species and *P. lozanoi* was additionally infected with *G. micropsi*-like species. First we collected the ITS rDNA sequences of several specimens isolated from all host species. Subsequently, we collected and compared morphological data from *G. rugiensis*-like species of all hosts. In order to quantify the morphological differentiation among the different host-associated populations, morphometric and statistical analyses have been carried out on 17 morphological features of the opisthaptoral hard parts. Since there was not sufficient material available for *G. micropsi* and *G. micropsi*-like species, only the molecular analysis is discussed. Finally, host and parasite phylogenies are compared to examine the ecological and phylogenetic processes involved in this particular host-parasite system. Phenomena such as co-evolution and host-switching are evaluated.

2. Material and methods

2.1. Sampling and sample preparation

Gobies were collected in the English Channel and across the North Sea in Belgium, France, The Netherlands and Norway (Table 1). Fish were brought alive to the laboratory and immediately screened for *Gyrodactylus* infection using a stereomicroscope. Some *Gyrodactylus* specimens were fixed in ammonium picrate glycerine (Malmberg 1970), to examine the haptoral sclerites by phase contrast microscopy. All parasites were identified morphologically to species level prior to DNA analysis. From the population of Texel, where *P. minutus* and *P. microps* co-occur and host-switching might be possible, the opisthaptor was separated from the body enabling simultaneous morphological and molecular analyses. No host-switching was suspected in Ostend where only *P. microps* occurs and in Bergen where *P. microps* was not reported. Each parasite specimen was individually placed in 5 μl of milli-Q water and stored at −20°C. DNA extractions were performed as described by Zietara et al. (2002). Drawings of *G. micropsi* were made from material provided by Dr Gläser and from specimens originating from the same population used for molecular analysis (Zietara et al., 2002).

2.2. Molecular analysis

About 1.200 bp of the rDNA complex spanning the 3′ end of the 18S subunit, ITS1, 5.8S subunit, ITS2, and the 5′ end of the 28S subunit were amplified from four to 10 specimens of each species (Table 1). The original ITS sequences of *G. micropsi* and *G. rugiensis* from *P. microps* were obtained in a previous study (Zietara et al., 2002, EMBL accession numbers AF328868 and AF328870); additional sequences from *G. rugiensis* were obtained from parasites collected in Ambleteuse (F), Texel and Yerseke (NL). ITS amplification and sequencing were performed as described by Zietara et al. (2002). *Gyrodactylus salaris* was used as outgroup in the phylogenetic analyses (Zietara et al., 2002, EMBL accession number AF328871). Three datasets were prepared: 5.8S + ITS1 + ITS2, and ITS1 and ITS2 separately. Sequences were aligned with the ClustalW (version 1.7) multiple sequence alignment program (Thompson et al., 1994). Modeltest 1.05 was used to select the model of DNA evolution that best fits the data based on log likelihood scores (Posada and Crandall, 1998). To infer a phylogeny based on ITS1, 5.8S and ITS2, we used maximum parsimony, maximum likelihood and distance-based methods (PAUP* v. 4.01b, Swofford DL., 2001, PAUP*: Phylogenetic Analysis Using Parsimony, (and other methods)

Table 1
species, host, locality and date of sampling of the specimens used for morphometric and molecular analysis[a]

Parasite	Host	Locality	Date, temperature, salinity	N/G
G. rugiensis	*P. microps*	Ostend, Belgium 51°14′ N, 2°57′ E	08/99, 16–18°C[b] 31.1 ppm	20/[c]
G. rugiensis	*P. microps*	Ambleteuse, France 50° N, 1° 36′ E	09/99, 15°C 16–30 ppm	–/1
G. rugiensis	*P. microps*	Texel, The Netherlands 53° N, 4°48′ E	11/00, 12°C 31.0 ppm	–/2
G. rugiensis	*P. microps*	Yerseke, The Netherlands 51° 30′ N, 4°4′ E	11/99, 16.7°C 30.1 ppm	3/2
G. rugiensoides	*P. minutus* *P. lozanoi*	Texel, The Netherlands 53°N, 4°48′ E	11/00, 12°C 31.0 ppm	21/3
G. rugiensoides	*P. minutus*	Texel, The Netherlands 53° N, 4°48′ E	05/99, 12°C 31. 0 ppm	6/–
G. rugiensoides	*P. lozanoi*	Belgian continental shelf 51°35′ N, 2°18′ E	10/99, 12°C 35.0 ppm	–/2
G. rugiensoides	*P. minutus*	Bergen, Norway 60°16′ N, 5°10′ E	06/00, 9–10°C 33.0 ppm	2/2
G. rugiensoides	*P. pictus*	Bergen, Norway 60°16′ N, 5°10′ E	06/00, 9–10°C 33.0 ppm	20/3
G. cf. *micropsi*	*P. minutus* *P. lozanoi*	Texel, The Netherlands 53° N, 4°48′ E	05/99, 12°C 31.0 ppm	–/4
G. micropsi	*P. microps*	Doel, Belgium 51°19′ N, 4°16′ E	09/98, 15°C 5–10 ppm	–/[c]

[a] N = number of specimens measured, G = number of specimens sequenced in this study.
[b] Fish were kept in the laboratory at a water temperature of about 18°C.
[c] See Zietara et al., 2002.

Sunderland, MA: Sinauer Associates). In maximum parsimony gaps were treated as missing data and all sites were equally weighted but different transition:transversion (ti/tv) ratios were applied; 10:5 for 5.8S and 1:5 for ITS1 and ITS2, to compensate for the difference in evolutionary rate between coding and non-coding regions. The maximum likelihood analysis was performed using the parameters estimated under the best-fit model and optimised through repeated estimation. We conducted the exhaustive search method and bootstrapped ($n = 1.000$) with the branch and bound algorithm. With the minimum-evolution distance method, the maximum likelihood genetic distances were calculated under the optimised model. The heuristic search method was applied and we bootstrapped ($n = 1000$) with the tree-bisection-reconnection branch-swapping algorithm in force.

2.3. Morphometric and statistical analyses on *G. rugiensis*-like species

In total, 72 specimens of *G. rugiensis*-like species were measured (Table 1). In analogy with Shinn et al. (1996) and Geets et al. (1999) 17 hook characteristics were selected for morphometric analysis (Fig. 1). Measurements were done using a Zeiss HBO50 microscope (magnification of $10 \times 40 \times$ for the anchors, $10 \times 100 \times$ oil for the marginal hook features, with phase contrast). Images were analysed with the program SigmaScan Pro 5. For the statistical analyses STATISTICA 5.0 was used, except for nearest neighbours and linear discriminant analysis which was done with S-PLUS 2000 for Windows. Drawings of the anchors and ventral bar were done using a magnification of $10 \times 90 \times$ oil; drawings of the marginal hook features were done using $16 \times 90 \times$ oil using the equipment from Malmberg (1970).

Significant differences between the second and eighth marginal hook within one group (Texel, $n = 21$, Nov. 2000) were tested using a *t*-test for dependent variables on the variables of the marginal hook. Correlations between all measured features were tested using Pearson's correlation coefficient. To test for host-dependent differences in hook morphology, Tukey's honest significant difference test for unequal sample sizes was performed. This test allows for post hoc multiple comparisons between the means of each group. The specimens were grouped according to their genotype and according to their respective host. Observations with missing variables or with a C.V. exceeding 12% were excluded to avoid measurement errors. A standard discriminant analysis was used to assess the contribution of each variable in the separation of the different groups. Finally, in analogy with Kay et al. (1999), statistical classifiers were tested for their ability to discriminate among *G. rugiensis*-like species from the different host groups. Again two datasets were prepared; in the first set the specimens were grouped by means of their genotype and in the second by means of their respective host. A measure of error was

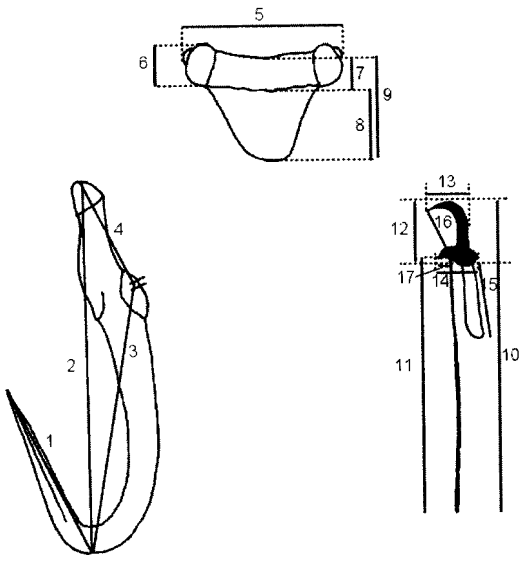

Fig. 1. Measurements of the opisthaptoral hard parts of *Gyrodactylus* spp. *Hamulus*: (1) LAP, length of anchor point; (2) LA, total length of anchor; (3) LAS, length of anchor shaft; and (4) LAR, length of anchor root. *Ventral bar*: (5) LVB, length of ventral bar; (6) BWVB, basal width of ventral bar; (7) MWVB, median width of ventral bar; (8) VBM, length of ventral bar membrane; and (9) TLVBM, total length of ventral bar membrane (median width of ventral bar + length of ventral bar membrane). *Marginal hook*: (10) LMH, total length of marginal hook; (11) LH, length of marginal hook handle; (12) LSI, length of marginal hook sickle; (13) DWSI, distal width of marginal hook sickle; (14) PWSI, proximal width of marginal hook sickle; (15) LOOP, length of marginal hook filament loop; (16) APERTURE, marginal hook sickle aperture distance; and (17) TOE, marginal hook toe length.

expressed using a misclassification matrix. The performance of the classifier was assessed by sevenfold cross-validation. Linear discriminant analysis and nearest neighbours were selected since they gave the best results in the study of Kay et al. (1999).

3. Results

3.1. Molecular identification

Both *G. rugiensis* and *G. micropsi* consisted of two host-dependent genotypes. The specimens found on *P. lozanoi* and *P. pictus* had the same genotype as found on *P. minutus*, hereafter named *Gyrodactylus rugiensoides*. *Pomatoschistus lozanoi* harboured also the same genotype of *G. micropsi* as found on *P. minutus*, hereafter named *G.* cf. *micropsi*. The genotypes found on *P. microps* will be referred to as *G. micropsi* and *G. rugiensis*, respectively. The alignment of the ITS sequences is shown in Fig. 2. The gene 5.8S was identical for all species. Genetic distances among the four species varied from 2.5 to 16.5% (Table 2). No intraspecific differences were found between ITS1 and ITS2 sequences of specimens from France, Norway, Belgium and the Netherlands. The phylogenetic relationships are visualised in a maximum likelihood phylogram (Fig. 3). The phylogeny of the host is inferred from the study of Wallis and Beardmore (1984). Comparison of the different models of evolution judged the HKY + Γ_4 model of substitution (Hasegawa et al., 1985), with gamma shape parameter = 0.7 as the most suited for the ITS1 and ITS2 data. Tree topologies generated by the different datasets and different tree building methods were identical and supported by high bootstrap values of 100%. Maximum parsimony analysis was based on 116 parsimony informative sites, length = 152, CI = 0.93, RI = 0.91. The ITS sequences of *G. rugiensoides* and *G.* cf. *micropsi* have been submitted to the EMBL nucleotide database under accession numbers AJ427414 and AJ427221, respectively.

3.2. Morphometric and statistical analyses on G. rugiensis-like species

A morphometric comparison between features from the second and eighth marginal hook showed that the total length (LMH, $P < 0.0001$), the shaft length (LH, $P < 0.0001$), the sickle distant width (DWSI, $P < 0.018$), sickle length (LSI, $P < 0.025$), the aperture ($P < 0.0002$) and the toe (TOE, $P < 0.016$) of both marginal hooks are significantly different. This is not the case for the sickle proximal width (PWSI, $P < 0.885$) and the filament loop (LOOP, $P < 0.14$). In order to exclude variation caused by these intra-individual differences, only measurements of the eighth marginal hook will be used in further analyses.

The mean, range and coefficient of variation of all 17 features are presented in Table 3. In general, measurements on the anchor resulted in low C.V. values. The median width of the ventral bar appeared to be the most variable structure of the ventral bar (C.V. 15%). Regarding the marginal hook, all features except the loop and the aperture displayed a C.V. less than 12%. If a comparison was made with the original species description of Gläser (1974a) (Table 3), the majority of measurements were most comparable with *G. rugiensis* collected from *P. microps* of Ostend. This is especially the case for the marginal hook features where the differences with the specimens collected from *P. minutus* and *P. pictus* are more pronounced. Regarding the anchors, the results of Gläser (1974a) show a lower range in total length of anchor and length of anchor shaft (LA and LAS) and length of anchor root (LAR) compared with our results. It should be noted that Gläser (1974a) made no discrimination between specimens collected from different host species, which were kept together in tanks for several days. Moreover, no specification is given regarding which marginal hook was used for measurements. This hampers the comparison between his results and the results from the present study. However, Dr Gläser kindly provided some of his material (*G. rugiensis* from fins of *P. minutus*, Breeger Bodden, Germany, 20/06/73), which allowed a re-examination. One specimen is redrawn (Fig. 4a) and measured (Table 3) according to our procedure. The drawings and

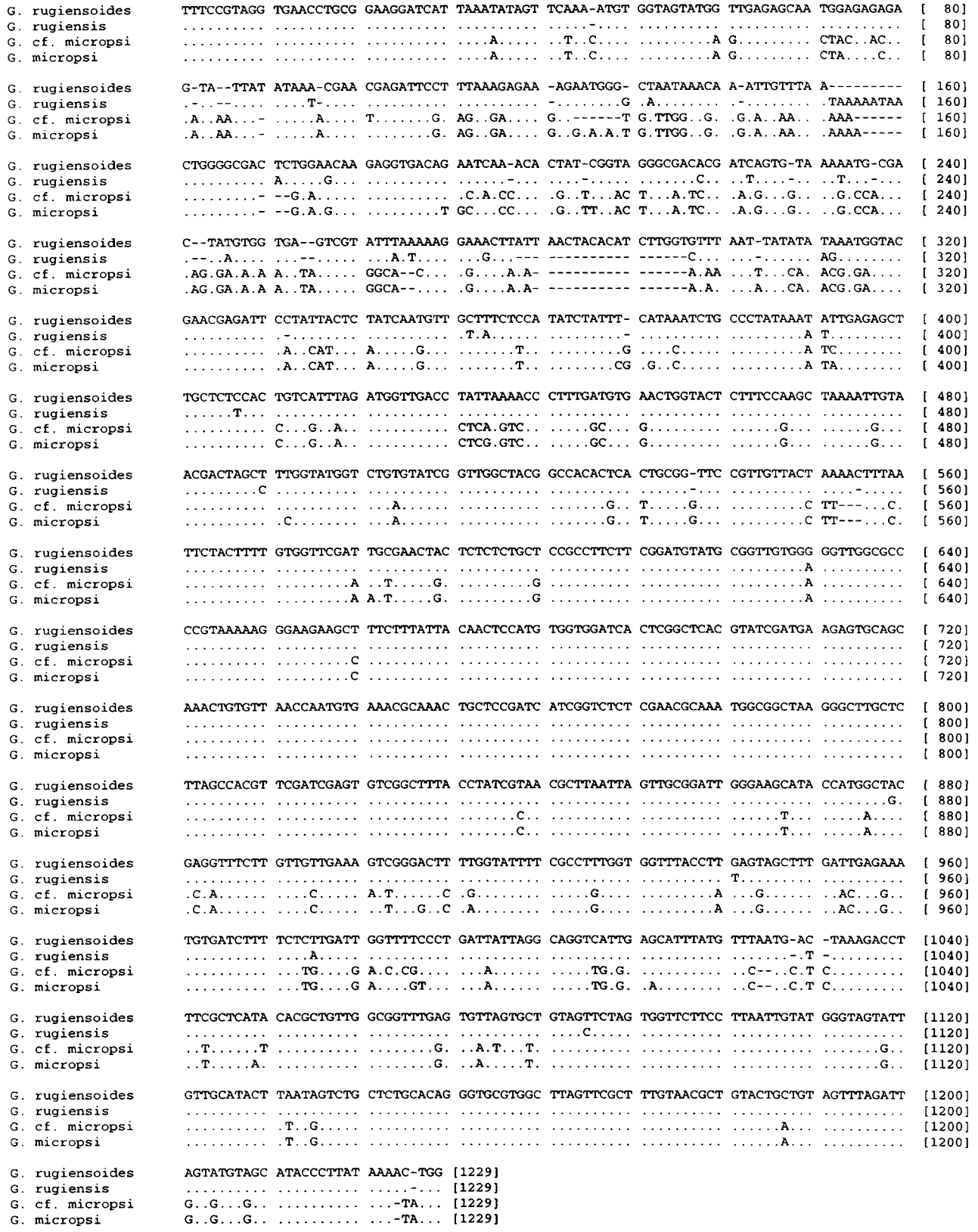

Fig. 2. Alignment of internal transcribed spacers (ITS) 1 and 2 and 5.8S gene sequences from *G. micropsi*, *G. rugiensis* (EMBL accession number AF328868 and AF328870), *G. rugiensoides* n. sp. and *G.* cf. *micropsi*. Dots (.) indicate nucleotides identical to *G. rugiensoides*; dashes (–) indicate alignment gaps.

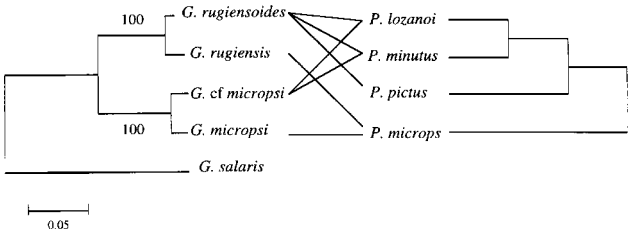

Fig. 3. Comparison of host and parasite phylogeny. The maximum likelihood phylogram of the parasites is constructed with the 5.8S and ITS2 sequences using the HKY + Γ_4 model of substitution with gamma shape parameter = 0.7; Ln L = −3207.3. The bootstrap values are identical for all treebuilding methods. The host cladogram is inferred from Wallis and Beardmore (1984). Lines connect hosts with their parasites.

measurements of the ventral bar and the marginal hook features of this specimen resemble most with *G. rugiensis* collected from *P. microps* of Ostend.

The highest Pearson's correlation coefficients are found between features measured on the same structure along the same direction (LA and LAS; LMH and LH) (Table 4). As a consequence, these variables are likely to produce redundant information. As shown in the study of Geets et al. (1999), features measured on different structures of the haptor show lower correlations, which might imply that they provide complementary information.

The results of the Tukey's honest significant difference test for unequal sample sizes are summarised in Table 5. Significant differences ($P < 0.05$) between both genotypes can be found in the length of anchor point (LAP), basal and median width of ventral bar (BWVB, MWVB) and total length of the ventral bar membrane (TLVBM). Regarding the marginal hook features, differences in the total length of marginal hook (LMH), length of handle (LH), length of sickle (LSI), proximal width of sickle (PWSI) and sickle aperture could be detected. These features of the marginal hook are highly responsible for the interspecific differences since they did not generate significant intraspecific variations. In contrast, the LAP, LAS and LA and likewise the length of the ventral bar (LVB), generated significant differences within *G. rugiensoides*, found on *P. pictus* and *P. minutus* respectively. In all cases, the largest values were found for the specimens from *P. pictus*. This population is caught in spring whereas most specimens from *P. minutus* were caught in autumn. Two specimens from *P. minutus* were caught together with the population from *P. pictus*. Those measurements appeared to be the maximum range found for the total population from *P. minutus* and were thus of comparable magnitude as the specimens from *P. pictus* (Table 3). Still, the differences in LAP and LVB are significant and can only be partly explained by seasonal variation.

Table 2
HKY distance matrix on ITS1 and ITS2 sequences of five *Gyrodactylus* species; rates are assumed to follow a gamma distribution with gamma shape parameter = 0.7

	1	2	3	4	5
1 *G. rugiensoides*	–				
2 *G. rugiensis*	0.025	–			
3 *G.* cf. *micropsi*	0.146	0.163	–		
4 *G. micropsi*	0.147	0.165	0.033	–	
5 *G. salaris*	0.352	0.353	0.365	0.373	–

A standard discriminant analysis was performed to detect the variables responsible for the differences between the groups. Three variables with a C.V. greater than 12% were eliminated: MWVB, the sickle filament loop and sickle aperture, as well as eight specimens with missing variables. The specimens are grouped according to their respective hosts. *G. rugiensis* on *P. microps* is clearly separated from *G. rugiensoides* found on *P. minutus* and *P. pictus* (Fig. 5). The variables mainly responsible for this separation are the marginal hook features (LMH, LH, LSI), the TLVMB and to a lesser extent the MWVB, BWVB and the proximal width of the marginal hook sickle. Intraspecific differences, between specimens from *P. minutus* and *P. pictus* respectively, can be found in the LVB and the LAS and LAP. The performances of the two statistical classifiers, namely linear discriminant analysis and nearest neighbours, are summarised in Table 6. In the nearest neighbours method nine neighbours were used. Both methods performed more or less equally well. The estimated misclassification rate was markedly lower for the dataset where the specimens were divided according to their genotype (3.1/4.7 versus 17.2). This difference can be explained by misclassifications between specimens from *P. minutus* and *P. pictus*. For example, nearest neighbours assigned 50% of the latter group as members of the first group. When grouped according to the respective genotype, *G. rugiensoides* was perfectly discriminated by nearest neighbours and one time misclassified by linear discriminant analysis.

3.3. Species description

Family Gyrodactylidae Cobbold, 1864
Genus *Gyrodactylus* Nordmann, 1832
Subgenus: *G. (Paranephrotus)* Malmberg, 1964
Species group: *G. rugiensis*-group Gläser, 1974
***Gyrodactylus rugiensis* Gläser, 1974**

Host: *P. microps* Krøyer, 1838 (Gobioidea), common goby; Table 1.
Location on host: Fins and skin, occasionally on gill arches.
Locality: Spuikom, Ostend, Belgium (51°14′ N, 2°57′ E); Table 1.
Water temperature, salinity and date of collection: 18°C, 31.1 ppm (18/8/1999); Table 1.
Morphological examination: 33 specimens collected live at Ostend (Belgium), Ambleteuse (France) and Yerseke (The Netherlands); Table 1.
Number measured: 23; Tables 1 and 3.
Number drawn: 5.

Table 3
Size range of characters of the opisthaptoral hard parts of *G. rugiensis* on *P. minutus* and *P. microps*, measured by Gläser (1974a) and measured in this study on a single drawing of material provided by Gläser. Mean, range and C.V. (C.V. = 100 × the square root of the variance divided by the mean) of *G. rugiensis* on *P. microps*(Ostend, 08/98); *G. rugiensoides* n. sp. on *P. minutus* (Texel, 11/00); *G. rugiensoides* n. sp. on *P. pictus* (Bergen, 06/00); and all *Gyrodactylus* species used in the present study pooled on all hosts. [a]

Gyr. species		*G. rugiensis*	*G. rugiensis*	*G. rugiensoides*	*G. rugiensoides*	All parasites
Host Species		*P. microps/P. minutus*	*P. microps*	*P. minutus/P. lozanoi*	*P. pictus*	All hosts
N specimens		33	23	29	20	72
Anchors						
LAP	Mean	29.4 (27–31)	30.2 (28.6–32.0)	28.6 (27.2–31.2)	30.3 (28.7–32.0)	29.6 (27.2–32.0)
	C.V.		3.5	3.1	3.0	4.3
LA	Mean	59.4 (50–59)	58.4 (54.7–61.2)	58.9 (54.6–64.3)	60.0 (56.6–62.1)	59.1 (54.6–64.3)
	C.V.		2.8	3.6	2.7	3.7
LAS	Mean	42.4 (39–43)	42.5 (39.0–44.4)	42.5 (39.4–48.1)	43.9 (40.8–47.7)	42.9 (39.0–48.1
	C.V.		2.9	3.6	3.9	4.1
LAR	Mean	19.9 (13–18)	19.7 (18.1–21.1)	19.5 (17.9–21.5)	19.6 (18.4–21.6)	19.6 (17.9–21.6)
	C.V.		4.6	4.3	3.7	4.8
Ventral bar						
LVB	Mean	25.7 (21–25)	25.9 (23.1–28.5)	26.1 (23.8–30.0)	28.2 (26.1–31.1)	26.4 (23.1–31.1)
	C.V.		5.7	5.9	4.8	6.8
BWVB	Mean	7.2	7.3 (6.1–8.3)	7.7 (6.2–8.9)	8.0 (6.7–9.0)	7.6 (6.1–9.0)
	C.V.		7.7	8.4	7.5	8.8
MWVB	Mean	5.6 (4.2–4.7)	5.5 (3.4–6.8)	6.6 (4.9–9.7)	6.1 (5.2–7.0)	6.1 (3.4–9.7)
	C.V.		16.1	13.4	8.1	15.0
VBM	Mean	12.3	12.1 (9.4–15.0)	12.6 (11.7–14.2)	13.4 (11.2–16.9)	12.5 (9.4–16.9)
	C.V.		11.6	5.8	11.9	12.0
TLVBM	Mean	18.0	17.6 (15.0–20.2)	19.1 (17.6–21.3)	19.4 (14.8–22.9)	18.5 (14.8–22.9)
	C.V.		8.6	5.3	10	9.5
Marginal hook						
LMH	Mean	29.7 (28–31)	29.4 (26.7–30.6)	32.5 (30.8–34.6)	33.0 (32.0–34.3)	31.7 (26.7–34.6)
	C.V.		3.6	3.7	1.7	5.9
LH	Mean	25.2 (21–25)	23.5 (21.4–24.8)	26.3 (24.4–28.4)	26.7 (25.9–27.6)	25.5 (21.4–28.4)
	C.V.		4.1	1.5	1.8	6.7
LSI	Mean	6.0 (5.5–6.7)	6.5 (5.7–6.9)	7.0 (6.4–7.4)	7.0 (6.2–7.8)	6.8 (5.7–7.8)
	C.V.		5.0	3.3	4.8	5.5
DWSI	Mean	3.7	3.7 (3.1–4.3)	3.7 (3.0–4.5)	3.6 (3.0–3.9)	3.7 (3.0–4.5)
	C.V.		8.6	9.2	8.0	8.7
PWSI	Mean	3.8	3.8 (3.4–4.2)	4.12 (3.8–4.6)	4.0 (3.4–4.2)	4.0 (3.4–4.6)
	C.V.		5.5	4.8	5.6	6.0
Aperture	Mean	5.2	5.0 (4.5–5.5)	5.3 (4.5–6.0)	5.4 (5.0–6.1)	5.2 (4.5–6.1)
	C.V.		4.9	6.0	5.6	15.6
Toe	Mean	1.5	1.4 (1.1–1.7)	1.5 (1.3–1.8)	1.5 (1.2–1.8)	1.5 (1.1–1.8)
	C.V.		10.6	8.7	8.2	9.4
LOOP	Mean	6.6	8.4 (5.1–104)	8.8 (7.1–11.7)	8.7 (6.5–11.1)	8.7 (5.1–11.7)
	C.V.		17.9	14.1	15	15.6

[a] All measurements are in μm. N = number of parasite specimens measured. For abbreviations see Fig. 1.

Deposited specimens: two slides: Fig. 4b and one extra are deposited in the Natural History Museum, London (Reg. No. 2002.2.14.4 and 2002.2.14.5, respectively).
Molecular analysis: Polymerase chain reaction (PCR) amplified ITS1 and 2 and 5.8S gene sequences were previously obtained of specimens from Ostend (Zietara et al., 2002, EMBL accession number AF328870). For this study five additional specimens were sequenced: two from Yerseke (The Netherlands), two from Texel (The Netherlands) and one from Ambleteuse (France).
Diagnosis
Pharynx with eight long processes. Cirrus with one large and five small spines arranged in a single arched row. Anchors and ventral bar reminding those of members of the *Gyrodactylus wageneri*-group, subgenus *G.* (*Limnonephrotus*) Malmberg, 1964. Anchors and anchor points longer than those of *G. micropsi* Gläser (1974a). Ventral bar with distinct processes. Length of marginal hook sickle shorter than in *G. rugiensoides* n. sp. Ventral bar membrane tongue-shaped, its posterior part more blunted and total length of ventral bar membrane shorter than in *G. rugiensoides* n. sp. Proximal and distal width of marginal hook almost equal, sickle point reaching further than marginal hook toe. Marginal hook sickle aperture

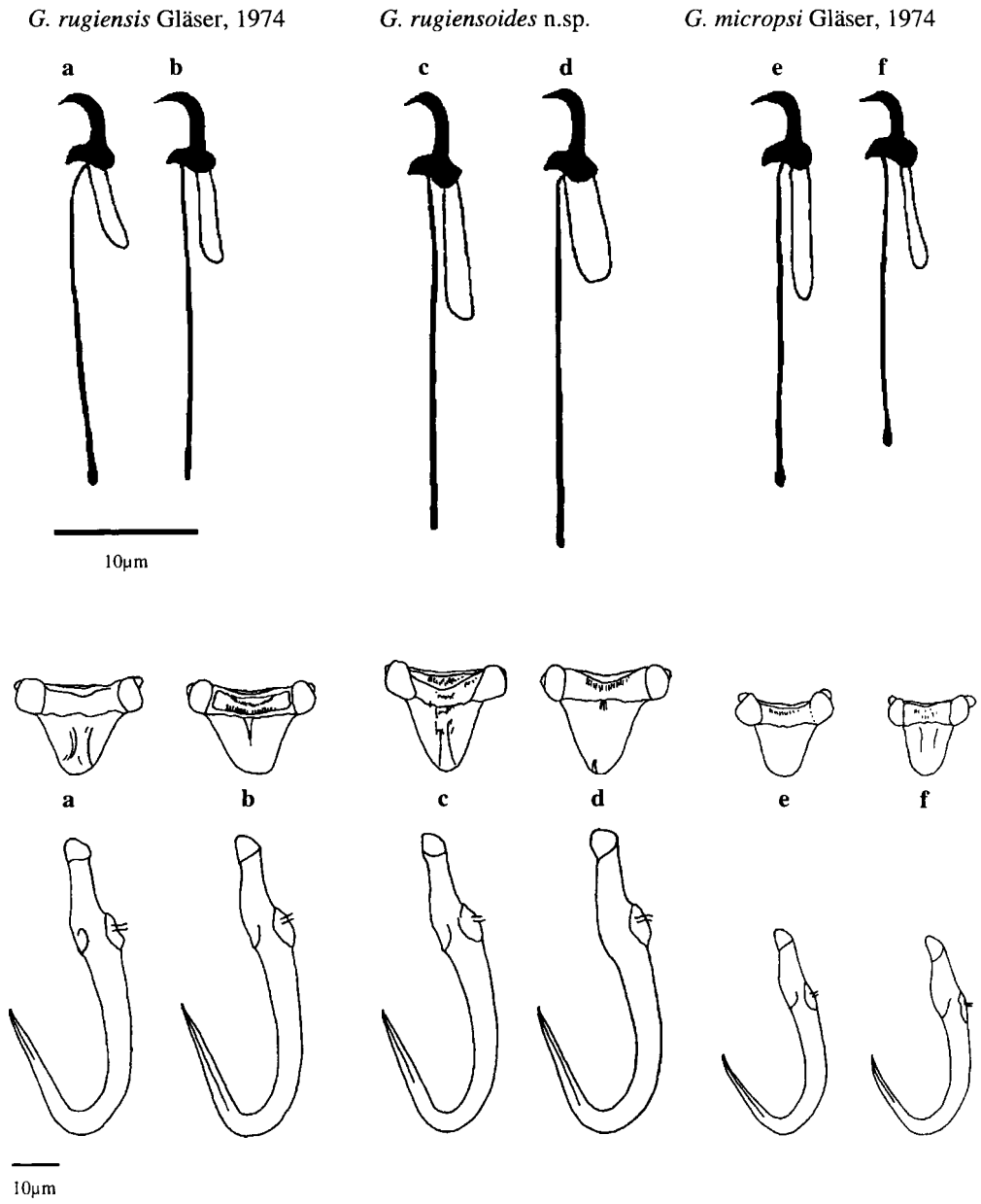

Fig. 4. Marginal hooks, ventral bars and anchors of *Gyrodactylus* species parasitising species of *Pomatoschistus*. (a and b) *G. rugiensis* Gläser (1974a): (a) specimen from Gläser's collection, fins of *P. microps* (Breeger Bodden, Germany, 20/06/73); (b) specimen from the fins of *P. microps* (Ostend, 08/99). (c and d) *G. rugiensoides* n. sp.: (c) specimen from the fins of *P. minutus* (Texel, 11/00); (d) specimen from the fins of *P. pictus* (Bergen, 06/00). (e and f) *G. micropsi* Gläser (1974a): (e) specimen from Gläser's collection, fins of *P. minutus* (Breeger Bodden, Germany, 27/06/73); and (f) specimen from the gills of *P. microps* (Doel, 09/98). Marginal hook number 2, 4, 5, 6, 1, 4, respectively, numbered according to Malmberg (1970).

smaller compared with *G. rugiensoides*. Total length of marginal hook about half the size of the total anchor length.

Molecular diagnosis

Genetic distance between *G. rugiensis* and *G. rugiensoides* amounts to 2.6% (ITS1 and 2; calculated under the HKY model with gamma shape parameter = 0.7). No intraspecific differences were found. The phylogenetic position is visualised in Fig. 3.

***Gyrodactylus rugiensoides* n. sp.**

Synonyms: *G. rugiensis* sensu Geets (1998. Doctoraatsthesis, Katholieke Universiteit Leuven), p 109

Host: *P. minutus* Pallas, 1970 (Gobioidea), sand goby; Table 1.

Other hosts: *P. lozanoi* de Buen, 1923 (Gobioidea), Lozano's goby; *P. pictus* Malm, 1865 (Gobioidea), painted goby; Table 1.

Location on hosts: Fins, skin, occasionally on gill arches.

Type-locality: Texel[1], The Netherlands (53° N, 4°48′ E); Table 1.

Other localities: Bergen[2], Norway (60°16′ N, 5°10′ E); Table 1.

Table 4
Pearson's correlation coefficients for 13 morphometric features measured on *G. rugiensis* and *G. rugiensoides* n. sp. on all host species ($n = 72$)[a]

	LAP 1	LA 2	LAS 3	LAR 4	LVB 5	BWVB 6	MWVB 7	LMH 8	LH 9	LSI 10	DWSI 11	PWSI 12	LOOP 13
1	1.00												
2	**0.42**	1.00											
3	**0.50**	**0.90**	1.00										
4	0.18	**0.63**	**0.37**	1.00									
5	**0.40**	**0.48**	**0.50**	**0.34**	1.00								
6	−0.04	0.07	0.09	−0.01	**0.29**	1.00							
7	**−0.32**	−0.01	−0.01	−0.10	0.07	**0.60**	1.00						
8	−0.05	**0.46**	**0.42**	0.10	**0.25**	0.18	**0.32**	1.00					
9	−0.05	**0.44**	**0.41**	0.08	0.22	0.10	**0.29**	**0.92**	1.00				
10	**0.29**	**0.25**	**0.34**	−0.09	**0.35**	**0.31**	0.10	**0.42**	**0.25**	1.00			
11	−0.07	0.19	0.09	0.18	−0.16	0.07	0.18	0.13	0.11	−0.07	1.00		
12	0.15	**0.26**	**0.23**	0.15	0.22	**0.34**	**0.32**	**0.31**	**0.29**	**0.40**	**0.44**	1.00	
13	0.08	**0.25**	0.21	**0.26**	0.16	**0.25**	0.22	**0.38**	**0.30**	0.17	0.03	0.15	1.00

[a] Correlations >0.70 are underlined. For abbreviations see Fig. 1.

Water temperature, salinity and date of collection: 12°C, 31.0 ppm (26/11/2000)[1]; 33.0 ppm, 9°C (26/6/2000)[2]; Table 1.
Number studied: 47 specimens collected live at Texel (The Netherlands), the Belgian continental shelf and Bergen (Norway); Table 1.
Number measured: 29 individuals of *P. minutus* and 20 of *P. pictus*; Tables 1 and 3.
Number drawn: 7; Figs. 4c,d.
Types: one holotype and two paratypes are deposited in the Natural History Museum, London (Reg. No. 2002.2.14.5, 2002.2.14.2 and 2002.2.14.3, respectively).
Molecular analysis: five specimens from Bergen (three from *P. pictus*; two from *P. minutus*); three from Texel and two from the North Sea. PCR amplified internal transcribed spacers (ITS) 1 and 2 and 5.8S gene sequences are submitted to the EMBL nucleotide database under accession number AJ427414; Fig. 2.

Diagnosis

Pharynx with eight long processes. Cirrus with one large and five small spines arranged in a single arched row. Anchors and ventral bar reminding those of members of the *G. wageneri* group, subgenus *G.* (*Limnonephrotus*) Malmberg, 1964. Anchors and anchor points longer than those of *G. micropsi* Gläser (1974a). Ventral bar with small processes, not always visible. Median width of ventral bar wider than in *G. rugiensis* Gläser (1974a). Ventral bar membrane triangular and longer than in *G.*

Table 5
Analysis of variance testing for differences in morphological traits of *G. rugiensis* and *G. rugiensoides* n. sp[a]

Groups	*P. microps-P. pictus/P. minutus*	*P. microps-P. minutus*	*P. minutus-P. pictus*	P. microps-P. pictus
Variables				
LAP	**0.0089**	**0.0001**	**0.0001**	0.9423
LA	0.2153	0.9163	**0.0027**	**0.0079**
LAS	0.4414	0.3825	**0.0002**	**0.0065**
LAR	0.4931	0.2862	0.2193	0.9612
LVB	0.1409	0.3333	**0.0001**	**0.0001**
BWVB	**0.0052**	**0.0316**	0.3443	**0.0011**
MWVB	**0.0021**	**0.0019**	0.6642	**0.0418**
LVBM	0.0936	0.4961	0.5374	0.0932
TLVBM	**0.0018**	**0.0092**	0.8732	**0.0057**
LMH	**0.0001**	**0.0001**	0.0563	**0.0001**
LH	**0.0001**	**0.0001**	0.0812	**0.0001**
LSI	**0.0001**	**0.0001**	0.7493	**0.0001**
DWSI	0.4103	0.9617	0.5202	0.3773
PWSI	**0.0004**	**0.0003**	0.5233	**0.0142**
APERTURE	**0.0001**	**0.0012**	0.0562	**0.0001**
TOE	0.1688	0.4521	0.9624	0.3532
LOOP	0.3227	0.7592	0.8633	0.4733

[a] Specimens are grouped according to genotype and respective host species. Correlations in bold are significant at $P < 0.05$. For abbreviations see Fig. 1.

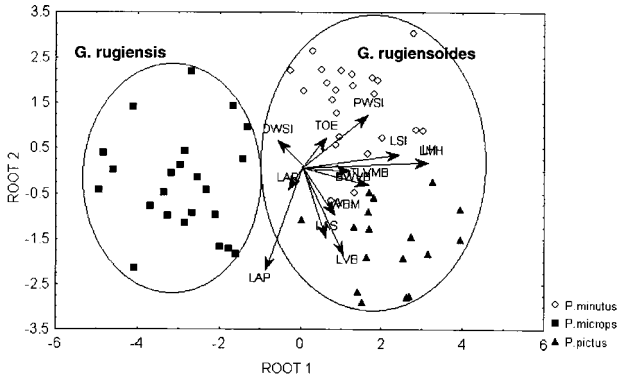

Fig. 5. Plot of standard discriminant analysis (Wilk's Lambda: 0.09, $P < 0.0001$) on morphological measurements of 72 *Gyrodactylus* species occurring on *P. minutus*, *P. pictus* and *P. microps*. For abbreviations see Fig. 1.

rugiensis. Length of marginal hook sickle longer than in *G. rugiensis*. Marginal hook sickle aperture more open; its aperture larger than in *G. rugiensis*. Proximal width of marginal hook sickle always wider than distal width; sickle point rarely reaching further than marginal hook toe. Total length of marginal hook longer than half the total anchor length.

Molecular diagnosis

Genetic distance between *G. rugiensis* and *G. rugiensoides* amounts to 2.6% (ITS1 and 2; calculated under the HKY model with gamma shape parameter = 0.7). No intraspecific differences were found. The phylogenetic position is visualised in Fig. 3.

Comments

As could be concluded from the PCA (Fig. 5), the marginal hook total length and the shaft length (LH and LMH) as well as the LSI are mainly responsible for the differences between *G. rugiensis* and *G. rugiensoides*. The length difference between LH and LMH of both species amounts to approximately 3 μm. Specimens of *G. rugiensoides* from *P. pictus* had longer anchors and longer ventral bars than specimens from *P. minutus* and *P. lozanoi*.

Host-specificity and prevalence

Pomatoschistus minutus is found to be infected throughout Norway, The Netherlands, Belgium and France. Its close relative *P. lozanoi* does not occur in Norway but appeared to be equally infected with the parasite in the Dutch and Belgian coastal waters. Due to its deep water niche in these latter areas, only a few specimens of *P. pictus* have been caught and examined. None of them were found to be infected with the particular species. However, off Bergen (Norway), *P. pictus* occurs close to the shore. Those specimens were found be to be highly infected with *G. rugiensoides*.

Etymology: The species was named *Gyrodactylus rugiensoides* for its similarity to *G. rugiensis*.

4. Discussion

Gyrodactylus rugiensis Gläser (1974a) was originally described as a parasite occurring on both *P. minutus* and *P. microps*. The present study, however, showed the existence of a host-associated species complex of *G. rugiensis*-like species. The study is based on independent data sets consisting of ITS rDNA sequences, multivariate analyses of morphometric data, and the use of statistical classifiers. As a consequence, we have divided *G. rugiensis* into two species: *G. rugiensis* Gläser (1974a) parasitising *P. microps* and *G. rugiensoides* n. sp. infecting *P. minutus*, *P. lozanoi* and *P. pictus*. Both species differ in 1.8 and 1.5% (uncorrected p-distances) in their ITS1 and 2 region, respectively; no intras-

Table 6
Misclassification matrices obtained using (A) nearest neighbours; and (B) linear discriminant analysis on 14 variables of the opisthaptoral hard parts of 64 specimens from *G. rugiensis* and *G. rugiensoides* n. sp[a]

(A) Nearest neighbours

Predicted class	True class	
(1) By genotype		
	G. rugiensis	*G. rugiensoides*
G. rugiensis	21	2
G. rugiensoides	0	41
Estimated misclassification rate = 3.1%		
(2) By host		
	P. microps	*P. minutus*	*P. pictus*
P. microps	22	1	0
P. minutus	1	20	3
P. pictus	0	6	11
Estimated misclassification rate = 17.2%			

(B) Linear discriminant analysis

Predicted class	True class	
	G. rugiensis	*G. rugiensoides*
G. rugiensis	21	2
G. rugiensoides	1	40
Estimated misclassification rate = 4.7%		
	P. microps	*P. minutus*	*P. pictus*
P. microps.	21	2	0
P. minutus	1	19	4
P. pictus	1	3	13
Estimated misclassification rate = 17.2%			

[a] Specimens are divided according to (1) their genotype; and (2) respective host species. The estimated misclassification rate was calculated using stratified sevenfold cross-validation. In nearest neighbours nine neighbours were used.

pecific variation among specimens from different sampling sites was found. It is known that the ITS region can vary greatly among species. Sequence variation between *Gyrodactylus* species as reported in the literature ranges from 2.7 to 56 to 1.5 to 38.7% for ITS1 and 2 respectively (Kimura distances from Matejusova et al., 2001). In a study on polystomatid monogeneans the ITS1 sequence variation ranges from 0.6 to 23.3% (Tajima-Nei distances, Bentz et al., 2001). Species differences found in the present study varied from 2.5 to 16.5% (HKY distances from ITS1 + ITS2, Table 2), and are thus falling within the lower range of the above results. However, it should be taken into account that our species were sampled within a single fish genus whereas in the above studies species were also collected from different fish families.

4.1. Morphometric and statistical analyses

The morphological differences between the new species *G. rugiensoides* and *G. rugiensis* can be mainly found in the shape and size of the marginal hook and ventral bar. The importance of the marginal hook features in discriminating among closely related *Gyrodactylus* species has also been reported in other studies (Malmberg, 1970; Shinn et al., 1996; Cunningham et al., 2001). However, caution has to be taken regarding the marginal hooks since this study confirmed earlier observations (e.g. Malmberg, 1970) that features of the second and eighth marginal hook significantly differ in length. Despite the small and relatively limited morphological differences, multivariate analysis could effectively separate both species. With the use of statistical classifiers, *G. rugiensoides* was clearly discriminated from *G. rugiensis* by nearest neighbours and one time misclassified by linear discriminant analysis. The resulting estimated misclassification rate was in both methods lower than the estimated misclassification rate reported by Kay et al. (1999). When we divided the specimens according to their respective host species, the estimated misclassification rate increased markedly. This indicates that the interspecific differences far exceed intraspecific differences. However, since this value is still comparable with the results of Kay et al. (1999), it might indicate some host-dependent variation in the morphology of *G. rugiensoides*. This is also suggested by the Tukey's honest significant difference test and the standard discriminant analysis (Table 5; Fig. 5). Specimens found on the host *P. pictus* are characterised by larger anchors, a significantly longer ventral bar, smaller median width of the ventral bar and a longer ventral bar membrane. It might be postulated that the populations are morphologically adapted to their respective host, which might be followed by genetic differentiation in the absence of gene flow. Gobies of the genus *Pomatoschistus* are very abundant and some species may occur in sympatry. These two features may create possibilities for accidental host-switching. Already a very low amount of gene flow is sufficient to prevent speciation (Slatkin, 1987). However, variation in size caused by different water temperatures has to be taken into consideration as well. Samples from *P. pictus* were taken in spring whereas the samples from *P. minutus* were taken in autumn. There is a tendency for larger opisthaptoral hard parts in colder periods (Malmberg, 1970; Mo, 1991; Geets et al., 1999). Still, this will only partly explain the observed size differences. Therefore it would be interesting to investigate *G. rugiensoides* from *P. pictus* from the Dutch and Belgian North Sea where it does not occur in sympatry with *P. minutus*. The fact that interspecific morphological variation succeeds intraspecific variation rejects the possibility that the morphological differences found between *G. rugiensis* and *G. rugiensoides* n. sp. only represent seasonal or host-dependent phenotypic plasticity. Moreover, the consistent molecular differentiation and the absence of intraspecific variation between populations from different regions justify the identification of two distinct species.

The existence of two host depending genotypes within *G. micropsi* found on *P. minutus* and *P. lozanoi*, and *P. microps*, respectively, points to the presence of host associated species complexes within *Gyrodactylus* parasitising *Pomatoschistus* species (see Fig. 3 and Table 2). The differentiation between both genotypes amounted to 2.4 and 2.6% in ITS1 and ITS2, respectively (uncorrected p-distances). The drawing of *G. micropsi* from material provided by Dr Gläser (Fig. 4e) resembles very much the drawing from material collected from Doel (Fig. 4f). The differences in size may be explained by seasonal variation since the former is collected in spring whereas the latter is collected in late summer. The difference between this species and *G. rugiensis/G. rugiensoides* is very clearly pronounced in the anchor and the ventral bar morphology. However, the marginal hooks are rather similar despite the fact that these features are mainly responsible for the interspecific differences between *G. rugiensis* and *G. rugiensoides*. This indicates that morphological parameters may have a different mode of evolution in different species groups.

4.2. Phylogenetic versus ecological influences

Parasite speciation is influenced by ecological and phylogenetic factors. To differentiate among the different speciation modes phylogenetic studies are needed (Brooks and McLennan, 1993). On the one hand, the direct life-cycle and the high host-specificity enforce a tight relationship of a *Gyrodactylus* species and its host, promoting co-evolution. On the other hand, the ability for auto-infection increases the chance for sympatric speciation and speciation by host-switching (Brooks and McLennan, 1993). Each of the investigated host species, except *P. pictus*, harbours a member of both species complexes (Fig. 3), which are clearly separated from each other (HKY distances about 15%). *Gyrodactylus* species infecting different host species cluster together and are thus more closely related to each other than to the parasites on the same host species. Therefore sympatric speciation could be ruled out. Two other explanations can be

proposed: the current host-parasite association represents an association by descent (co-speciation) or an association by colonisation. Since the hosts *P. lozanoi* and *P. minutus* speciated only recently (Fonds, 1973; Wallis and Beardmore, 1984), their similar parasite fauna could be explained by delayed co-speciation (Brooks and McLennan, 1993). However, since they live sympatrically in the North Sea, host-switching might provide another explanation. It should be noted that despite this sympatric lifestyle *P. lozanoi* harbours a highly host-specific gill parasite *G. longidactylus* (Geets et al., 1999). The other host pair sharing the same *Gyrodactylus* species is *P. minutus* and *P. pictus*. They are more distantly related (Wallis and Beardmore, 1984; Fig. 4), but in Norway both hosts occur in sympatry. In this situation host transfer is the most probable explanation for the occurrence of *G. rugiensoides* on both hosts. In Norway only one catch (June 2000) was checked for the presence of *G.* cf. *micropsi* and only very few *P. pictus* specimens from Belgium were examined. Thus, no conclusion can yet be made on the role of *P. pictus* as a potential host for *G.* cf. *micropsi*.

Species diversity and host-specificity of *Gyrodactylus* species infecting the *Pomatoschistus* species have been underestimated. However, the species here presented have only been reported from this host group, despite extensive research on the *Gyrodactylus* fauna of other fish species sharing the same habitats, e.g. gasterosteids and pleuronectids (Gläser, 1974b; Geets, 1998. Doctoraatsthesis, Katholieke Universiteit Leuven, België; Zietara et al., 2000). As such, we may assign these parasite species a phylogenetic host-specificity towards gobies of the genus *Pomatoschistus*. Besides phylogenetic factors, also ecological factors such as host habitat seem to play an important role in this *Gyrodactylus-Pomatoschistus* system.

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