



## Four new species of *Gyrodactylus* von Nordmann, 1832 (Monogenea, Gyrodactylidae) on gobiid fishes: combined DNA and morphological analyses

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### Abstract

Four *Gyrodactylus* species parasitising four closely related gobiid species in European coastal waters were studied and compared with *G. arcuatus* Bychowsky *sensu* Bychowsky & Poljansky (1953) from *Gasterosteus aculeatus*. These were *G. gondae* n. sp. from *Pomatoschistus minutus* and *P. lozanoi*, *G. flavescens* n. sp. from *Gobiusculus flavescens*, *G. arcuatoides* n. sp. from *P. minutus* and *G. branchialis* n. sp. from *P. microps*. Combined molecular and morphological analyses, as well as morphometric and statistical methods, were used. The *ssrRNA* V4 region and the complete ITS rDNA region were sequenced. Genetically the four new species are clearly distinct from *G. arcuatus*. From a morphological point of view, the haptor hard parts of *G. gondae* n. sp., *G. flavescens* n. sp. and *G. arcuatoides* n. sp. are related to those of *G. arcuatus*, while these parts of *G. branchialis* n. sp. are different, but related to those of *G. quadratidigitus* Longshaw, Pursglove & Shinn, 2003. For the latter two species, a new species group is formed. The V4 and ITS sequence analyses, however, indicate a close relationship between *G. branchialis* and the three *G. arcuatus*-like species.

### Introduction

Species of *Gyrodactylus* von Nordmann, 1832 are common ectoparasites on fish in Eurasian fresh and salt waters. According to Cable et al. (1999), progenesis and viviparity have led to a low number of useful taxonomic characteristics. A morphological identification method has been developed by Malmberg (1970), mainly based on the hard parts of the haptor, with the marginal hook features being crucial for discrimination of very closely related species. Cunningham et al. (1995) introduced molecular markers, namely the rDNA region with the V4 region and the internal transcribed spacers as a new tool for species identification. These molecular tools, combined with morphological analysis, have been applied by many researchers (Cunningham et al., 2001; Huyse & Volckaert, 2002; Ziętara & Lumme, 2003).

\*TH takes responsibility for the molecular, morphometric and statistical parts and GM for the morphological aspects of this work.

In a previous study by Geets et al. (1999), *Gyrodactylus arcuatus*-like specimens were found on the gills of three goby species, *Pomatoschistus minutus* (Pallas), *P. lozanoi* (de Buen) and *P. pictus* (Malm), from the Voordelta Area (SW Netherlands). These closely related sympatric goby species are the most abundant benthic fish species in the North Sea (Fonds, 1973; Miller, 1986). *Gyrodactylus* specimens were collected in different seasons, from the three goby hosts and from *Gasterosteus aculeatus* L. (Geets et al. 1999). A morphometric multivariate analysis on 17 hook characters demonstrated both seasonal and host-related variation. The authors could not establish, however, whether these host-related forms belonged to the same species or represented three distinct species. In this paper, on the basis of combined molecular, morphological and statistical analyses, the two *Gyrodactylus arcuatus*-like species in Geets et al. (1999), *G. sp. 1* from *P. minutus* and *P. lozanoi* and *G. sp. 2* from *P. pictus* are described together with another un-

Table 1. *Gyrodactylus* species analysed: *G. arcuatus*, *G. gonda* n. sp., *G. flavescens* n. sp., *G. arcuatooides* n. sp. and *G. branchialis* n. sp.

<i>Gyrodactylus</i>	Host spp.	Locality	Date, water temperature, salinity	N	P*	A*	Site	nS
<i>G. arcuatus</i>	<i>Gasterosteus arcuatus</i>	Edesö, Sweden 59°22' N, 18°27' E	11/06/01, 10 °C, 5.0 ppm	4	3/4	12	F/G	1
<i>G. gonda</i>	<i>Pomatoschistus minutus</i>	Texel, The Netherlands 53°N, 4°48' E	15/06/99, 12 °C 31.0 ppm	11	91	330	G/F	2
<i>G. gonda</i>	<i>P. minutus</i>	Texel, The Netherlands	25/11/00, 12 °C	23	61%	372	G/F	3
<i>G. gonda</i>	<i>P. lozanoi</i>	53°N, 4°48' E	31.0 ppm	7	5/7	40	G/F	2
	<i>P. lozanoi</i>	North Sea, Belgium 51°35' N, 2°18' E	26/10/99, 12 °C 35.0 ppm	31	19%	14		2
<i>G. gonda</i>	<i>P. minutus</i>	Trondheim, Norway 63° 32' N, 10° 26 E	13/06/00, 12 °C 32.0 ppm	3	2/3	14	F	2
<i>G. gonda</i>	<i>P. minutus</i>	Bergen, Norway 60°16' N, 5°10' E	21/05/01, 9–10 °C 33.0 ppm	10	60%	18	F	3
<i>G. flavescens</i>	<i>Gobiusculus flavescens</i>	Bergen, Norway 60°16' N, 5°10' E	22/06/00, 9–10 °C 33.0 ppm	6	6/6	70	F/G	3
<i>G. flavescens</i>	<i>G. flavescens</i>	Bergen, Norway 60°16' N, 5°10' E	21/05/01, 8–9 °C 33.0 ppm	12	83%	78	F/G	1
<i>G. flavescens</i>	<i>G. flavescens</i>	Trondheim, Norway 63°32' N, 10°26 E	13/06/00, 12 °C 32.0 ppm	3	2/3	8	F/G	3
<i>G. arcuatooides</i>	<i>P. pictus</i>	Bergen, Norway 60°16' N, 5°10' E	22/06/00, 9–10 °C 33.0 ppm	16	81%	98	G/F	3
<i>G. arcuatooides</i>	<i>P. pictus</i>	Bergen, Norway 60°16' N, 5°10' E	21/05/01, 8–9 °C 33.0 ppm	10	70%	29	G/F	2
<i>G. branchialis</i>	<i>P. microps</i>	Spuikom Ostend, Belgium 51°14' N, 2°57' E	18/08/99, 16–18 °C* 31.1 ppm	25	84%	248	G	4
<i>G. branchialis</i>	<i>P. microps</i>	Ambleteuse, France 50°N, 1°36' E	23/09/99, 15 °C 16–30 ppm	15	47%	75	G	2
	<i>P. microps</i>	Texel, The Netherlands 53°N, 4°48' E	15/06/99, 12 °C 31.0 ppm	8	50%	9	G	2
	<i>P. microps</i>	Yerseke, The Netherlands 51°30' N, 4°4' E	06/11/99, 16.7 °C 30.1 ppm	8	88%	53	G	1
<i>G. rarus</i>	<i>Spinachia spinacha</i>	Trondheim, Norway 63°32' N, 10°26 E	13/06/00, 12 °C 32.0 ppm	3	2/3	8	G	3

N, number of host specimens collected.

P, prevalence; A, abundance.

Sites: F, fins; G, gills; G/F, >50% on gills, but also found on fins; F/G, >50% on fins, but also found on gills.

nS, number of sequences.

P\*, prevalence, calculated as the ratio of the number of infected fish specimens to the total number of fish (if  $N \geq 10$ ) examined; A\*, abundance, calculated as the total number of *Gyrodactylus* specimens per fish population.

described *G. arcuatus*-like species from *Gobiusculus flavescens*. *Gyrodactylus* sp. from *P. microps* in Geets et al. (1999) is described as a *G. quadratidigitus*-like (see Longshaw, Pursglove & Shinn, 2003) species.

## Materials and methods

### Hosts and parasites

The material for the present study was collected along the Eastern Atlantic seaboard, from Trondheim, Norway (the northernmost sampling point) to Ambleteuse, France (the southernmost sampling point). Data regarding host, locality, date of collection, water tem-

perature, salinity, number of host specimens, prevalence, abundance, site on host and number of the included *Gyrodactylus* specimens are listed below and in Table 1. Fish were transported alive in local water to the laboratory and killed by pithing prior to investigation. Using a stereomicroscope, *Gyrodactylus* specimens were individually removed from the fish by means of preparation needles. Whenever possible, the same specimen was used for both morphological and molecular analysis. If so, the haptor was separated from the body and fixed between slide and coverslip in ammonium picro-glycerine according to Malmberg (1970), and the body was transferred into 5  $\mu$ l of milli-Q water and stored at  $-20^{\circ}\text{C}$  for further DNA analysis. The excretory bladders were studied in some live specimens of all four new species.

#### Molecular analysis

Amplification and sequencing of the complete ITS region, including the 5.8S gene and the V4 region of the ssrRNA, have been performed previously (Huysse et al., 2003; Accession Nos AY338432- AY338434). Sequences were aligned with the Clustal X multiple sequence alignment program (version 1.81, Thompson et al., 1997); regions with an ambiguous alignment were excluded from further analyses. To infer phylogenetic relationships, maximum parsimony (MP), maximum likelihood (ML) and distance-based methods were applied using PAUP\* v. 4.01b (Swofford 2001). Sequences from *Gyrodactylus rarus* Wegener, 1910 (AY338445) were used as outgroup since it belongs to another subgenus (Malmberg, 1970; Huysse et al., 2003). MP trees were inferred with the branch and bound algorithm (1000 replicates). In these analyses gaps were treated both as fifth character and as missing data; all sites were equally weighted. Modeltest 3.0 was used to select the model of DNA evolution that best fits the data based on log likelihood scores (Posada & Crandall, 1998). The ML analysis was performed using the parameters estimated under the best-fit model. With the minimum-evolution distance method, the distance matrix was calculated using the ML parameters. Trees were statistically tested by calculating *P* values for the ML tree and by using 1,000 bootstrap samples for the minimum-evolution tree.

#### Morphological analysis

The morphological analyses of *Gyrodactylus* specimens were carried out at the Laboratory of Aquatic

Ecology, Catholic University Leuven, Belgium and the Department of Zoology, Stockholm University, Sweden. In Belgium measurements of ventral bars and anchors were made using a Zeiss HBO50 microscope ( $\times 100$  oil immersion objective, with  $\times 10$  micrometer eyepieces). Digital images were analysed with the program SigmaScan Pro 5. In Stockholm the microscopical analyses were carried out using oil immersion ( $\times 90$  objective), phase contrast and drawing prism equipment (Malmberg, 1970) connected to a Leica DC 300 digital camera and archiving system. Images of the haptoral hard parts of all specimens and of large embryos in the uterus, when present, were stored and printed (LazerPrint system; Reality Imaging System, Munich, Germany) for further analysis. The results were compared to drawings made by means of a drawing tube (Malmberg, 1970). Measurements of marginal hook sickles were performed by means of image analysis (Leica Q500IW with a Hamamatsu 3 CCD camera, C5810), the sickle area by detection and the other measurements by interactive measuring on a computer screen. Drawings of the holotype and the paratype specimens of the *Gyrodactylus* species in the senior author's collection were compared to drawings of *G. arcuatus* specimens in the 'Malmberg collection'. In total, 10 *G. arcuatus* specimens (Swedish Museum of Natural History, SMNH, Acc. No. 48440) were studied: 10 digitally recorded, three drawn by means of a drawing apparatus and 12 marginal hooks measured. For data on the four new species, see below. The classification of species based on morphological characteristics follows Malmberg (1970).

In order to study geographical variation, specimens of *G. gondae* n. sp. were collected off Texel, The Netherlands, late in November and in Bergen, Norway, late in May (see Table 1). The analyses were run both with and without the inclusion of these different specimens.

For the statistical analyses Statistica 6.0 was used. Correlations between all measured features were tested using Pearson's correlation coefficient. To test for species-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. Factor analysis and discriminant analyses were used to assess the contribution of each variable in the separation of the different species. Observations with missing variables were substituted by their means.

For scanning electron microscopy (SEM), performed at the Royal Belgian Institute of Natural

Sciences in Brussels, live specimens were fixed in glutaraldehyde (2% solution in sodium cacodylate buffer), rinsed in sodium cacodylate buffer, dehydrated in acetone and dried in a Balzers Union Critical Point Dryer. The specimens were subsequently sputter coated with gold in a Balzers Union Sputter Coater Device and scanned in a Philips-515 scanning electron microscope.

## Results

### Molecular identification

About 950 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, and about 350 bp of the *ssrRNA* V4 region were amplified from four to nine specimens of each species (Table 1; Huyse et al., 2003). The 5.8S gene was identical for all species, with the exception of *G. gonda* n. sp. which exhibited one unique substitution. Since this gene is not informative below the subgeneric level (Ziętara et al., 2002), it has been excluded from further analyses. Based on the V4 and ITS region, *G. arcuatus* differs in 11% (13% ITS) from the four species described in the present study. The genetic differentiation between the four species ranges from 0.7 to 1.8% (ITS: 0.5–1.6%). *G. arcuatus* from *Gasterosteus aculeatus* from Stockholm, Sweden, (V4 region not compared) exhibited one transition compared to *G. arcuatus* from the North Sea off Belgium.

Comparison of the different models of evolution revealed that the HKY +  $\Gamma_4$  model (Hasegawa et al., 1985), with the gamma shape parameter = 0.5, was the most suited to the V4 and ITS dataset. The phylogenetic relationships are presented in Figure 2; the cladogram is rooted with *G. rarus*. The MP tree (tree length = 48; C.I. = 0.88; R.I. = 0.84) was identical with the ML tree ( $-Ln$  likelihood = 2644.24) and the minimum-evolution tree. *G. arcuatus* clustered most basally and outside the monophyletic group constituted by the four species found on the gobies (*Pomatoschistus* and *Gobiusculus*). The exact position of *G. flavescens* n. sp. and *G. gonda* n. sp. could not be resolved; *G. branchialis* n. sp. clustered fairly strong with *G. arcuatoides* n. sp. Table 2 presents the uncorrected pair-wise differences.

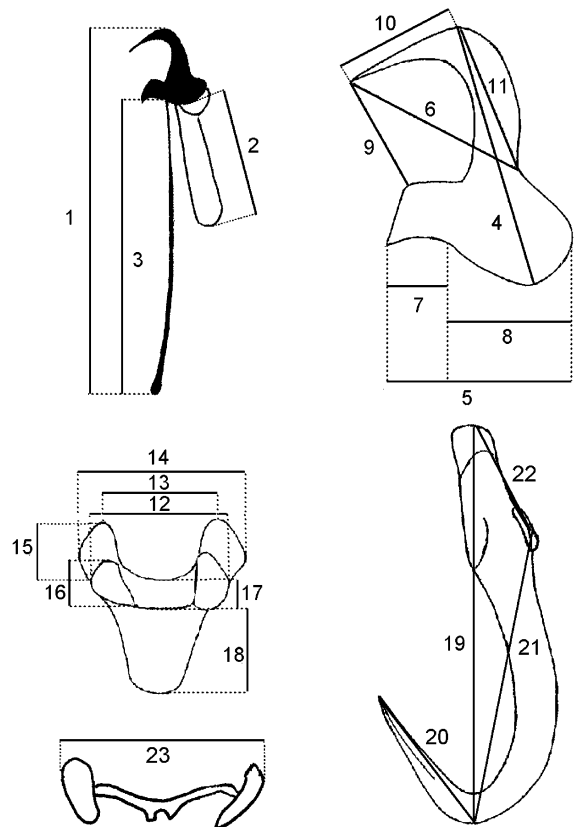


Figure 1. Method of measuring the haptoral hard parts of *G. arcuatus*, *G. gonda* n. sp., *G. flavescens* n. sp., *G. arcuatoides* n. sp. and *G. branchialis* n. sp. Marginal hook: 1. Total length of marginal hook. 2. Length of marginal hook filament loop. 3. Length of marginal hook handle. Marginal hook sickle: 4. Length of sickle. 5. Proximal width of sickle. 6. Distal width of sickle. 7. Toe length of sickle. 8. Heel length of sickle. 9. Aperture distance of sickle. 10. Point length of sickle. 11. Shaft length of sickle (area of sickle measured by detection at image analysis). Ventral bar: 12. Length of ventral bar. 13. Distances between tips of ventral bar processes. 14. Maximum distance between ventral bar processes. 15. Length of ventral bar processes. 16. Basal width of ventral bar. 17. Median width of ventral bar. 18. Ventral bar membrane, length. Anchor: 19. Total length of anchor. 20. Length of anchor point. 21. Length of anchor shaft. 22. Length of anchor root. Dorsal bar: 23. Length of dorsal bar.

### Microscopical analysis

The marginal hook sickles of *G. arcuatus*, *G. gonda* n. sp., *G. flavescens* n. sp., *G. arcuatoides* n. sp. and *G. branchialis* n. sp. are all less than 2.5  $\mu\text{m}$ , i.e. among the smallest described for *Gyrodactylus* (see e.g. Malmberg, 1970). However, small but constant differences in shape between the marginal hook sickles of these five species were found. Differences were also found between the ventral bars and anchors of the species. Furthermore, size differences

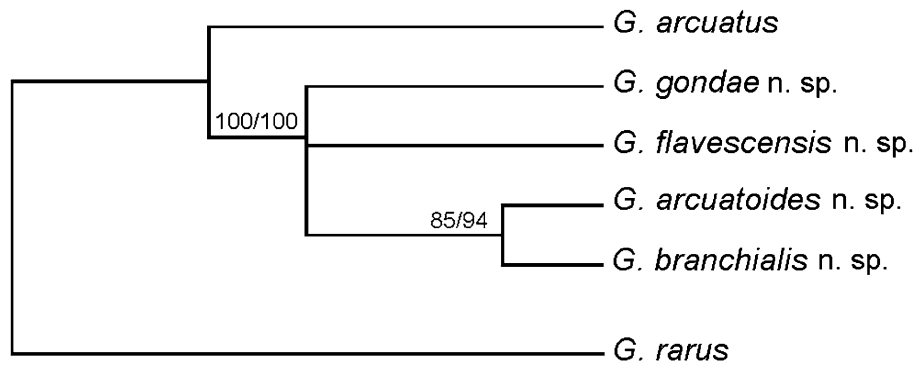


Figure 2. Consensus tree constructed from the ssrRNA V4 region and complete ITS region (1,124 bp) of six *Gyrodactylus* species. Bootstrap support (1,000 replicates) is presented for the maximum parsimony/minimum evolution analyses.

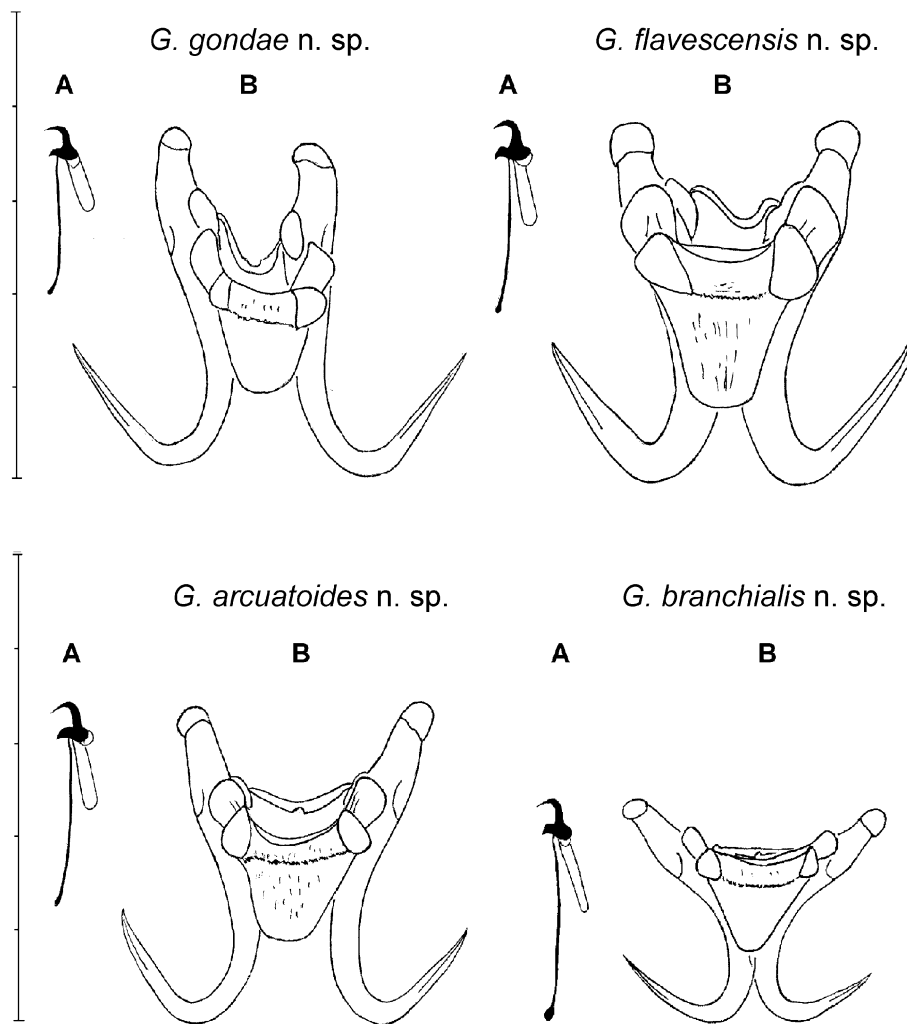


Figure 3. Haptor hard parts of the holotypes of four *Gyrodactylus* species. A. marginal hook; B. anchors, dorsal bar and ventral bar, Parasite data: *G. gondae* n. sp., host *Pomatoschistus minutus*, gills, 25.xi.2000, Texel, The Netherlands; *G. flavescens* n. sp., host *Gobiusculus flavescens*, fins, 21.v.2001, Bergen, Norway; *G. arcuatoides* n. sp., host *P. pictus*, gills, 21.v.2001, Bergen, Norway; *G. branchialis* n. sp., host *P. microps*, gills, 18.viii.1999, Ostend, Belgium. Scale-bar: 50  $\mu$ m.

Table 2. Uncorrected pair-wise distances between *Gyrodactylus arcuatooides* n. sp., *G. flavescens* n. sp., *G. branchialis* n. sp., *G. gonda* n. sp. and *G. arcuatus* calculated from the sequences of the ITS and V4 region are shown in the upper right triangle (outgroup not included); squared Mahalanobis distances from the standard discriminant analysis on morphological measurements on 123 marginal hook sickles of the *Gyrodactylus* species are shown in the lower left triangle.

	<i>G. arcuatooides</i>	<i>G. flavescens</i>	<i>G. branchialis</i>	<i>G. gonda</i>	<i>G. arcuatus</i>
<i>G. arcuatooides</i>	0.00	1.6	0.7	0.9	11.0
<i>G. flavescens</i>	6.48	0.00	1.8	1.1	11.2
<i>G. branchialis</i>	23.49	42.16	0.00	1.3	11.0
<i>G. gonda</i>	10.36	7.21	59.71	0.00	10.9
<i>G. arcuatus</i>	7.55	16.91	22.71	25.40	0.00

Table 3. Slide specimens with a cirrus, a cirrus and an embryo or only an embryo in *Gyrodactylus gonda* n. sp., *G. flavescens* n. sp., *G. arcuatooides* n. sp. and *G. branchialis* n. sp.

<i>Gyrodactylus</i>	Number studied	Cirrus	Cirrus + Embryo	Embryo
<i>G. gonda</i>	22	5	8	6
<i>G. flavescens</i>	13	1	0	7
<i>G. arcuatooides</i>	22	3	3	1
<i>G. branchialis</i>	12	0	0	3

were found between specimens of *G. gonda* from the Netherlands and from Norway, respectively (Figures 4 and 6), which may represent geographical variation. Comparative drawings of the marginal hooks, ventral bars and anchors of the species are presented in Figures 4 and 7. Measurements of these haptor hard parts are presented in Table 4. Information on the presence of a cirrus/penis and/or an embryo in the studied specimens is presented in Table 3.

#### Morphometric and statistical analysis

For each specimen two marginal hooks were measured. The C.V. values of the marginal hook features were fairly low, with minor exceptions for the toe length and the length of the filament loop. These two features were excluded from further statistical analyses. Thus in total, nine of 11 measured marginal hook features (Figure 1) were used in the analyses. A factor analysis included these seven marginal hook features, measured on a total of 123 marginal hook sickles. Three factors had an eigenvalue above 1.00, of which the first two explained 59.2% of the total variance. Factor one is mainly correlated with the proximal width of the sickle and the length of the heel, with factor loadings exceeding 0.70. The second factor

is mainly determined by the total length of the sickle and the distance of the sickle aperture. The backward and forward discriminant analysis revealed that the proximal and distal width of the sickle, the aperture between the apex of the sickle point and the most distal part of the toe, and the length of the handle were mainly responsible for the separation between species. This was confirmed by Tukey's honest significant difference test for unequal sample sizes, which found significant ( $P < 0.01$ ) interspecific differences for these features.

The classification matrix misclassified *G. flavescens* n. sp. once as *G. arcuatooides* n. sp., and it misclassified *G. arcuatooides* n. sp. once as *G. flavescens* and once as *G. gonda* n. sp. Based on the F-values and the squared Mahalanobis distances, *G. branchialis* appeared most distinct, being most dissimilar to *G. gonda*. The smallest distances (all significant at  $P < 0.01$ ) were found between *G. gonda* and *G. flavescens*, and *G. arcuatus* and *G. arcuatooides*, respectively (Table 2).

Marginal hook, ventral bar and anchor features of all four species were measured of approximately 8-23 specimens per species. These numbers are rather low for proper statistical analyses, but the aim of this study was to compare our results with those of Geets et al.

Table 4. Mean, range and C.V. ( $100 \times$  the square root of the variance divided by the mean) of the body, pharynx and features of the haptor hard parts of *G. gondae* n. sp., *G. flavescens* n. sp., *G. arcuatooides* n. sp. and *G. branchialis* n. sp. All measurements are in micrometres.

Gyrodactylus species	<i>G. gondae</i>		<i>G. flavescens</i>		<i>G. arcuatooides</i>		<i>G. branchialis</i>	
	<i>P. lozanoii/P. minutus</i>		<i>Gobiusculus flavescens</i>		<i>P. pictus</i>		<i>P. microps</i>	
<b>Body</b>								
Total length	mean 623.6 (509.5-718.5)	9	594.6 (551.5-662.5)	3	723.5 (654-851.5)	8	488.7 (363-584.5)	9
	C.V. 11.1		10.0		9.0		13.9	
Haptor, length	mean 102.0 (71.5-128)	14	106.7 (90.5-122.5)	7	107.7 (81.5-134)	11	79.3 (66.5-109)	10
	C.V. 17.1		10.8		14.2		16.0	
Haptor, width	mean 118.2 (82.5-153)	10	127.9 (93-147.5)	6	143.4 (97.5-177.5)	11	103.6 (87-124)	8
	C.V. 18.2		14.69		16.3		12.3	
<b>Pharynx</b>								
Length	mean 52.2 (41-61.5)	10	57.2 (52-64)	3	61.3 (50.5-76)	8	40.3 (30.5-45)	7
	C.V. 12.7		10.9		14.6		14.3	
Width	mean 47.6 (38.5-58.5)	11	58.2 (54.5-61)	3	61.3 (57-72)	9	41.8 (28-50.5)	7
	C.V. 11.4		5.7		7.5		17.7	
<b>Marginal hook</b>								
1. Total length of marginal hook	mean 20.3 (19-22)	22	21.5 (20-23)	14	21.5 (19-23)	18	24.6 (23.5-26.5)	14
	C.V. 3.0		6.4		3.1		4.2	
2. Length of marginal hook filament loop	mean 7.1 (5.5-10)	35	7.9 (6.5-10)	21	7.2 (6-8.5)	34	7.1 (5-10)	24
	C.V. 11.0		10.7		10.0		17.9	
3. Length of marginal hook handle	mean 16.3 (15-18)	38	17.9 (16-20)	22	17.7 (15.5-19)	36	20.9 (19-23)	24
	C.V. 3.0		6.7		3.3		5.3	
4. Length of marginal hook sickle	mean 4.1 (3.5-5)	38	4.6 (4-5)	22	4.4 (4-5)	36	4.5 (4-5)	24
	C.V. 3.9		5.4		3.8		6.7	
5. Proximal width of marginal hook sickle	mean 3.3 (3-4)	38	3.5 (3-4)	22	3.4 (3-4)	36	2.8 (2.5-3)	24
	C.V. 6.3		7.3		6.1		8.1	
6. Distal width of marginal hook sickle	mean 3.4 (2.5-4)	38	3.8 (3.5-4)	22	3.5 (3-4)	36	3.4 (3-4)	24
	C.V. 5.3		5.4		6.7		6.3	
7. Marginal hook toe length	mean 1.2 (0.5-1.5)	38	1.2 (1-1.5)	22	1.3 (1-1.5)	36	0.9 (0.5-1)	24
	C.V. 13.5		12.5		13.3		11.8	
8. Marginal hook heel length	mean 2.1 (1.5-2.5)	38	2.4 (2-3)	22	2.1 (1.5-2.5)	36	1.8 (1.5-2)	24
	C.V. 8.3		9.1		7.5		11.3	
9. Marginal hook sickle aperture distance	mean 2.0 (1.5-2.5)	38	2.1 (1.5-3)	22	2.1 (1.5-2.5)	36	2.2 (2-2.5)	24
	C.V. 8.6		10.7		7.8		6.4	
10. Marginal hook sickle shaft length	mean 2.8 (2.5-3)	38	3.1 (2.5-3.5)	22	2.9 (2-3.5)	36	3.0 (2.5-3.5)	24
	C.V. 6.2		8.0		7.2		7.6	

Table 4 continued.

Gyrodactylus species	G. gondae		N. G. flavescens		N. G. arcuatooides		N. G. branchialis	
	P. lozanoi/P. minutus		Gobiusculus flavescens		P. pictus		P. microps	
Host species	mean	C.V.	mean	C.V.	mean	C.V.	mean	C.V.
11. Marginal hook sickle point length	2.2 (1.5-2.5)	13.5	2.2 (1.5-2.5)	10.3	2.3 (2-3)	9.5	2.0 (1.5-2.5)	8.9
Area of marginal hook	7.2 (5.5-9.5)	8.0	8.7 (7-11.5)	11.9	7.6 (6.5-9)	10.0	6.5 (5-8)	11.7
<b>Ventral bar</b>								
12. Length of ventral bar	18.5 (18.5-23)	4.3	19.1 (13-23)	9.8	17.1 (14-21.5)	6.3	13.3 (11.5-14.5)	5.3
13. Distance between tip of processes	17.5 (12.5-21.5)	8.4	16.2 (12.5-19)	13.3	17.0 (13.5-20)	7.5	15.4 (13-17)	6.9
14. Outermost distance between processes	21.3 (17.5-25)	5.2	21.9 (17.5-24)	11.6	20.8 (17.5-24)	7.0	16.9 (14-18.5)	7.8
15. Length of ventral bar processes	6.9 (5-9)	12.2	7.8 (5.5-9)	11.3	5.9 (4.5-8.5)	11.3	3.4 (2.5-5)	14.2
16. Basal width of ventral bar	6.7 (4-9)	6.1	7.6 (5-10.5)	6.4	6.5 (4-8)	8.7	4.5 (4-5.5)	8.0
17. Median width of ventral bar	4.6 (3.5-5.5)	12.2	5.0 (4-6)	13.1	4.4 (3-5.5)	9.9	3.3 (2-3.5)	9.5
18. Length of ventral bar membrane	10.8 (7.5-13)	7.5	10.3 (7.5-12.5)	14.5	9.0 (6.5-11)	10.7	6.1 (5-7)	11.6
<b>Anchors</b>								
19. Total length of anchor	42.4 (36-49)	2.9	39.6 (36-41)	3.8	36.4 (30-44)	7.0	25.8 (23.5-27.5)	3.8
20. Length of anchor point	20.7 (17-24)	2.6	19.5 (17-21)	5.5	16.8 (14-21)	8.5	12.6 (11-13.5)	6.5
21. Length of anchor shaft	32.9 (27.5-38)	3.0	31.6 (29.5-34)	4.2	28.4 (23.5-33.5)	8.4	19.9 (18-21.5)	6.2
22. Length of anchor root	12.0 (10-14.5)	7.1	11.1 (10-12.5)	6.0	10.9 (8-13)	8.7	9.9 (8.5-11.5)	11.5
<b>Dorsal bar</b>								
23. Length of dorsal bar	19.0 (15.5-21.5)	8.7	17.0 (14-20.5)	10.4	16.7 (14-18.5)	8.6	16.8 (15.5-17.5)	4.4

N, the number of *Gyrodactylus* specimens measured.

Mean, mean + range in parentheses.

The numbers preceding the marginal hook, ventral bar and anchor features relate to Figure 1.

(1999) – see the ‘Introduction’. Most features had a C.V. value <12%, with the exception of a few ventral bar features measured for *G. flavescens* (Table 4). All measured features (Figure 1) were used in the statistical analyses. Two factors had an eigenvalue >1.00, of which the first one explained 73.56% of the total variance. Factor one is correlated with most anchor and ventral bar features, with factor loadings exceeding 0.70. The second factor is determined by the distance between the tip of the ventral bar processes. Backward discriminant analyses selected the length of the anchor point, total anchor length, the length of the ventral bar processes and the distance between the tips of the ventral bar processes as the most discriminating features. Figure 8 shows the plot of the backward discriminant analysis. The species were never misclassified. Based on the F-values and the squared Mahalanobis distances, *G. branchialis* appeared most distinct; the highest distance was found in comparison with *G. gonda*. The smallest distances (all significant at  $P < 0.01$ ) were found between *G. gonda* and *G. flavescens* followed by the distance between *G. gonda* and *G. arcuatoides*. When all *G. gonda* specimens, collected late in November in Texel (The Netherlands) and late in May in Bergen (Norway), were included in the discriminant analysis, it was more difficult to separate this species from *G. arcuatoides*. This was also reported by Geets et al. (1999). In Figure 8, only the specimens collected in Norway were included in the analysis, except for *G. branchialis* for which only specimens of Belgium were measured.

#### Family Gyrodactylidae Cobbold, 1864

#### Genus *Gyrodactylus* Nordmann, 1832

#### Subgenus *Mesonephrotus* Malmberg, 1964

#### Morphological species group: *G. arcuatus*-group

##### Diagnosis

Species with marginal hook sickles of a type similar to that in *G. arcuatus* Bychowsky *sensu* Bychowsky & Poljansky (1953). Anchor roots relatively short. Ventral bars with long processes. Dorsal bars may have a postero-medial notch. Small cirrus/penis spines in one arched row. (See also Malmberg, 1970)

#### *Gyrodactylus gonda* n. sp.\*

*Type-host*: *Pomatoschistus minutus* Pallas.

*Other host*: *P. lozanoi* de Buen.

\* Previously recorded as *G. sp.* 1 in Geets et al. (1999) and Ziętara et al. (2002) and as *G. gonda* n. sp. in Huyse (2002).

*Site*: Gills and fins, mostly pelvic and pectoral fins.

*Type-locality*: Off Texel, The Netherlands (53°N, 4°48' E).

*Water temperature, salinity and date of collection*: 12 °C, 31.0 ppm, 25.xi.2000.

*Other localities*: *P. minutus*, off Trondheim and Bergen, Norway; *P. lozanoi*, North Sea, off Belgium.

*Specimens studied for molecular analysis*: *P. minutus*, Texel 5, Trondheim 2, Bergen 3. *P. lozanoi*, Texel 3, North Sea 2. DNA sequences are deposited in GenBank under accession nos AY338432-AY338434, AY339769 (Huyse et al., 2003). See also AF328866 (Ziętara et al., 2002, under the name *G. sp.*, North Sea).

*Specimens studied for morphological analysis*: The holotype specimen and 11 other specimens from Texel, 10 specimens from Bergen and 2 specimens from *P. lozanoi* from North Sea were studied. Specimens (22) were digitally recorded and the haptoral hard parts of 2 specimens from Texel and of 1 from Bergen were drawn by means of a drawing apparatus. The 22 specimens were measured (marginal hook sickles 38, ventral bars 21, anchors 7). The holotype specimen (Acc. No. 5782) and 15 paratypes (Acc. No. 5783-5797) are deposited in the Swedish Museum of Natural History (SMNH). See Table 1.

*Etymology*: The species is named for Dr Gonda Geets, who first detected the species by means of morphometric analysis.

#### Molecular diagnosis

The DNA sequence of ITS1 was 364 bp, 5.8S rDNA 157 bp and ITS2 394 bp; the total segment was 915 bp long; 317 bp of the V4 region were sequenced. All sequences studied were identical (Huyse et al., 2003). Genetic distances between *G. gonda* n. sp. and *G. branchialis* n. sp., *G. flavescens* n. sp. and *G. arcuatoides* n. sp. amount to 1.3%, 1.1% and 0.9%, respectively (ITS and V4 region; uncorrected p-distances, Table 2). No intraspecific differences were found. The phylogenetic arrangement/relationship is illustrated in Figure 2.

#### Microscopical diagnosis (Figures 3, 4B1-B3, 5B, 6B1-B37, Tables 1–4)

Coverslip-flattened specimens in ammonium picroglycerine 624 (510-719)  $\mu\text{m}$  long. Haptor delineated from body. Pharynx with short processes. Cirrus/penis situated posteriorly to pharynx, with 1 large spine and small spines in single arched row. Protonephridial system with small bladders. Marginal hook sickle: shaft

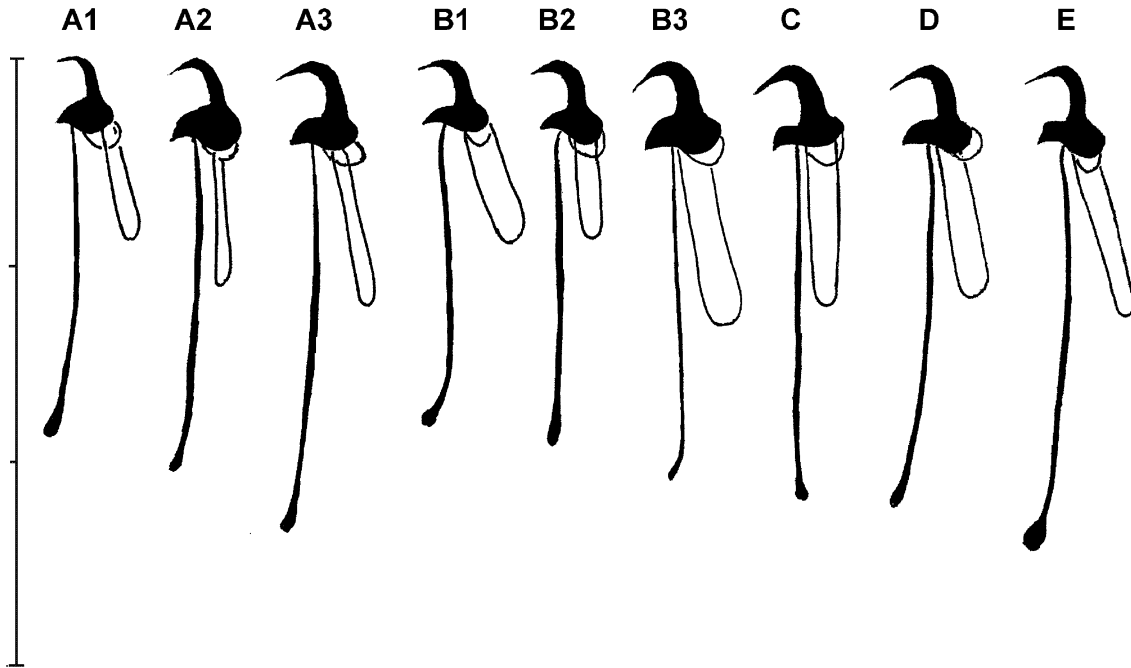


Figure 4. Comparative drawings of the marginal hooks of five *Gyrodactylus* species. A1-A3. *G. arcuatus* from *Gasterosteus aculeatus*; A1 and A2, from Malmberg (1964); A1, host specimen length 1.35 cm, Stockholm Archipelago, Nämdö, 30.vii.1957; A2, host specimen length 6 cm, Kieler Hafen, Germany, 19.vi.1958; A3, specimen from Stockholm Archipelago, Edesö, 11.vi.2001. B1-B3. *G. gondae* n. sp.; B1, holotype specimen, Figure 3; B2, specimen from *P. minutus*, Texel, The Netherlands, 25.xi.2000 (anterior part of the body used for DNA); B3, specimen from *P. minutus*, Bergen, Norway, 21.v.2001. C. *G. flavescens* n. sp., holotype specimen, Figure 3. D. *G. arcuatooides* n. sp., holotype specimen, Figure 3. E. *G. branchialis* n. sp., holotype specimen, Figure 3. Species in A-D are members of the *G. arcuatus*-group: marginal hook sickles of a similar type and different to that in E, i.e. *G. branchialis* of the *G. quadratidigitus*-group. Scale-bar: 30  $\mu$ m

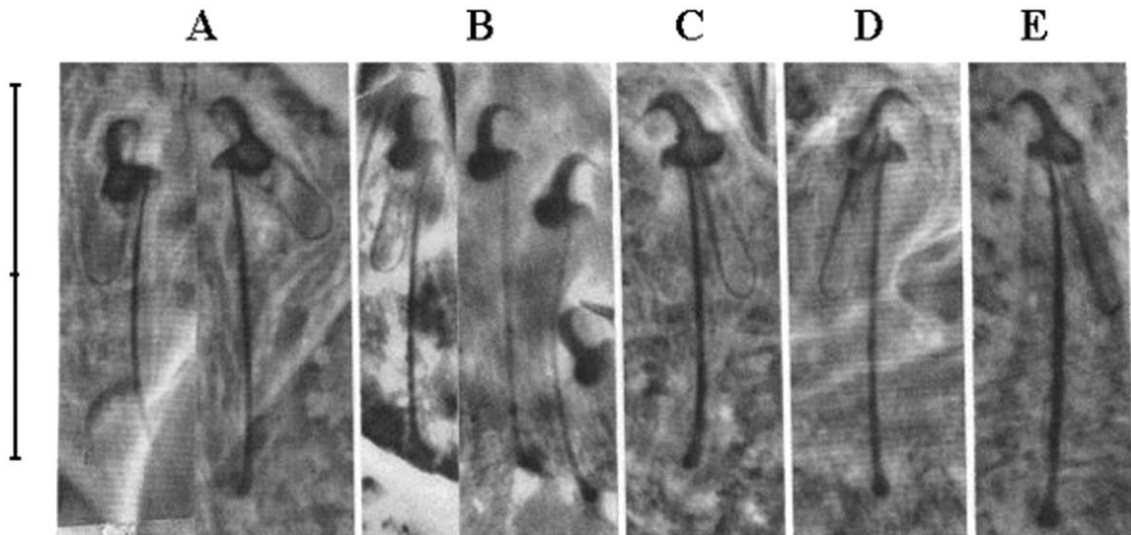


Figure 5. Comparative micrographs (phase contrast) of the marginal hooks of five *Gyrodactylus* species. A. *G. arcuatus* from *P. microps*, Bergen, Norway 21.v.2001. B-D. The holotype specimens in Figure 3: B, *G. gondae* n. sp.; C, *G. flavescens* n. sp.; D, *G. arcuatooides* n. sp. E. *G. branchialis* n. sp. Scale-bar: 20  $\mu$ m.

shorter than in *G. arcuatus*, *G. flavescens* and *G. arcuatoides*. Proximal region (toe) of sickle less prominent than in these 3 species. Distal region (point) not extending beyond toe. Furrow present on each lateral side of point (see Mo, 1993, Figure 4). Marginal hook sickle of specimens from Bergen larger than those from the Netherlands. Marginal hook handle shorter than in *G. arcuatus*, *G. flavescens* and *G. arcuatoides*. Shape of ventral bar similar to that of *G. arcuatus* with laterally pointing processes. Anchor roots not slightly divergent laterally from median line, as in *G. arcuatus*, but slightly curved towards median line, as in *G. flavescens* and *G. arcuatoides*. Anchor point longer than in *G. arcuatus*, with grooves between lateral furrows (Figure 7). Dorsal bar may have median notch.

### *Gyrodactylus flavescens* n. sp.\*

*Type-host:* *Gobiusculus flavescens* Fabricius.

*Site:* Gill-arches, gill-filaments; in heavy infections also on pelvic and pectoral fins.

*Type-locality:* Off Bergen, Norway (60°16' N, 5°10' E).

*Water temperature, salinity and date of collection:* 8–9 °C, 33.0 ppm, 21.v.2001.

*Other localities:* Off Trondheim, Norway.

*Specimens studied for molecular analysis:* From Bergen 4, Trondheim 3 (Table 1). DNA sequences are deposited in GenBank under accession nos AY338437, AY338438 and AY339770 (Huyse et al., 2003).

*Specimens studied for morphological analysis:* The holotype specimen and 12 other specimens from Bergen were studied. All 13 specimens were digitally recorded and the haptor hard parts of 5 specimens were drawn by means of a drawing apparatus. The 13 specimens were measured (marginal hook sickles 22, ventral bars 12, anchors 6). The holotype specimen (Acc. No. 5798) and 11 paratypes (Acc. Nos 5799–5809) are deposited at the Swedish Museum of Natural History (SMNH). See Table 1.

*Etymology:* The specific name *flavescens* refers to the Latin name of the host.

### *Molecular diagnosis*

The DNA sequence of ITS1 was 364 bp, 5.8S rDNA 157 bp and ITS2 394 bp; the total segment was 915 bp long; 317 bp of the V4 region were sequenced.

\*Previously recorded as *G. flavescens* n. sp. in Huyse (2002).

All sequences studied were identical (Huyse et al., 2003). Genetic distances between *G. flavescens* n. sp. and *G. gondae* n. sp., *G. branchialis* n. sp. and *G. arcuatoides* n. sp. amount to 1.1%, 1.8% and 1.6%, respectively (ITS and V4 region; uncorrected p-distances, Table 2). No intraspecific differences were found. The phylogenetic arrangement/relationship is illustrated in Figure 2.

### *Microscopical diagnosis* (Figures 3, 4C, 5C, 6C; Tables 1–4)

Coverslip-flattened specimens in ammonium picroglycerine 595 (551–663) μm long. Haptor delineated from body. Pharynx with short processes. Cirrus/penis situated posteriorly to pharynx, with 1 large spine and small spines in single arched row. Protonephridial system with small bladders. Marginal hook sickle: shaft longer than in *G. gondae*. Proximal region (toe) more prominent than in *G. gondae*, and with distinct knee. Distal region (point) extends beyond toe. Dorsal proximal region (heel) less prominent than in *G. arcuatus* and *G. branchialis*. Marginal hook handle shorter than in latter 2 species. Ventral bar broad, with broad processes of similar shape to those of *G. arcuatoides* and pointing more anteriorly than those of *G. arcuatus* and *G. gondae*. Anchors more robust than in *G. arcuatoides*. Anchor roots slightly curved towards median line, as in *G. arcuatoides*.

### *Gyrodactylus arcuatoides* n. sp.\*

*Type-host:* *Pomatoschistus pictus* Malm.

*Site:* Gill-arches, gill-filaments; in heavy infections also on pelvic and pectoral fins.

*Type-locality:* Off Bergen, Norway (60°16' N, 5°10' E).

*Water temperature, salinity and date of collection:* 8–9 °C, 33.0 ppm, 21.v.2001.

*Specimens studied for molecular analysis:* 5 from Bergen (Table 1). DNA sequences are deposited in GenBank under accession nos AY338429 and AY339771.

*Specimens studied for morphological analysis:* The holotype specimen and 24 other specimens from Bergen were studied. All 25 specimens were digitally recorded and the haptor hard parts of 6 specimens were drawn by means of a drawing tube. The 25 specimens were measured (marginal hook sickles 36,

\*Previously recorded as *G. sp. 2* in Geets et al. (1999) and as *G. arcuatoides* n. sp. in Huyse (2002).

ventral bars 24, anchors 7). The holotype specimen (Acc. No. 5810) and 23 paratypes (Acc. Nos 5811-5833) are deposited at the Swedish Museum of Natural History (SMNH). See Table 1.

*Etymology*: The species is named *arcuatoides* because its haptoral hard parts resemble those of *G. arcuatus*.

#### *Molecular diagnosis*

The DNA sequence of ITS1 was 364 bp, 5.8S rDNA 157 bp and ITS2 394 bp; the total segment was 915 bp long; 317 bp of the V4 region were sequenced. All sequences studied were identical (Huyse et al., 2003). Genetic distances between *G. arcuatoides* n. sp. and *G. gonda* n. sp., *G. flavescens* n. sp. and *G. branchialis* n. sp. amount to 0.9%, 1.6% and 0.7%, respectively (ITS and V4 region; uncorrected p-distances, Table 2). The phylogenetic arrangement/relationship is illustrated in Figure 2.

#### *Microscopical diagnosis* (Figures 3, 4D, 5D, 6D; Tables 1–4)

Coverslip-flattened specimens in ammonium picroglycerine 724 (654–852)  $\mu\text{m}$  long. Haptor delineated from body. Pharynx with short processes. Cirrus/penis situated posteriorly to pharynx, with 1 large spine and small spines in single arched row. Protonephridial system with small bladders. Marginal hook sickle: shaft longer and proximal region (toe) more prominent than in *G. gonda*. Distal region (point) extends beyond toe. Dorsal proximal region (heel) less prominent than in *G. branchialis*. Marginal hook handle shorter than in latter species. Ventral bar smaller than in *G. flavescens* but with processes of similar shape and pointing more anteriorly than in *G. arcuatus* and *G. gonda*. Anchors similar to those in *G. gonda*. Anchor roots slightly curved towards median line, as in *G. flavescens*. Dorsal bar may have median notch.

#### **Morphological species group: *G. quadratidigitus*-group**

##### *Diagnosis*

Species with marginal hook sickles of similar type, with blunt toe, distinct knee and long heel, as in *G. quadratidigitus* Longshaw, Pursglove & Shinn, 2003. Ventral bar with laterally pointing processes. Anchors small, with curved shaft.

#### ***G. branchialis* n. sp.\***

*Type-host*: *Pomatoschistus microps* Krøyer.

*Site*: Gills/Gill-filaments.

*Type-locality*: Off Spuikom, Ostend, Belgium (51°14' N, 2°57' E).

*Water temperature, salinity and date of collection*: 16–18°, 31.1 ppm, 18.viii.1999.

*Other locality*: Off Ambleteuse, France; Texel and Yerseke, The Netherlands.

*Specimens studied for molecular analysis*: Ostend 4, Ambleteuse 2, Texel 2, Yerseke 1 (Table 1). DNA sequences are deposited in GenBank under accession no: AY338430, AY338450, AY338451 and AY339773 (Huyse et al., 2003).

*Specimens studied for microscopical analysis*: The holotype specimen and 11 other specimens from Spuikom, Ostend, were studied. All 12 specimens were digitally recorded and haptoral hard parts of 4 specimens were drawn by means of a drawing apparatus. The 12 specimens were measured (marginal hook sickles 24, ventral bars 12, anchors 12). The holotype specimen (Acc. No. 5834) and 11 paratypes (Ostend) (Acc. Nos 5835–5845) are deposited in the Swedish Museum of Natural History (SMNH). See Table 1.

*Etymology*: The name *branchialis* refers to the site of attachment of this species.

#### *Molecular diagnosis*

The DNA sequence of ITS1 was 365 bp, 5.8S rDNA 157 bp and ITS2 394 bp; the total segment was 916 bp long; 317 bp of the V4 region were sequenced. All sequences studied were identical (Huyse et al. 2003). Genetic distances between *G. branchialis* n. sp. and *G. gonda* n. sp., *G. flavescens* n. sp. and *G. arcuatoides* n. sp. amount to 0.9%, 1.6% and 1.6%, respectively (ITS and V4 region; uncorrected p-distances, Table 2). No intraspecific differences were found. The phylogenetic arrangement/relationship is illustrated in Figure 2.

#### *Microscopical diagnosis* (Figures 3, 4E, 5E, 6E; Tables 1–4)

Coverslip-flattened specimens in ammonium picroglycerine 489 (363–585)  $\mu\text{m}$  long. Haptor delineated from body. Pharynx with short processes. Cirrus/penis situated posteriorly to pharynx, with 1 large spine and

\*Previously recorded as *G. sp.* in Geets et al. (1999) and as *G. branchialis* n. sp. in Huyse (2002).

small spines in single arched row. Protonephridial system with small bladders. Marginal hook (sickle + handle) longer than anchors. Marginal hook handle longer than in *G. gondae*, *G. flavescens* and *G. arcuatoides*, but of similar length as in *G. arcuatus*. Marginal hook sickle of same type and size as that of *G. quadratidigitus* Longshaw, Pursglove & Shinn, 2003, thus differing from those of *G. arcuatus*, *G. gondae*, *G. flavescens* and *G. arcuatoides*. Marginal hook sickle length similar to that of *G. flavescens* and *G. arcuatoides*, but shaft more slender and heel longer and pointing more anteriorly than in these two species. Toe blunt, as in *G. quadratidigitus*, with distinct knee and pointed ventrally. Sickle point extends beyond toe. Ventral bar small with small processes pointing laterally. Ventral bar membrane triangular. Anchors small, resembling those of *G. arcuatus*-group, with curved shafts and diverging roots; roots are longer and more slender than in *G. quadratidigitus*. Dorsal bar may have median notch.

## Discussion

### *Molecular analyses*

It is known that the ITS region can vary greatly between species, but it is accepted as a species diagnostic marker for *Gyrodactylus* species, since it exhibits a very low level of intraspecific and geographical variation (Ziętara & Lumme, 2002). In the present molecular analysis, only minor geographical variation was found between specimens of *G. arcuatus* collected in the Belgian North Sea and the Baltic Sea off Sweden. The highest level of geographical variation described in literature has been found for *G. arcuatus*, where Ziętara et al. (2000) compared freshwater, brackish and marine isolates from Overpelt (Belgium), Gdansk (Poland), Doel (Belgium) and Aberdeen (Scotland) and found eight variable sites in the complete ITS region (3 transitions, two transversions and 3 double-state sites). Despite its frequent use as a diagnostic tool, there is no consensus yet regarding a 'cut-off value' of ITS differentiation to be considered as indicating a distinct species. Recently, Ziętara & Lumme (2003) suggested a threshold of 1% variation in the ITS region. However, it is obvious that a single rule cannot be applied for all species of *Gyrodactylus*, since the evolutionary rate can differ even between closely related species (e.g. Huyse et al., 2002; Huyse et al., 2003). Additional

morphological and ecological information also has to be taken into consideration. The choice of marker is another important issue. For example, although *G. thymalli* Zitnan, 1960 is described as a distinct species, based on morphological, behavioural and pathological grounds (Sterud et al., 2002), no variation could be found throughout the ITS region when compared with *G. salaris* Malmberg, 1957. Recently, however, genetic differentiation between both species has been found in the COI mtDNA region (Meinila et al., 2002).

The four new gyrodactylid species found on species of *Gobiusculus* and *Pomatoschistus* cluster strongly together as a monophyletic group. This has been confirmed in a molecular phylogenetic study by Huyse et al. (2003), which included all available *Gyrodactylus* species in GenBank. They were clearly separated from *G. arcuatus*, with a genetic distance of up to 13%. The genetic differentiation between the four new species (0.7-1.8%, uncorrected p-distances constructed on the complete data-set) is low compared to the differentiation of *Gyrodactylus* spp. reported in literature (2.7-6% and 1.5-38.7% for ITS1 and ITS2; Kimura distances from Matejusova et al., 2001), but it is of about the same magnitude as described by Huyse & Volckaert (2002) for *G. rugiensis* Gläser, 1974 on the fins of *P. microps* and *G. rugiensoides* Huyse & Volckaert, 2002 on the fins of *P. pictus* and *P. minutus* (differing by 2.5% and 1.3% in the ITS and V4 region, respectively; uncorrected distances). The present study and that of Huyse & Volckaert (2002) are the first papers describing the molecular variation of *Gyrodactylus* species found on closely related hosts of the same genus<sup>†</sup>. As such, it appears that, in this particular system, closely related hosts are parasitised by closely related *Gyrodactylus* species.

### *Morphometric analyses*

An elaborate morphometric analysis was carried out by Geets et al. (1999) on 17 hook characters of *G. arcuatus*, *G. sp.1* (here *G. gondae* n. sp.) and *G. sp. 2* (here *G. arcuatoides* n. sp.). The investigated specimens were collected in different seasons and a total of 268 specimens were analysed. Geets et al. (1999) were able to demonstrate both seasonal and species-specific variation by means of multivariate analyses. In the present paper, two more species, *G. flavescens* n. sp. and *G. branchialis* n. sp. were analysed,

<sup>†</sup>Although *Gobiusculus flavescens* is currently placed in another genus, it clusters strongly within *Pomatoschistus* based on its ITS1 rDNA and 12S and 16S mtDNA (Huyse et al., 2004).

with special attention being paid to the marginal hook features. Based on seven of the nine measured features of the marginal hook sickle, all species could be fairly well separated from each other. All statistical analyses pointed to the proximal and distal width of the marginal hook sickle, the distance of the marginal hook sickle aperture and the length of the marginal hook handle as the strongest morphometric diagnostic characters. Perfect discrimination was obtained by multivariate analyses on anchor and ventral bar features. In particular, the length of the anchor point, the total length of the anchor, the length of the ventral bar processes and the distance between the tip of the ventral bar processes had a high discriminatory value.

#### *Morphology, biology and DNA*

The marginal hooks, ventral bars and anchors of *G. gondae* n. sp. are very similar to the same haptoral hard parts in *G. arcuatus*. Because of this, the species was initially named *G. cf. arcuatus* or *G. sp. 1* (Geets et al., 1999). The fact that *G. arcuatus* can, at least temporarily, survive on several fish species other than its host *Gasterosteus aculeatus* (see Malmberg, 1970), made its presence on *Pomatoschistus minutus* and *P. lozanoi* plausible. The subsequent morphometric analyses, however, suggested that the *Gyrodactylus* specimens from the two gobiids belonged to an undescribed species. Preliminary data on the genetic variability of *G. sp. 1* from *P. minutus* and *P. lozanoi* confirmed the presence of one and the same *Gyrodactylus* species (Geets, 1998). Renewed morphological analyses by means of image analysis, together with statistical analyses, revealed clear differences in the size of the marginal hook sickles (Figure 4), ventral bars and anchors (Figures 6, 8) between *G. arcuatus* and *G. sp. 1/G. gondae*. Then the molecular differences between these two species clearly confirmed the existence of two different species.

Rather large size difference between specimens of *G. arcuatus* parasitising *Gasterosteus aculeatus* (also in the same locality) are described. Thus the body, marginal hooks, ventral bars and anchors were distinctly smaller in *G. arcuatus* specimens from a small host fish (< 2 cm) than in *G. arcuatus* specimens from larger host fish (Malmberg, 1964, figure 17; Figure 4 A1, 6A1). The differences described above in the size of the haptoral hard parts in *G. gondae* (Figure 6B1-B3) might suggest that the differences between *G. gondae* and *G. arcuatus* are examples of intraspecific variation, but our molecular, statistical

and morphological investigations show that this is not the case.

Two more species presented in Geets (1998) and Geets et al. (1999), namely *G. sp. 2* and *G. sp.*, were characterised at a molecular level and are here described as *G. arcuatooides* n. sp. and *G. branchialis* n. sp., respectively. The differences in the shape of the marginal hooks, anchors and ventral bar processes, together with the molecular analysis, indicate two different species (Figures 3,4,6). This is also valid for *G. flavescens* n. sp. from *Gobiusculus flavescens*.

*G. arcuatus*, *G. gondae*, *G. flavescens* and *G. arcuatooides* have the same type of marginal hooks, ventral bars and anchors. Thus morphologically they belong to the same species group, the *G. arcuatus*-group (Malmberg 1970). This, however, does not apply to *G. branchialis*. The shape of the marginal hook sickles with a blunt toe, the ventral bar with laterally pointing processes and the small, curved anchors are of the same type as those described for *G. quadratidigitus* Longshaw, Pursglove & Shinn, 2003. From a morphological point of view, these two species belong to the same species group, here presented as the *G. quadratidigitus*-group. Unfortunately, no molecular data for *G. quadratidigitus* are yet available. From a molecular point of view, however, *G. branchialis* appears to be very closely related to *G. arcuatooides*. This may imply that the morphological type of marginal hooks of the *G. quadratidigitus*-group arose from the type occurring in the *G. arcuatus*-group.

By contrast, *G. arcuatus* and *G. gondae* are genetically two very distinct species; yet, without a detailed morphological analysis, they can be confused. This shows that there may have been differences in the pace of molecular and phenotypic evolution. This can also be seen in the fact that the morphometric resemblance between the species was not always reflected in the genetic distances (Table 2).

The checking of slides of the present material (Table 4) revealed a cirrus/penis in five of 22 *G. gondae* specimens, in one of 13 *G. flavescens* specimens and in three of 22 *G. arcuatooides* specimens. A cirrus/penis and a vesicula seminalis with sperm were also present in the *G. arcuatus* specimens studied. Thus, at least in the life-cycle of these particular species, sexual reproduction was likely involved. This suggests that the observed molecular and morphological differentiation is not a result of clonal variation.

The *Gyrodactylus* species described above from the gills of species of *Pomatoschistus* and *Gobiusculus* might be a result of host-switching events, with the

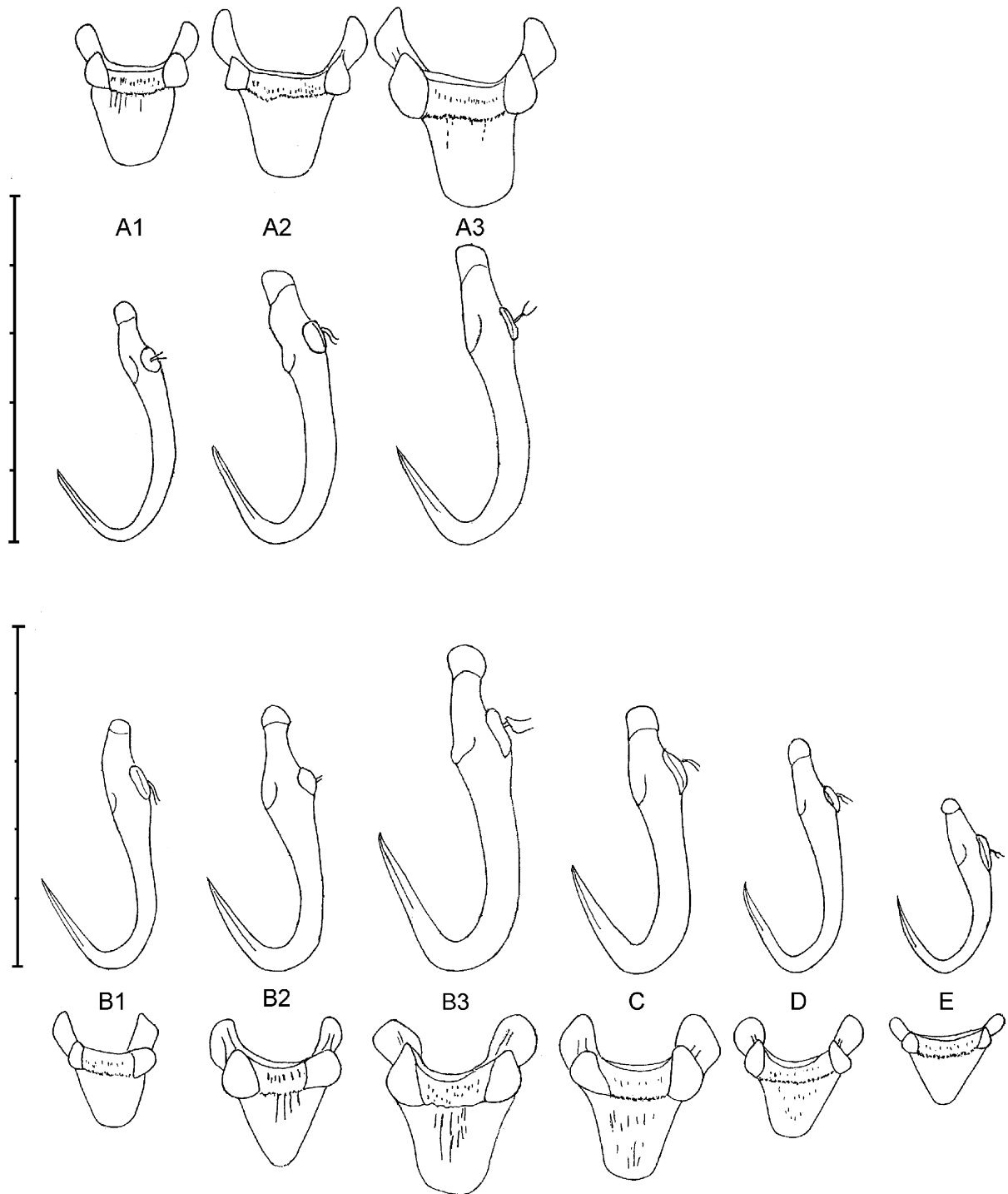


Figure 6. Comparative drawings of the ventral bars and anchors of the specimens in Figure 4: A1-A3. *G. arcuatus*; B1-B3. *G. gondaie* n. sp.; C. *G. flavescens* n. sp.; D. *G. arcuatoides* n. sp.; E. *G. branchialis* n. sp. Scale-bar: 50  $\mu$ m.

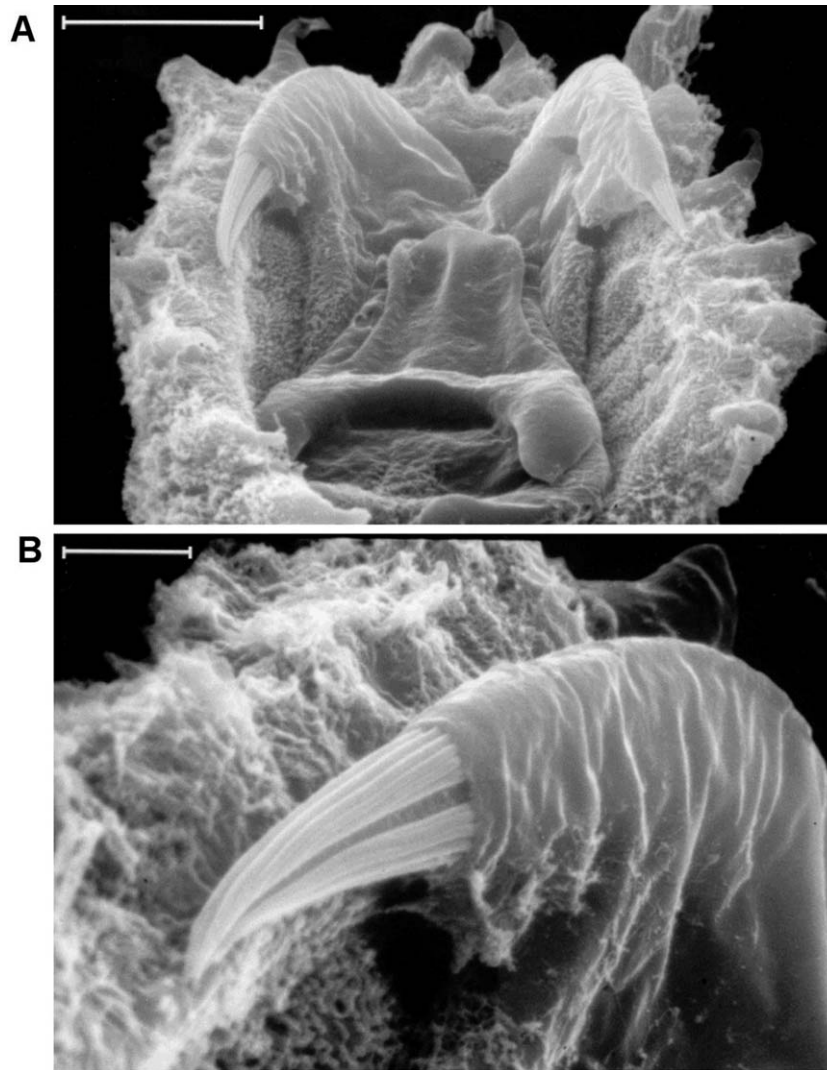


Figure 7. SEM micrographs of *Gyrodactylus gondae* n. sp. A. Ventral view of the haptor showing the ventral bar and the posterior region of the anchors. The ventral bar and the anchors, except for the outermost region of the anchors, are covered by the anchor membrane. A furrow is visible in one of the anchor points (left). B The anchor point (left) in A further enlarged. The outermost part of the point is protruding from the anchor membrane. The lateral furrow of one side of the point and grooves on both sides of the furrow are visible. Scale-bar A, 10  $\mu\text{m}$ ; B, 2  $\mu\text{m}$ .

worms transferring from *Gasterosteus aculeatus*, the three-spined stickleback, onto the three gobiids, and resulting in the evolution of *G. gondae*, *G. arcuatooides* and *G. flavescensis*, which are all members of the *G. arcuatus*-group. Initially, host-switching between the gobiids may also have been involved, followed by speciation on their 'new' gobiid host (Huyse et al., 2003).

The four species have small excretory bladders, thus they belong either to the subgenus *Mesonephrotus* Malmberg, 1970 or the subgenus *Metanephrotus* Malmberg, 1970. The molecular phylogenetic ana-

lysis suggests that they are members of *Mesonephrotus*. Gobies are also parasitised by members of the subgenus *Paranephrotus* Malmberg, 1970 (see Gläser, 1974; Huyse & Volckaert, 2002), which represents a more evolved state than *G. (Mesonephrotus)* (Malmberg 1970, 1998). In some cases, closely related fish species are parasitised by closely related *Gyrodactylus* species. Furthermore, more primitive fish species appear to be parasitised predominantly by more primitive *Gyrodactylus* species, and more advanced fish species by more advanced *Gyrodactylus* species (Malmberg 1970, 1998). *Pomatoschistus* is

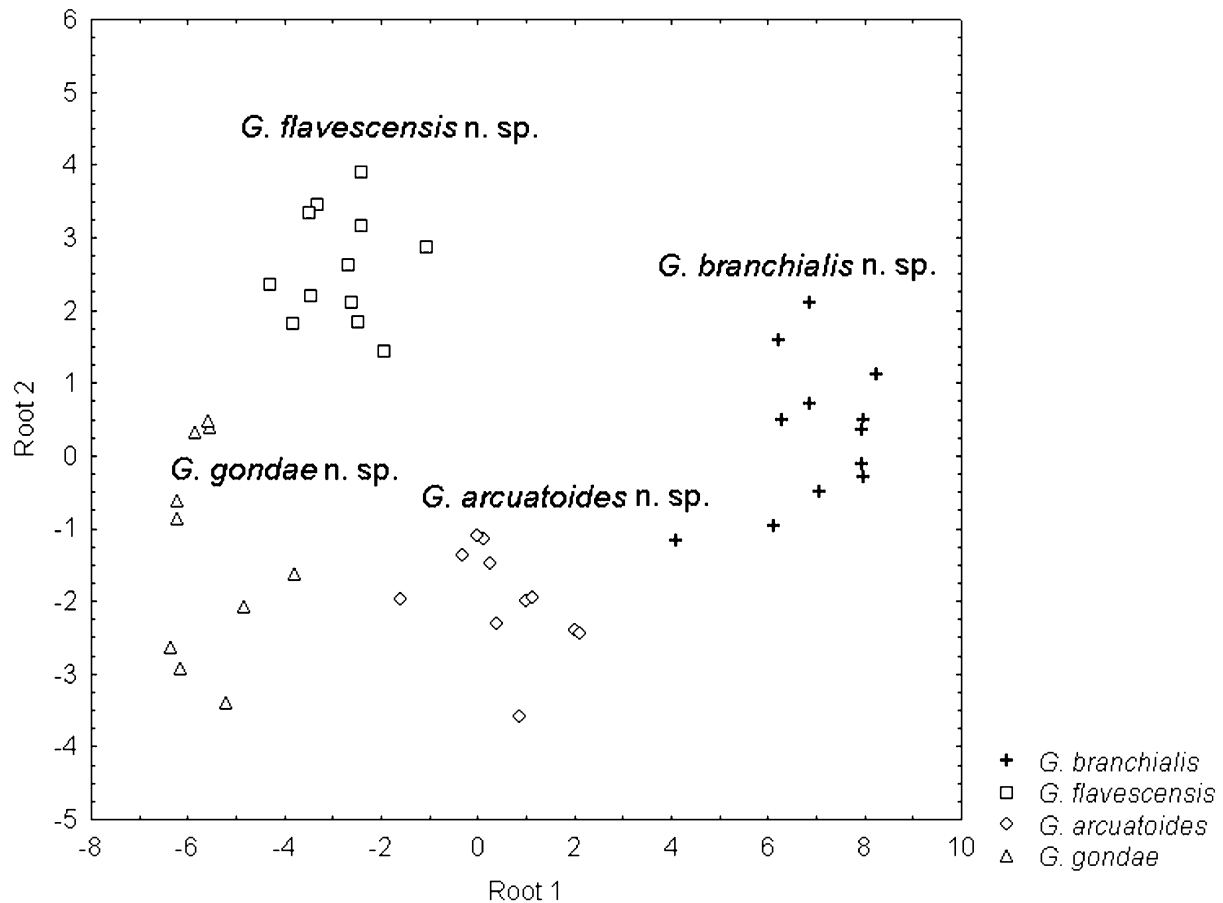


Figure 8. Plot of the standard discriminant analysis (root 1 vs root 2) based on morphological measurements of the anchors and ventral bar features of *G. gonda n. sp.*, *G. flavescens n. sp.*, *G. arcuato n. sp.* and *G. branchialis n. sp.* All included specimens were collected in Norway, except for those of *G. branchialis*, which were collected in Belgium.

more advanced than most fish groups parasitised by species of *G. (Mesonephrotus)*, e.g. members of the *G. arcuatus*-group. Thus the fact that gobies are parasitised by species of *G. (Paranephrotus)* fits to the above correlations, while their infection by members of *G. (Mesonephrotus)* might be secondary, supporting the above hypothesis of host-switching.

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