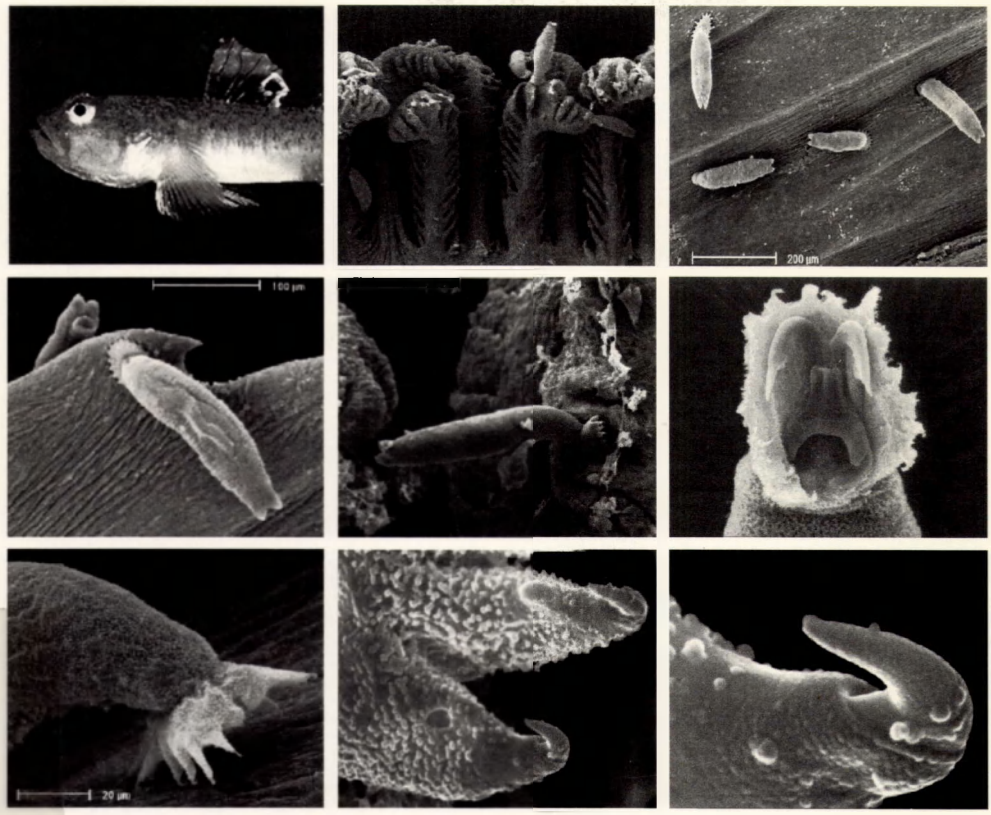
Evolutionary assocations between *Gyrodactylus* and its goby host:

Bound forever?



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KATHOLIEKE UNIVERSITEIT LEUVEN
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Laboratorium voor Aquatische Ecologie

Evolutionary associations between *Gyrodactylus* and its goby host:

bound forever?

Evolutionaire relaties tussen *Gyrodactylus* en zijn grondel gastheer:

eeuwig samen?

Promotor:
Prof. Dr. F. Volckaert

Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Wetenschappen door:

Tine Huyse

2002

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Tine

Content

CHAPTER 1: INTRODUCTION

Virtually all biological questions require an evolutionary context: they can not be answered outside the framework in which we consider how organisms have evolved to the state in which we observe them today. Phylogenetic relationships therefore play a crucial role in most, if not all aspects of biology. All the events of biological evolution are played out somewhere along the branches of phylogenetic trees (Pagel, 1999). Recent advances in molecular biology and phylogenetic analyses help to infer the historical patterns of evolution responsible for the diversity of contemporary species. Phylogenetic co-evolution can result in phylogenies of closely interacting taxa showing more similar topologies than can be expected by chance. Such pairs of groups include hosts and their parasites, organisms and their genes, and geographical areas and the species that inhabit them (Page, 1994; Page and Charleston, 1998). Here we focus on host-parasite systems; more specifically, we use the ectoparasitic *Gyrodactylus* spp. and their goby host as a model system.

Host-parasite systems are intrinsically interesting because they signal a long and intimate association between organisms that are distantly related and quite dissimilar biologically (Page and Hafner, 1996). The prerequisites for reconstructing these historical associations, have been summarized by Page et al. (1996): (1) a sound alpha taxonomy of both host and parasite, (2) wide taxon sampling, (3) robust phylogenies of hosts and parasites, preferentially reconstructed from molecular data, (4) quantitative comparison of host and parasite trees by means of explicit statistical tests and (5) host transfer experiments. The following paragraphs are built upon these aspects but first we briefly discuss the parasitic life-style and its consequences for speciation. After that we go deeper into the biology of both groups constituting our host-parasite system. At the end of this chapter the thesis outline will be presented.

1. Host-parasite systems as outstanding systems for studies of speciation modes

In the past, researchers assumed that parasites were so different from the majority of free-living organisms that it was almost impossible to make overall generalizations between them. Confusion partly originated from the assumption that parasites are the passive members of the association, highly dependent on their host. However, Brooks and McLennan (1993) showed that, once speciation is viewed from the parasite's perspective, speciation modes

become analogous to those proposed for free-living organisms (Fig. 1). Vicariant speciation occurs when an ancestral species is geographically separated into two or more relatively large and isolated populations, followed by lineage divergence in both populations. By analogy, geographical separation of the ancestral host population together with its parasite may lead to the speciation of both host and parasite, or only one member of the association will speciate. Peripheral isolate allopatric speciation involves the separation of a small subset at the periphery of the ancestral population, followed by speciation. Thinking of hosts as geographic areas equals speciation by host-switching to peripheral isolate allopatric speciation. In free-living organisms the reduction in gene flow depends on their dispersing capabilities and the magnitude of the geographical barrier, while in parasites the magnitude of gene flow depends on the transmission mode of the parasite and the degree of sympatry between the old and the new host species.

Sympatric speciation occurs when species arise in absence of a geographical barrier. This mode of speciation has been fiercely debated in the past and remains controversial, but the underlying mechanisms are becoming increasingly intelligible (see e.g. Dieckmann and Doebeli, 1999; Tregenza and Butlin, 1999; Via, 2001). Although allopatric speciation seems to be the norm in free-living species (Barraclough and Vogler, 2000), sympatric speciation may occur relatively frequently in parasitic organisms (Gusev, 1995; de Meeüs et al., 1998; Poulin, 2002). Gene flow might initially be severed by hybridisation or the evolution of asexual or parthenogenetic populations (Brooks and McLennan, 1993) or niche differentiation and specialisation (de Meeüs et al., 1998). The term sympatric speciation has been, and still is being used for speciation followed by host-switching (Price, 1980; and for example in the literature on phytofagous insect parasites (Bush et al., 1998; Emelianov, 2001; Craig et al., 2001). This again traces back to the fact that from a host's perspective, speciation takes place in the same area, but not so for the parasite. In the following parts we adopted the definition of sympatric speciation described by Brooks and McLennan (1993, see Fig. 1), implying speciation on the same host species. Other terms frequently used in co-evolutionary studies to describe this kind of speciation are intra-host speciation or parasite duplication (see below; Page, 1993; Hafner and Page, 1996; Page and Charleston, 1998; Poulin, 2002).

Because the host constitutes the principal environment of a parasite, speciation modes are more readily inferred in host-parasite systems. Host-switching is not influenced by post-speciation dispersal that plagues free-living organisms (Brooks and McLennan, 1993). Therefore host-parasite systems are outstanding systems for studies of speciation modes (de Meeüs et al., 1998).

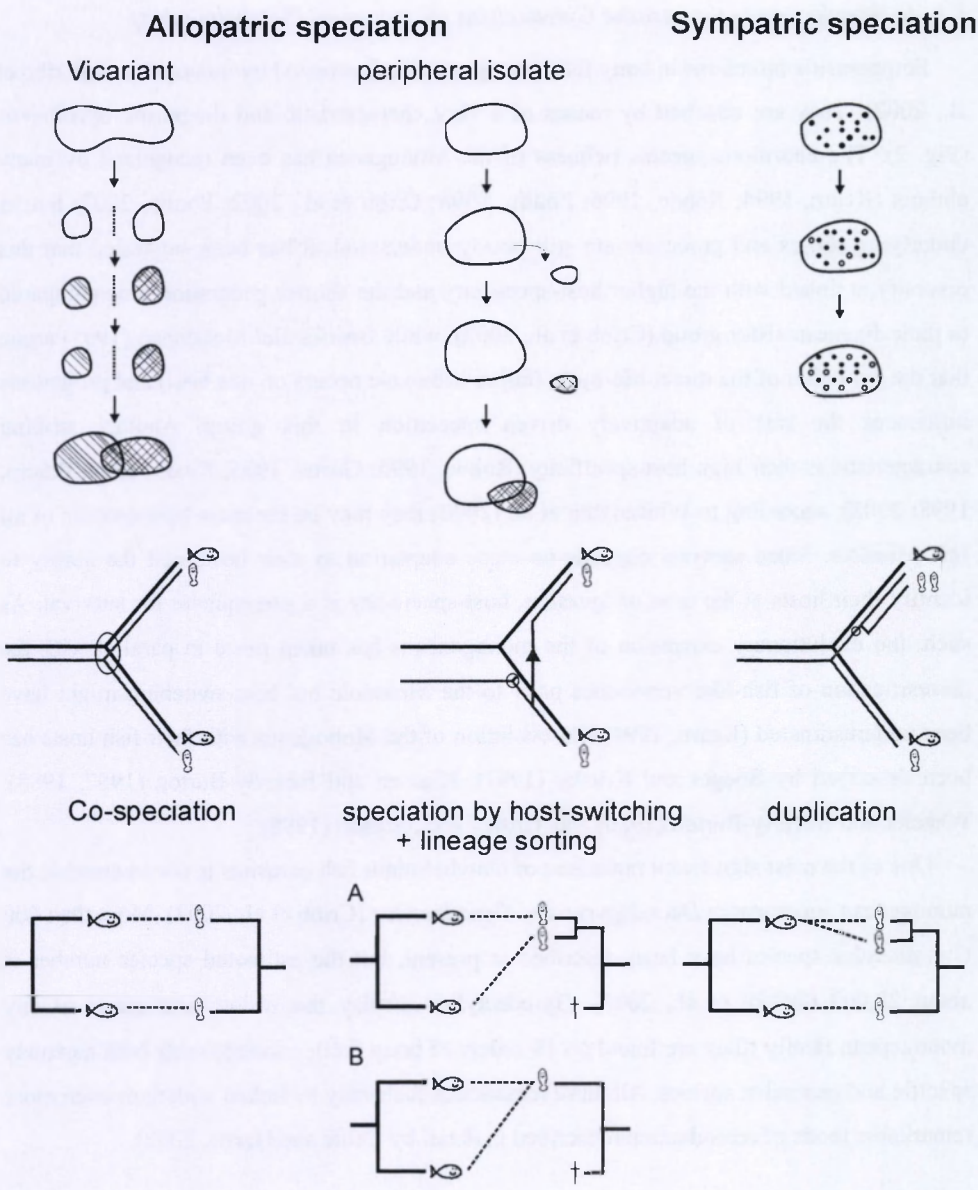

Fig. 1. The three major speciation modes (according to Brooks and McLennan, 1991) and their phylogenetic correlates, applied to parasites. An ancestral parasite species can be geographically subdivided together with its ancestral host species (vicariance). If both the host and parasite (repeatedly) speciate, this will result in mirror-image phylogenies (vicariant allopatric co-speciation), but two more possibilities exist (see text). Host-switching involves the movement of a small subset of a species into a new "geographical area". This can (A) be followed by speciation (via a peripheral isolate mode) or (B) the new host will be added to the species range of the parasite. Finally, speciation occurring in the same "area" or on the same host species, is referred to as sympatric speciation.

1.1. An introduction to the parasite Gyrodactylus (Monogenea, Platyhelminthes)

Ectoparasitic infections in bony fishes are generally dominated by monogeneans (Cribb et al., 2002). They are attached by means of a very characteristic and diagnostic opisthaptor (Fig. 2). The enormous species richness of the Monogenea has been recognized by many authors (Kearn, 1994; Rohde, 1996; Poulin, 1998; Cribb et al., 2002; Poulin, 2002) but its underlying causes and processes are still poorly understood. It has been suggested that this diversity is linked with the higher host-specificity and the shorter generation time compared to their digenean sister-group (Cribb et al., 2002), while Brooks and McLennan (1993) argue that the evolution of the direct life-cycle (entire life-cycle occurs on one host) and progenesis influences the rate of adaptively driven speciation in this group. Another striking characteristic is their high host-specificity (Rohde, 1996; Gusev, 1995; Kearn, 1994; Poulin, 1998; 2002); according to Whittington et al. (2000) they may be the most host-specific of all fish parasites. Since survival depends on close adaptation to their hosts and the ability to identify their hosts at the time of invasion, host-specificity is a prerequisite for survival. As such, the evolutionary expansion of the monogeneans has taken place in parallel with the diversification of fish-like vertebrates prior to the Mesozoic but host-switching might have been underestimated (Kearn, 1994). Co-evolution of the Monogenea with their fish hosts has been described by Boeger and Kritsky (1997); Klassen and Beverly-Burton (1987, 1988); Wheeler and Beverly-Burton (1989) and Tinsley and Jackson (1998).

One of the most significant radiations of platyhelminth fish parasites is documented in the monogenean supergenera *Dactylogyrus* and *Gyrodactylus* (Cribb et al., 2002). More than 400 *Gyrodactylus* species have been described at present, but the estimated species number is about 20,000 (Bakke et al., 2002). Gyrodactylids display the widest host range of any monogenean family (they are found on 19 orders of bony fish), encompassing both narrowly specific and generalist species. All these remarkable facts may be linked with their even more remarkable mode of reproduction (described in detail by Cable and Harris, 2002).

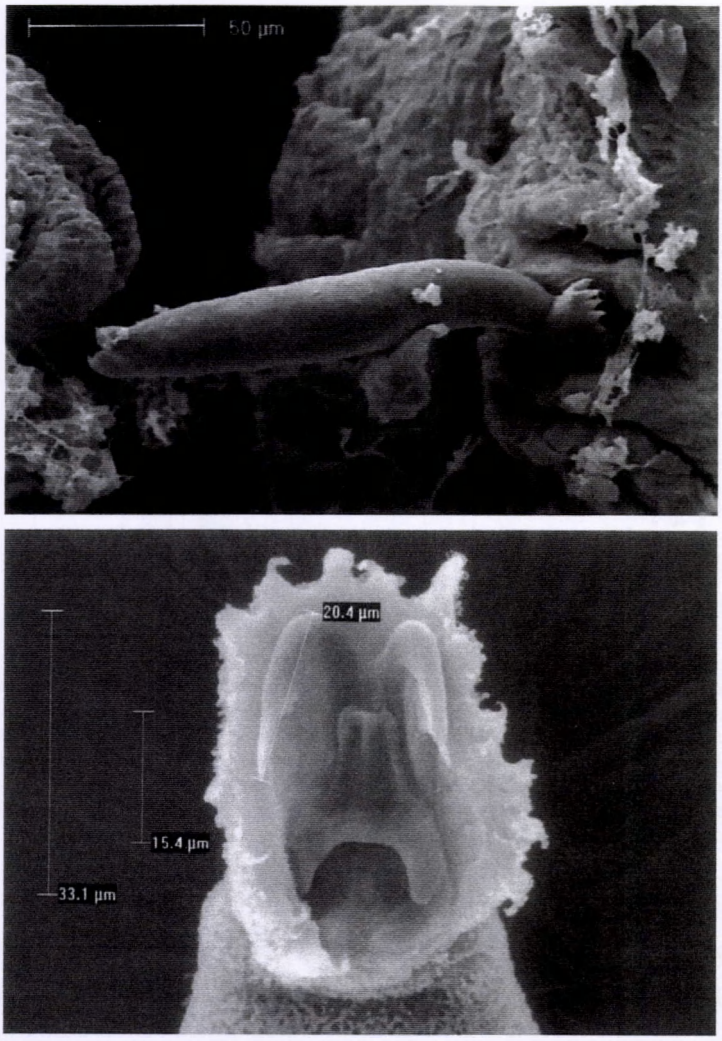

Fig. 2. *Gyrodactylus* displays more anatomical conservatism in its attachment and copulatory apparatus than any other monogenean parasite (Kearn, 1994). The number of useful taxonomic characteristics is limited due to adaptations for viviparity and progenesis (Cable et al., 1999). Above shows a scanning electron micrographs of a *Gyrodactylus* specimen detaching itself from the gill tissue, only a few fingers with the marginal hooks are visible. Below shows the opisthaptor, constituted of a pair of anchors (some measurements are indicated) and the ventral bar, surrounded by 16 fingers, each with a protruding marginal hook sickle point.

Gyrodactylus spp. contain a fully-grown daughter *in utero*, which in turn encloses a developing embryo, boxed inside one another like 'Russian dolls'. The reproduction follows a specific pattern (Fig. 3), including asexual, parthenogenetic and sexual reproductive modes. The combination of viviparity, progenesis and protogyny is unique in the Animal Kingdom Cable and Harris, 2002).

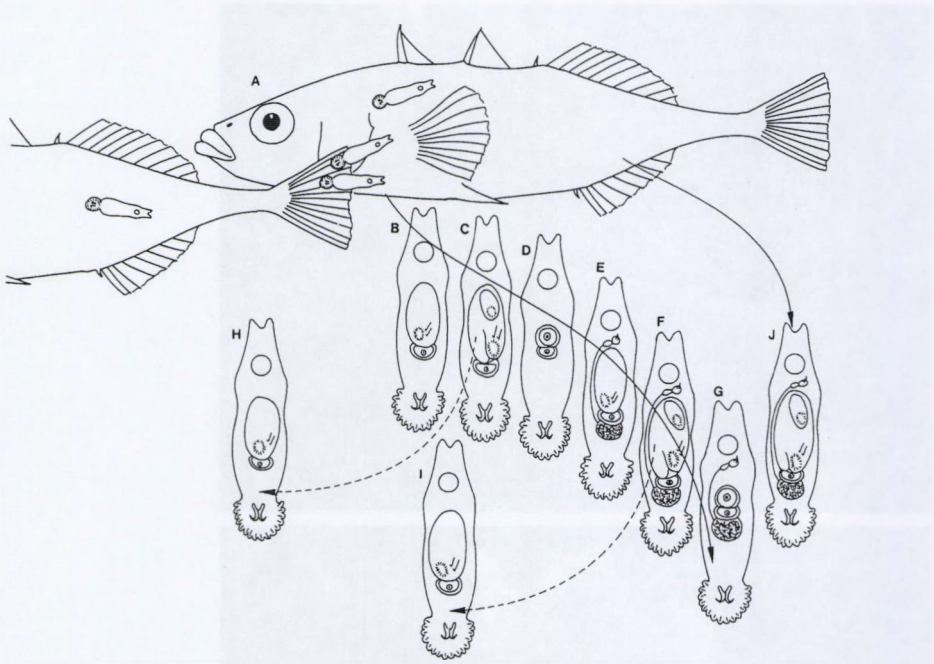

Fig. 3. Life-cycle of *Gyrodactylus*. A: gyrodactylids transfer from fish to fish when hosts make contact with each other. B–I represent successive stages of a newborn parasite. At stages C and F the mother gives birth. The successive daughters have different origins. The first-born daughter develops asexually while its parent is still an embryo. The second-born daughter develops from an oocyte that commences development before the parent's male reproductive system is fully mature. E: development of the male reproductive system. Subsequent daughters develop either parthenogenetically or sexually after mating between post-second birth individuals (G) and another adult (J) (From Kearn, 1995; Cable and Harris, 2002).

They furthermore represent the only example where responsibility for embryo nutrition is devolved to an embryonic parent. Advanced progenesis allows the first-born daughter to be produced within 24 h of her parent's birth. This may result in an explosive population growth, especially when transmission is favored under aquacultural conditions. The epidemic spreading of *G. salaris* from fish farms to wild fish caused major salmon losses (for a summary see Malmberg, 1993; Bakke et al., 2002). The population dynamics resemble those of microparasites rather than that of typical helminth macroparasites (Cable and Harris, 2002).

One of the pressures that may operate against early sex is that recombination may compromise the fitness of sexually produced offspring, so that their survival may be suboptimal relative to that of the parent when infecting the same host (Kearn, 1994). According to Harris (1993) this may be very important because of the low fecundity of gyrodactylids and may explain why colonizing parasites have often given birth once, leaving behind an identical copy of the genome on a suitable host. The degree of sexual reproduction

is species-specific, depends on the population-age structure, and is negatively correlated with host-specificity (Harris, 1993). In species where sexual reproduction is an occasional event, sex is usually associated with epidemic population expanses. Survival following transfer to new hosts may be enhanced by the generation of new gene combinations (Kearn, 1994). In some species mating occurs also between different species. Recently sperm transfer has been observed between *G. arcuatus* and *G. gasterostei* both parasitizing three-spined stickleback (Scott et al., 2001). Nothing is known yet about the possible offspring resulting from such pairings, but it highlights the possibility of hybridisation.

During asexual and automictic parthenogenetic phases, the whole genome behaves almost as a single linkage group and the neutral or even slightly deleterious alleles hitch-hike with the genes selected for. Sexual episodes might counteract inbreeding by creating new genotypes, unless mating occurs within the same clone. It might be assumed that inbreeding is strong and the bottleneck effect extreme under such mode of speciation (Zietara and Lumme, in press).

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 (Poulin, 1992; Kearn, 1994). On the other hand, the ability to produce a viable deme from a single 'pregnant' female, increases the chance for sympatric speciation and speciation by host-switching (Brooks and McLennan, 1993). The latter process seems to have played an enhanced role in the gyrodactylid speciation as many instances of ecological radiation onto distant-related hosts are described (Harris, 1993). However, experimental studies have shown that their reproduction is regulated by parental physiological state and host identity, suggesting an important role in controlling host-specificity. For example, when parasites are detached from the host for short periods embryo development is severely retarded and even ceased when the parent is kept on a novel host (Cable and Harris, 2002).

Altogether, it can be concluded that the above described features make gyrodactylids an ideal target for comparative studies on the mechanisms of speciation and the evolution of host-specificity (Bakke et al., 2002).

2.2. An introduction to the host Pomatoschistus

The gobies (Gobiidae, Teleostei) are regarded as one of the most speciose families of fish occupying marine, brackish and freshwater habitats in the tropical and temperate seas of the world (Hoese, 1984; Miller, 1986). Among the eastern Atlantic-Mediterranean region, a so-

called 'sand goby' group can be recognized, consisting of four phenetic genera: *Pomatoschistus*, *Gobiusculus*, *Knipowitschia* and *Economidichthys*. The sand gobies possess many interesting biological features such as courtship behavior, sneaking, cannibalism and sound production, making them an ideal subject of ecological, evolutionary and behavioral studies (Lugli and Torricelli, 1999; Lindstrom and Lugli, 2000; Jones et al., 2001a,b; Pampoulie et al., 2001; Mazzoldi et al., 2002).

A prerequisite for evolutionary studies is a sound taxonomy and classification. In the past, many systematic difficulties have arisen, due to their small body size and superficial resemblance to each other (Webb, 1980). Simonovic (1999) felt the controversy regarding goby classification was generated because most relationships have been based on phenetic methodology, rather than cladistics. Several allozyme studies have been carried out (Wallis and Beardmore, 1984 (see Fig. 4); 1984; McKay and Miller, 1997; Miller et al., 1994), resulting in conflicting phylogenies. Therefore DNA sequence analysis should provide meaningful insights into the inter- and intrageneric relationships within the 'sand goby' group. To date, the only molecular analysis on European gobiids available included only five out of the 17 putative 'sand goby' species (Penzo et al. 1998). Fragments of the 12S and 16S mtDNA appeared useful markers for phylogeny reconstruction.

For most vertebrate species, speciation dates back to the Pleistocene (Avise et al., 1998), triggered by allopatric speciation in separated refugia. Poikilotherm fishes constitute an exception to this; although many controversies exist regarding the molecular clock, the mtDNA clock is assumed to tick slower in fishes (Martin et al., 1992; Cantatore et al., 1994), and the speciation peak has been shifted to the Pliocene-Miocene period (Avise et al., 1998). Cichlid radiation in the African rift lakes (Sturmbauer and Meyer, 1992) is a famous exception to this.

Until now, not much attention has been paid to their role as a host for *Gyrodactylus*. Four *Gyrodactylus* species are known to parasitise *Pomatoschistus* spp.: *G. longidactylus* Geets, Malmberg and Ollevier, 1998 (on the gills of *P. lozanoi*), *G. rugiensis* Gläser, 1974 (on fins of *P. micropsi* and *P. minutus*) and *G. micropsi* Gläser, 1974 (on fins and gills of *P. microps* and *P. minutus*). Yet, several other still undescribed *Gyrodactylus* spp. are suspected to live on *Pomatoschistus* spp. (Geets, 1998). The high abundance and sympatric life-style of certain sand goby species have important consequences for the evolution of their parasites. For example, the likelihood of a close co-evolution between host and parasite is expected to be smaller if the parasite infects a host species belonging to a large family in which case host-switching would be more frequent (Poulin, 1992).

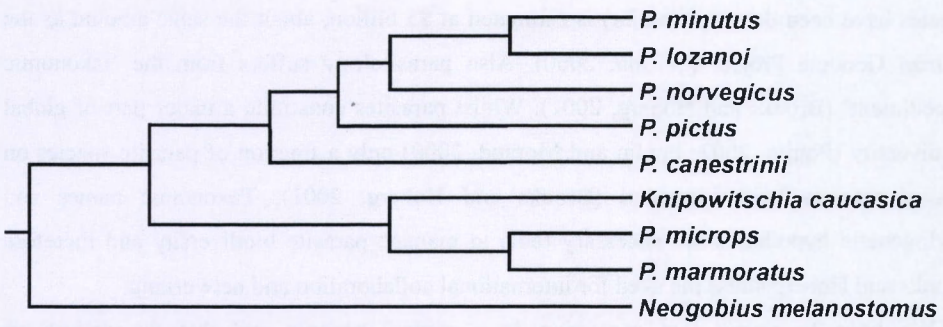

Fig. 4. Phenetic dendogram of gobies of the genera *Pomatoschistus*, *Knipowitschia* and *Neogobius* produced using the UPGMA procedure on Nei's *D* values, based on 31 allozyme loci (adapted from Wallis and Beardmore, 1984).

2. Naming and classifying species: taxonomy and phylogeny.

2.1. When it all comes down to species

One and a half centuries after the publication of Darwin's *Origin of species,* there appears more disagreement about the term species than ever before (Kunz, 2002). This is e.g. illustrated by the two dozen different species concepts proposed (for a summary see Hey, 2001) and the numerous debates led in literature (Graybeal, 1995; Avise and Wollenberg, 1997; Wiens and Servedio, 2000; Ferguson, 2002). Connected to this, Avise and Johns (1999) pointed to the lack of standardized criteria for taxonomic ranking. Whereas the great diversity of life precludes standardized organismal-level comparisons, molecular characters are nearly universal. Therefore they proposed that the approximate dates of nodes in a phylogenetic tree should be the universal taxonomic criterion for taxonomic classifications. An additional advantage is that genetic divergence is not bound to any of the current species concepts (Ferguson, 2002). However, besides the questionable reliability of molecular dating, it is clear that this procedure won't be operational until calibrated phylogenies of all groups are available. Others argue that genetic divergence on its own is not useful for identifying species and it cannot be used consistently across taxa (Ferguson, 2002). Combining both opinions, Tautz et al. (2002) suggest to match existing taxonomic information with DNA sequences of new specimens. Specialized DNA sequencing facilities should routinely handle species identification and multiple DNA sub-samples should be sent to museums as backups.

One thing that could not be agreed upon more is that there is a global shortage of professional taxonomists and systematicists (Wilson, 2000; Brooks and Hoberg, 2001,

Blackmore, 2002). The cost of a global inventory of life on Earth (only 2% of the living species have been described so far) is estimated at $5 billion, about the same amount as the Human Genome Project (Wilson, 2000). Also parasitology suffers from the 'taxonomic impediment' (Brooks and Hoberg, 2001). Whilst parasites constitute a major part of global biodiversity (Poulin, 2002: Poulin and Morand, 2000) only a fraction of parasite species on this planet has been identified (Brooks and Hoberg, 2001). Taxonomic names and phylogenetic hypotheses are necessary tools to manage parasite biodiversity and therefore Brooks and Hoberg stress the need for international collaboration and networking.

We have to accept that speciation is a gradual process and that the underlying evolutionary processes and outcomes are so complex that their reality cannot be fully captured by a necessarily simplified binomial summary (Milinkovitch, 2000; Hey, 2001). Avoiding to choose sides, we feel that the value of molecular data in describing species is still exploited best if combined with morphological and ecological data.

2.2. Trees and more trees

Recent advances in molecular biology and phylogenetic analyses make it possible to interpret the 'history written in the genes' (Stevens and Gibson, 1999). Phylogenetic trees provide an indirect record of the speciation events that have led to the present-day species (Barraclough and Nee, 2001). Since Mayr's *Systematics and the Origin of Species* (1942), the mode of speciation has been fiercely debated. By constructing species-level phylogenies and comparing the geographical distribution of sister taxa, the relative contribution of the different speciation modes can be inferred (Barraclough and Nee, 2001). There are however, a large number of methods for building a phylogeny, each of which uses a different model of evolution and potentially yields a different tree for the group studied. Therefore, it is very important to conduct elaborate data-analyses. However, the first step is the choice of marker, which depends on the question to be answered (relationships between species or genera or families). The next step, sequence alignment, is crucial since it determines the homologous sites, thus influencing all further analyses. Using multiple alignment software (e.g. ClustalX, Thompson et al., 1997) the sensitivity of the alignment to different alignment parameters can be explored. The program MALIGN (Wheeler and Gladstein, 1994) has options for automatically removing alignment ambiguous regions and assembling "grand" alignments from several individual alignments, while SOAP (Loytynoja and Milinkovitch, 2001),

automates the process of identifying alignment unambiguous positions from different multiple alignments.

Each phylogeny method has its strengths and its Achilles Heel. Using them in parallel will help to discover what evolutionary factors are at work in the sequence data. We will briefly highlight the three main methods, although we must refer to the literature for more details (Hillis, Mable and Moritz, 1996; Page and Holmes, 1998). Maximum parsimony holds faith to the principle that "simpler hypotheses are preferable to more complicated ones", thus selecting for minimal tree length. It has been extensively studied mathematically, and some very powerful software implementations are available. As it is does not make use of an evolution model, it is sensitive to rate heterogeneity among lineages (known as "long-branch attraction"). Distance based and maximum likelihood methods can correct for multiple substitution problems, on the condition that the 'right' models are chosen. More complex models are not always better, because uncertainty increases, as more parameters have to be estimated. Thus there is a trade-off between more realistic and complex models and their power to discriminate between alternative hypotheses. The program ModelTest 3.06 (Posada and Crandall, 1998) can help to select the model of DNA evolution that best fits the data by comparing the likelihood scores. Neighbor-joining and minimum evolution are computionally very fast, but the conversion of discrete data into distances results in a loss of information. In contrast to the other two methods, maximum likelihood makes use of all available information. It is an inductive statistical procedure that maximizes the probability of observing the data obtained with respect to some explicit model of evolution. If all methods result in the same tree topology, then the inferred interrelationships are fairly robust. The Shimodaira-Hasegawa test implemented in PAUP* (Shimodaira and Hasegawa, 1999) can be used to test whether one tree is supported significantly less by the data than a second tree.

Phylogenetic analyses have become an indispensable tool in evolutionary biology and epidemiology. By mapping biological characteristics onto the tree, inferences can be made regarding the evolution of a suite of comparative data like virulence, host-specificity, ecological shifts, etc. (Schluter, 2001). For example, the evolution and emergence of new bacterial pathogens can be reconstructed by tracing the history of the acquisition of virulence genes in *E. coli* (Reid et al., 2000).

3. Is there evidence for co-evolution? A quantitative comparison between host and parasite phylogenies.

The term co-evolution describes the natural process in which two or more species interact so intimately that their evolutionary fitness depends on each other (Ridley, 1996). Phylogenetic analysis is a crucial component of co-evolutionary studies. Congruent patterns of host and parasite phylogenies identify co-evolutionary phenomena, but it is not a proof of reciprocal interactions. The pattern can equally evolve from a one-way interaction, where speciation of the host induces speciation of the parasite, without parasite-induced speciation of the host. By strict definition, co-evolution requires reciprocal evolutionary change in the interacting species, but here we refer to co-evolution at the macro-evolutionary scale, where a complete agreement between host and parasite phylogenies represents the equivalent of co-evolution (Page, 1994; Hafner and Page, 1995; Page and Charleston, 1998). These mirror-image phylogenies are referred to as Fahrenholz's rule; it serves as a null model of host-parasite co-evolution against which other evolutionary scenarios can be tested (Poulin, 1998; Paterson and Banks, 2001).

Figure 1 shows the influence of the different parasite speciation modes on the phylogenetic branching pattern, and thus on the degree of congruence between host and parasite phylogenies. Besides speciation by host-switching, also sorting or duplication events produce incongruent patterns. Sometimes colonization of a new host might be disguised as 'false' congruence. For example, the apparent co-divergence between primate lentiviruses and their hosts appeared to be the result of frequent cross-species transmission of these lentiviruses between closely related host (Charleston and Robertson, 2002). This result has obviously an important impact on understanding lentivirus evolution. The opposite may occur as well: false incongruence between host and parasite phylogenies may arise when parasite duplication or parasite losses occur independent of the host phylogeny (Page, 1993; 1994).

Several statistical methods have been developed to tackle these pitfalls. An excellent review is provided by Paterson and Banks (2001). Basically, most methods are topology based and do not take genetic distances into account (Brooks' Parsimony Analysis: Brooks 1981; Component: Page, 1993; TreeMap 1.0: Page, 1994). The need of fully resolved phylogenies is an important disadvantage because, as pointed out above, phylogenies greatly depend on the quality of the data and the tree-building method used. There are however, maximum-likelihood methods available (Huelsenbeck et al., 1997; 2000) that can test the robustness of the molecular data used for phylogeny reconstruction. One major drawback

inherent to those programs, is that duplications and sorting events are not considered. The program ParaFit (Legendre et al., 2002) is also alternative in its approach by using genetic distances instead of phylogenetic trees. Again, only by using the different methods in parallel, evolutionary patterns between host and parasites might become disentangled.

4. Outline

In order to reconstruct the history of host-parasite associations between the monogenean ectoparasite *Gyrodactylus* and its gobiid hosts, several conditions have to be fulfilled: (1) a sound alpha taxonomy of both host and parasite taxa, (2) wide taxon sampling, (3) robust phylogenies of hosts and parasites, preferentially reconstructed from molecular data, and (4) quantitative comparison of host and parasite trees by means of explicit statistical tests (Page et al., 1996). These prerequisites will be addressed progressively throughout the thesis.

Cunningham et al. (1995, 1997) were the first to use ribosomal DNA sequences to distinguish three closely related *Gyrodactylus* species of economic importance, namely *G. salaris, G. derjavini* and *G. truttae.* The next step involved the use of the ITS marker in phylogeny reconstruction. Cable et al. (1999) studied eleven *Gyrodactylus* spp. based on a small ITS1 fragment and the combined 5.8S and ITS2 data. Besides sequencing problems, the phylogeny reconstruction was hampered in several ways. The 5.8S gene alone was not found phylogenetically informative while the ITS1 region appeared too variable for confident alignment. Therefore we felt it was necessary to first conduct an elaborate data analysis in order to estimate the perspectives and limitations of the ITS region as phylogenetic marker of such a speciose genus (**Chapter two**).

Geets (1998) pointed to several undescribed *Gyrodactylus* spp. living on gobies. Traditionally, species discrimination is based on the shape and size of the opisthaptor, which consists of a single pair of hamuli and 16 marginal hooks (Malmberg, 1970; 1998, see Fig. 2). **Chapter three** and **Chapter four** describe five new species, combining classical morphology and molecular markers (ssrRNA V4 region and the complete ITS rDNA region). In addition, comparative morphometric analyses and statistical classifiers have been used to discriminate these closely related species.

In accordance with the next point raised above, taxon sampling has to be be extended over space. Phylogeographical literature on *Gyrodactylus* is scarce (Zietara et al., 2000). Moreover, Bakke et al. (2002) pointed to the need for an increase of sampling effort of *Gyrodactylus* spp, since most gyrodactylids have been described from temperate northern

freshwater. **Chapter five** reports a first exploration of the *Gyrodactylus* fauna on gobies in the Mediterranean Sea.

In Chapter six and seven extensive molecular data analyses have been conducted on both the host and parasite in order to construct robust phylogenies. In **Chapter six**, the molecular phylogeny is compared with morphological phylogeny proposed by Malmberg (1970; 1998), based on the six types of excretory systems. In **Chapter seven**, the molecular phylogeny of European gobiids (Penzo et al., 1998) has been extended with several new sand goby species and comparisons have been made with the phylogenies based on morphological and allozyme data (McKay and Miller, 1994). Correlations between speciation and geological or hydrographic events (e.g. Messinian salinity crisis) are discussed.

Chapter eight brings us to the final goal of this thesis. Here, the obtained phylogenies of the previous two chapters have been quantitatively compared by several explicit statistical tests of co-speciation. This enabled us to finally reconstruct the evolutionary associations between *Gyrodactylus* and its goby host.

CHAPTER 2

DEEP DIVERGENCE AMONG SUBGENERA OF *GYRODACTYLUS* INFERRED FROM rDNA ITS REGION

Marek S. Ziętara, Tine Huyse, Jaakko Lumme and Filip A.M. Volckaert

Abstract: This paper adds new insight to a molecular phylogeny of *Gyrodactylus*, based on a complete sequence of the ITS rDNA region of 4 subgenera and a more detailed molecular analysis. We propose a hierarchical approach in elucidating the phylogeny of this species-rich genus. A total of 37 sequences (915-1239 bp) from 10 representative species from 4 out of 6 subgenera, as defined by Malmberg (1970), are included in the analysis. Genetic differences observed at the 5.8S locus provide objective criteria to separate (sub)genera, while deep genetic differences of the spacers form a sound basis for species-specific identification. We demonstrate that each *Gyrodactylus* subgenus possesses a unique sequence of the 5.8S gene. Thus, there is concordance between the 5.8S gene and the excretory system used by Malmberg (1970) as a diagnostic character of subgenus status. At the species level, there is a discrepancy between morphological and molecular variation. Whereas the morphological variation, expressed in the shape and size of the attachment apparatus, is very low, the molecular variation, expressed at the ITS1 and ITS2 regions, is very high. This can be attributed either to the fast evolving ITS region or to the fact that the genus consists of groups of a higher taxonomic level than previously recognised. Perspectives and limitations of using the ITS rDNA region for a molecular phylogeny of this genus are discussed.

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Contribution: limited lab work, complete data analysis and equal contribution to writing.

1. Introduction

With more than 400 species named (Williams and Jones, 1994), the genus *Gyrodactylus* is extremely rich in species. It is only recently that *Gyrodactylus* research has been given more attention, especially since *G. salaris* caused major losses in the salmon industry (Johnsen and Jensen, 1991). It is likely that many more species will be added to this long list. The high taxonomic diversity seems predictable from evolutionary models that include the high host and habitat specificity, and the combination of clonal and sexual reproduction (Suomalainen, Saura and Lokki, 1987; Harris, 1993; Kearn, 1994; Page and Hafner, 1996). According to Brooks and McLennan (1993) the high species diversity in comparison with their sister group the Cestodaria can be attributed to putative key innovations displayed by the Monogenea. They hypothesise that the evolution of the direct life-cycle and progenesis influences the rate of adaptively driven speciation in this group. The gyrodactylids display the extreme condition of this developmental trend, viviparity. A high number of species within a genus may also indicate that the genus is too loosely defined. In either case, more taxonomic attention is required.

In contrast to high species diversity, *Gyrodactylus* shows considerably more anatomical conservatism in its attachment apparatus (haptor) and copulatory apparatus than any other monogenean parasite, which might reflect the highly successful nature of the special combination of viviparity and progenesis (Kearn, 1994: Cable et al., 1999). Consequently, the number of useful taxonomic characteristics is extremely reduced. Nevertheless, a morphological phylogeny of the genus has been proposed by Malmberg (1970), based on characteristics of the excretory system studied in living specimens and the attachment apparatus. The genus has been subdivided into six subgenera: *G.* (*Gyrodactylus*), *G.* (*Mesonephrotus*), *G.* (*Metanephrotus*), *G.* (*Paranephrotus*), *G.* (*Neonephrotus*) and *G.* (*Limnonephrotus*). The division of the subgenera into species groups is based on morphological differences of the sclerites forming the haptor. However, identification based solely on morphometry is difficult because of intraspecific variation induced by climate and habitat (Harris, 1993; Appleby, 1996; Shinn et al., 1996; Geets et al., 1999). By using characters independent of morphology, molecular phylogeny can add a new dimension that promotes 'a total evidence' approach towards obtaining a true phylogeny.

Nowadays, more and more phylogenies are constructed based upon sequence comparisons (Pagel, 1999) but at the same time many questions arise on the interpretation of the molecular data and their translation into functional phylogenies. Page and Charleston

(1997) point to the potential danger in obtaining a gene tree different from the species tree. This can be overcome by using more than one gene and by comparing several evolutionary models and tree-building methods.

Cable et al. (1999) presented a first molecular phylogeny of 11 *Gyrodactylus* species based on a short 278 bp ITS1 fragment and a combination of the 5.8S gene and ITS2. Species were divided into two groups having either a short or long ITS1 sequence, which matched with the subgenera *G.* (*Mesonephrotus*) and *G.* (*Metanephrotus*) *vs. G.* (*Limnonephrotus*). The analysis based on the 5.8S gene alone was found to be phylogenetically uninformative and the ITS1 was too variable for confident alignment.

In this paper, we compare Malmberg's (1970) division of the genus with the molecular phylogeny constructed with the ITS sequences of 4 different subgenera. We show that the subgenus division of Malmberg is "natural" and indeed follows the phylogeny, but the subgenus is a very low systematic rank to describe this variation. The genetic divergence presented in this paper seems to be much deeper in comparison to other animal groups. Avise and Johns (1999) argue that current classifications fail to carry useful comparative information because of the lack of standard criteria for taxonomic ranks. The genus *Gyrodactylus* seems to be one of the extremes in this respect. To minimise problems with alignment of the ITS region, a new hierarchical approach for constructing the molecular phylogeny is proposed. Perspectives and limitations in using the ITS rDNA region for molecular phylogeny of this species-rich genus are discussed.

2. Material and methods

Specimens of 7 *Gyrodactylus* species were collected from the wild in Belgium, Sweden and Finland in 1997-1999. Host fish species, locality, habitat (salty, brackish or freshwater) are given in Table 1. The ITS region encompassing the internal transcribed spacers (ITS1 and ITS2) and the small ribosomal subunit (5.8S rRNA) gene was amplified. Three to six specimens of each species were analysed. All parasites were identified morphologically to species level prior to DNA analysis. Each specimen was individually placed in 5 µl of milli-Q water and stored at -20°C until required.

Table 1. Species list indicating sampling site, longitude and latitude, date and type of environment.

Parasite species	Host species	Site	Nr	Longitude, Latitude	Date	Habitat
G. arcuatus Bychowsky, 1933	*G. aculeatus*, fins	Lumijoki, Fi	5	64°55'N; 25°05'E	October'99	Brackish
G. gasterostei Gläser, 1974	*G. aculeatus*, fins	Overpelt, B	5	51°14'N; 5°25'E	June '98	Freshwater
G. micropsi Gläser 1974	*P. microps*, gills	Doel, B	3	51°19'N; 4°16'E	March, August '98	Brackish
G. pungitii Malmberg, 1964	*P. pungitius*, fins	Overpelt, B	5	51°14'N; 5°25'E	June '98	Freshwater
G. rugiensis Gläser, 1974	*P. microps*, fins	Oostende, B	5	51° 14' N; 2° 57' E	August '99	Marine
G. salaris Malmberg, 1957	*O. mykiss*, fins	Farm, Fi	4	Confidential	June '99	Freshwater
	S. salar, fins	Rönne, Sv	1	56° 04'N; 13°10'E	November '97	Freshwater
G. sp.1*	*P. lozanoi*, gills	North Sea, B	5	51°27'N; 3°02'E	June '98	Marine
	P. minutus, gills	Doel, B	1	51°19'N; 4°16'E	September'97	Brackish

Nr = number of *Gyrodactylus* specimens sequenced B = Belgium, Fi = Finland, Sv = Sweden, *Geets, Appleby and Ollevier (1999)

DNA extraction, amplification and sequencing were as described by Ziętara et al. (2000). The ITS1 primer of Cunningham (1997) 5'-TTTCCGTAGGTGAACCT-3' was used as the forward primer. To avoid amplification of the fish rRNA region, a new primer called ITS2R5'-GGTAATCACGCTTGAATC-3' was designed based on a comparison of the 28S rDNA 5' ends between *Gyrodactylus arcuatus* and two fish, *Gasterosteus aculeatus* and *Pomatoschistus minutus*. Eight sites out of 18 were different. The primer was designed so that there were three divergent nucleotides at the 3' end of the primer preventing the amplification of fish DNA.

Two or three additional primers, complementary to either 5.8S or ITS1, were used for sequencing: ITS1R 5'-ATTTGCGTTCGAGAGACCG-3', ITS2F 5'-TGGTGGATCACTCGGCTCA-3' and ITS18R 5'-AAGACTACCAGTTCACTCCAA-3'. The dideoxy terminator cycle sequencing method was used. Both strands of the DNA were sequenced. DNA from *G. arcuatus* and *G. salaris* was sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (PE Biosystems) and ABI 377 DNA sequencer. *G. rugiensis* DNA was sequenced following the protocol of SequiTherm EXCEL II (Epicentre Technologies). Samples were denatured for 4 min at 97°C, then incubated for 30 s at 95°C, 30 s at 58°Cand 45 s at 70°C for 30 cycles, followed by a final extension step at 70°C for 7 min. The reaction products were separated on a LICOR 4200 system and visualized on a 6% Long Ranger gel (FMI BioProducts).

Additional sequences were obtained from GenBank. Species names and accession numbers are for the Digenea: *Dolichosaccus symmetrus* (L01631), *Echinostoma caproni* (U58098), *E. hortense* (U58101), *E. liei* (U58099), *E. paraensei* (U58100), *E. revolutum* (consensus from AF067850, U58102), *E. trivolvis* (consensus from AF067851, AF067852, U58097), *Schistosoma intercalatum* (Z21717), *S. haematobium* (Z21716), *S. mansoni* (consensus from L03658, U22168, X85246, AA525615, AA528926, AI559064) and *S. mattheei* (Z21718); Monogenea: *Gyrodactylus arcuatus* (AF156668, AJ001839), *G. branchicus* (AF156669), *G. derjavini* (AJ132259, AJ001840), *G. gasterostei* (AJ001841), *G. pungitii* (AJ001845), *G. salaris* (Z72477, AJ001841) and *G. truttae* (AJ132260); Turbellaria: *Schmidtea mediterranea* (AF047853) and Porifera: *Hymeniacidon sanguinea* (X00132).

Sequences were aligned with the Clustal W (version 1.7) multiple sequence alignment program (Thompson, Higgins and Gibson, 1994). The conserved secondary structure elements that were inferred for each sequence independently using the algorithms as implemented in the program MFOLD (Zuker, 1989) were taken into consideration while adjusting the alignment. A DNA dot matrix comparison was performed to visualize the alignable sites of the ITS region, using the Dotlet software (Junier and Pagni, 2000). Based on this information, three sets of aligned sequences were prepared. One consisted of the 5.8S rDNA only, the second consisted of 5.8S and the ITS2 rDNA and the third consisted of the two separate spacer regions ITS1 and ITS2. All ambiguities and gaps longer than one nucleotide were excluded from further phylogenetic analysis. In a first step we used Modeltest 3.06 to select the model of DNA evolution that best fitted the data based on log likelihood scores (Posada and Crandall, 1998). Secondly, we compared the base composition for all sequences using a 5% χ^2 test on the average composition (PUZZLE, Strimmer and Von Haeseler, 1996). The molecular-clock hypothesis was tested assuming the TrN model (Tamura and Nei, 1993) with γ-distributed rates across sites, with the likelihood ratio test for the clock hypothesis implemented in PUZZLE. Maximum likelihood (ML), using the parameters estimated under the best-fit model, and maximum parsimony (MP) analysis were carried out on the 5.8S rDNA sequences with PAUP* v. 4.01b (Swofford, 2001). With MP all characters were weighted equally and gaps were treated as missing data, for bootstrapping (n=1000) the branch and bound algorithm was applied.

To infer a phylogeny based on 5.8S and ITS2, we used maximum parsimony (MP), maximum likelihood and distance-based methods (PAUP*). With maximum parsimony the following models were used: unweighted parsimony with all characters unordered and gaps

treated as both missing data and fifth character; weighted parsimony with transition:transversion (ti/tv) ratios 10:5 for 5.8S and 1:5 for ITS2; and transversion parsimony (ti/tv weight 0:1). The maximum likelihood analysis was performed using the parameters estimated under the best-fit model. The maximum parsimony and maximum likelihood trees were inferred using the branch and bound algorithm, and statistically tested by means of bootstrapping (1000 and 100 replicates respectively). With the minimum-evolution distance method, the distance matrix was calculated using the paralinear/LogDet distances. We conducted the heuristic search method and bootstrapped (n=1000) with the tree-bisection-reconnection (TBR) branch-swapping algorithm in force.

A third dataset was prepared for the 5 *G.* (*Limnonephrotus*) species, consisting of the ITS1 and ITS2 rDNA, with all ambiguities and gaps longer than one nucleotide excluded (a total of 901bp). The data were treated like the second dataset. In order to test the presence of saturation in the ITS1 and ITS2 sequences, DAMBE 4.0.75 (Xia and Xie, 2001) was used to compare the saturation index expected when assuming full saturations with the observed saturation index. A *t*-test with infinite degrees of freedom was used to assess statistical significance.

3. Results

3.1. General characteristics of the rDNA sequences

Thirty-four parasite sequences of the ITS region from 7 species and 7 localities were obtained (Table 1). The amplified fragments encompassing the 3' end of 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 5' end of the 28S rRNA gene varied in size from 939 bp in the not-yet-described *Gyrodactylus* sp. 1 from *Pomatoschistus lozanoi* and *P. minutus* to 1236 bp in *G. pungitii.* The sequences have been deposited in GenBank under accession numbers *G. arcuatus* (AF328865), *G. gasterostei* (AF328867), *G. micropsi* (AF328868), *G. pungitii* (AF328869), *G. rugiensis* (AF328870), *G. salaris* (AF328871) and *G.* sp. 1 (AF328866).

Coding regions were identified by comparative alignment with previously published sequences (Kane and Rollinson, 1994; Cunningham, Aliesky and Collins, 2000; Ziętara et al., 2000). All 5.8S rDNA sequences had the same length of 157 bp. The short fragments of 18S rDNA and 28S rDNA (15 bp and 9 bp, respectively) were invariable. The length of ITS2 varied from 392 bp in *G. arcuatus* to 428 bp in *G. gasterostei, G. derjavini, G. pungitii, G. salaris* and *G. truttae.* The pronounced length differences in the total amplified fragments

were mainly due to different lengths of ITS1, varying from 364 bp in *G.* sp. 1 to 654 bp in *G. truttae*. The GC content was slightly biased towards AT in the spacers (41% to 48%) and towards GC in 5.8S rDNAs (51% to 52%) (Table 2), which is in agreement with related taxa (Luton, Walker and Blair, 1992; Morgan and Blair, 1995; Ziętara et al., 2000).

Table 2. Length of components of the ITS region and base composition for *Gyrodactylus* species analysed in this study.

Species	ITS1		5.8S		ITS2		Total	
	Bp	GC%	Bp	GC%	bp	GC%	bp	GC%
G. arcuatus	392	48	157	52	392	47	941	48
G. branchicus[1]	372	47	157	52	402	43	931	45
G. derjavini[2]	654	44	157	52	428	42	1195	44
G. gasterostei	612	44	157	52	428	42	1197	44
G. micropsi	607	45	157	52	401	43	1165	45
G. pungitii	627	44	157	52	428	42	1212	44
G. rugiensis	599	39	157	52	401	41	1157	41
G. salaris	623	43	157	52	428	42	1208	44
G. sp.1	364	48	157	51	394	46	915	48
G. truttae[2]	654	44	157	52	428	41	1239	44

1. Ziętara et al., 2000; 2. Cunningham, Aliesky and Collins, 2000.

Table 3. Intraspecific variation in the complete ITS region of *G gasterostei* and *G.* sp. 1.
(The numbers correspond to the position counted from first nucleotide after the primer located at the 5' end of the 18S gene. R - A, G; W - A, T; Y - C, T.)

Species	Number of individuals	Substitution	
G. gasterostei		581	971
	1	W	C
	4	W	Y
G. pungitii		340	
	1	W	
	4	T	
G. sp. 1		209	210
	4	C	G
	1	C	R
	1	Y	R

All sequences obtained were consistent within a species although some intra-specific variation was also observed. There were two variable sites within the species of *G. gasterostei* and *G.* sp. 1 and one in *G. pungitii* (Table 3). No intra individual length variation of ITS was detected.

The rDNA of the following species *G. arcuatus* and *G.* sp 1. (*G.* (*Mesonephrotus*)); *G. micropsi*, and *G. rugiensis* (*G.* (*Paranephrotus*)) and *G. derjavini* (AJ132259); *G. gasterostei*, *G. pungitii*, *G. truttae* (AJ132260); and *G. salaris* (*G.* (*Limnonephrotus*)) were aligned separately. The alignment of five species of subgenus *G.* (*Limnonephrotus*) was 1259 bp long with 291 (23.1%) variable sites, 244 (36.3%) in ITS1 and 47 (10.9%) in ITS2. There was a 176 bp long fragment within ITS1 that hampered alignment considerably as visualised in Fig. 1. The alignments of only two species from subgenus *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*) were more reliable than the alignment of the 5 species belonging to the subgenus *G.* (*Limnonephrotus*). There were no regions with very long gaps; the longest gap of 11 bp was observed in ITS1 of *G.* (*Mesonephrotus*) and the other gaps were not longer than 4 nucleotides. The final alignment of *G.* (*Mesonephrotus*) resulted in a length of 954 bp. Altogether; there were 120 (12.6%) variable sites, 83 (20.9%) in ITS1, 1 (0.6 %) in 5.8S rDNA and 36 (9.0%) in ITS2. The ITS region alignment of *G.* (*Paranephrotus*) was 1179 bp long with 196 (16.6%) variable sites, 145 (23.3%) in ITS1 and 51 (12.7%) in ITS2.

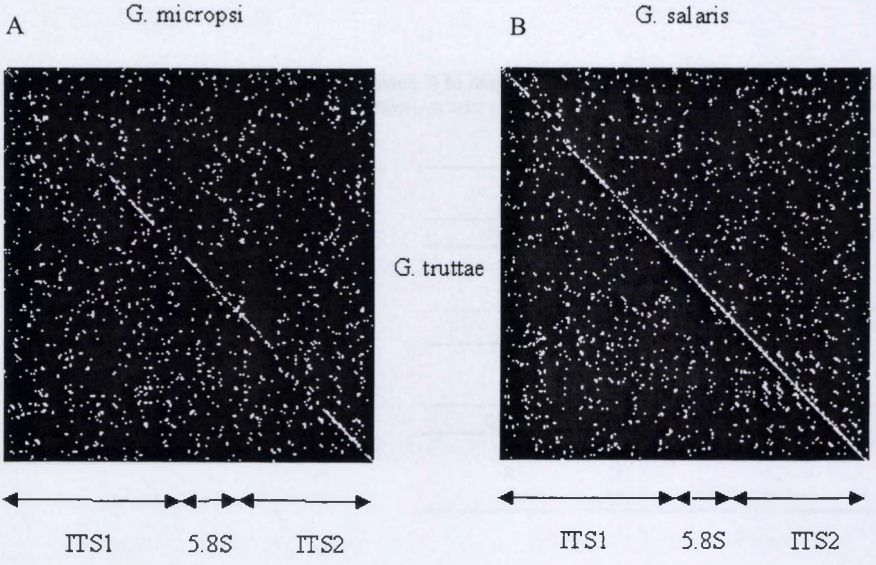

Fig. 1. DNA dot matrix of the ITS region (ITS1, 5.8S and ITS2): *G. truttae versus G. micropsi* (a); *G. truttae versus G. salaris* (b). The analysis was performed using the Dotlet software (Junier and Pagni, 2000). Every *dot* represents an eleven-nucleotide stretch with a similarity greater than 60%.

Table 4. Comparison of sequences obtained from different laboratories.

Species and accession numbers	Number of substitutions		
	ITS1	5.8S	ITS2
G. arcuatus			
(AF328865)[1]/(AF156668)[2]	0	0	1
(AF328865)[1]/(AJ001839)[3]	3 (2)	4 (3)	1
G. derjavini			
(AJ132259)[4]/(AJ001840)[3]	7 (4)	30 (6)	28 (6)
G. gasterostei			
(AF328867)[1]/(AJ001841)[3]	1 (1)	5 (3)	3
G. pungitii			
(AF328869)[1]/(AJ001845)[3]	12	9 (1)	2 (1)
G. salaris			
(AF328871)[1]/(AJ001847)[3]	0	1	1 (1)
(AF328871)[1]/(Z72477)[4]	1	0	0

1. This study; 2. Zietara et al. (2000); 3. Cable et al (1999); 4. Cunningham (1997), numbers in brackets refer to insertions/deletions within the variation.

3.2. Evaluation of GenBank sequences

We checked the quality of sequences deposited in GenBank. This is a delicate task because even if a region is recognised as highly conserved, substitutions might occur. There are 4 sources of *Gyrodactylus* ITS sequences, which have been processed either by Cunningham (1997), Cable et al. (1999), Zię tara et al. (2000), or in this paper. We compared all the sequences reported for each species. The sequences obtained by these authors differed intraspecifically by 1 to 65 nucleotides. Because this is far beyond the expected intraspecific variation, additional measures were taken to evaluate the correctness of the sequences.

First, 5.8S rDNA sequences were compared (Table 4). The subgenera differ from each other by one or several nucleotides, but within the subgenera *G.* (*Limnonephrotus*) (5 species) and *G.* (*Paranephrotus*) (2 species), no variation was detected. Among *G.* (*Mesonephrotus*), a G/T substitution in position 112 separates the two species (Appendix, Fig. 1). All 5.8S rDNA sequences submitted by Cunningham (1997) were invariable and identical with ours unlike those submitted by Cable et al. (1999).

Secondly, the spacer sequences were compared (Table 4). Some of the observed variation could be explained as intraspecific variation such as for example the sequence of *G. arcuatus* (AF 156668 *vs.* ours) or *G. salaris* (Z72477 *vs.* ours). However, it is obvious that some of the variation must be due to misinterpretation, e.g. the deletions or insertions. They were never observed within the same species in the subset including our samples and those of Cunningham (AJ132259, Z72477, AJ132260). Similarly, unique substitutions in regions with

several unidentified nucleotides were considered unreliable, for example in *G. derjavini* (AJ001840). Our final conclusion was that all the GenBank sequences with an aberrant 5.8S genotype were judged less reliable and therefore excluded from further phylogenetic analysis.

3.3. DNA dot matrix of the ITS sequences

Since the spacer sequences are known to be highly variable among different *Gyrodactylus* species (Cable et al., 1999; Zięta ra et al., 2000), problems arise during data analysis, especially in regards to sequence alignment. A dotplot is a graphical representation of the regions of similarity between two sequences and hence visualizes the useful sections for further data analysis. Fig. 1 shows a dotplot of two sequences of species belonging to different subgenera (a) and the same (b) subgenus. The ITS region consists of an alternation of highly variable and more conservative regions (Fig. 1a). The most conserved region is understandably the 5.8S gene and the 3' end of ITS2 that folds in a long unbranched loop in the secondary structure of all subgenera. The second most conserved regions are the 3' end of ITS1 and 5' end of ITS2. There is a shift in the middle of ITS2 which points to an insertion or deletion event. This region corresponds to the second loop that is the most variable one in the secondary structure of both species. Other fragments of ITS1 near the 5' end are almost impossible to align unambiguously due to large insertions/deletions and repeats (Fig. 1a). However, when we deal with species belonging to the same subgenus, the proportion of difficult alignable regions decreases considerably (Fig. 1b). We therefore propose a novel approach by preparing a hierarchical phylogeny. First, the sequences are analysed by the 5.8S part, which permits a subdivision into subgenera. Subsequently they could be divided in species-groups using the combined 5.8S and ITS2 sequences. Finally, for increasing the resolution up to species level, ITS1 and ITS2 can be used. These fragments might even include intraspecific variation between geographically separated populations (Zięta ra et al., 2000).

3.4. A deep division based on the 5.8S rDNA

The deepest division can be recognised using the 5.8S sequences. Each subgenus is characterised by diagnostic sequence features from this highly conserved gene. The five species of subgenus *G.* (*Limnonephrotus*) and the two species of subgenus *G.* (*Paranephrotus*) respectively, each shared a unique gene sequence. The two species of *G.*

(*Mesonephrotus*) differed in one nucleotide (Appendix, Fig. 1). The overall variation of the 5.8S gene among the different *Gyrodactylus* subgenera varied from 0.6% to 6.4%.

To compare this variation with published data, a phylogenetic reconstruction with other Platyhelminthes was prepared. Sequences of Platyhelminthes 5.8S rDNA were extracted from GenBank and aligned for phylogenetic analysis. Identical sequences were included as a single representative one. As such, five species of *Echinostoma* were represented as 2 sequences, one for the 37-collar-spined group (*E. caproni, E. liei, E. paraensei, E. revolutum and E. trivolvis*) and the other for the 28-collar-spined group (*E. hortense*). Four species of *Schistosoma* were also represented as 2 sequences although a deletion in position 141 may be an artefact because all Digenea have a double C in position 140 - 141 except *S. mansoni.* The final 5.8S rRNA alignment including 12 sequences was 157 bp long; the sponge *Hymeniacidon sanguinea* was used as outgroup. Only one gap was needed to align the Platyhelminthes sequences with the sponge, which proves that the 5.8S rRNA gene is highly conserved in length. Appendix, Fig. 1 shows the overall variation within the 5.8S rDNA of the Platyhelminthes studied here. The base composition for all sequences was compared with PUZZLE (Strimmer and Von Haeseler, 1996) and revealed a significant difference for the turbellarian *Schmidtea mediterranea.* Therefore this species was excluded from further analysis, which left the more distant sponge *H. sanguinea* as the only possible outgroup. Modeltest assigned the SYM + Γ_4 model (submodel of the general-time-reversible model) with gamma shape parameter = 0.99 and equal base frequencies as the most suited for the 5.8S data. Tree topologies constructed with ML and MP are generally in agreement, although ML generated lower bootstrap support (Fig. 2). Digenean and monogenean sequences are clearly separated from each other. The position of *D. symmetrus* remains unclear and is represented by a polytomy. The genetic diversity in the genus *Gyrodactylus* is higher compared to the other genera, as evidenced by the longer branch lengths. The "most advanced" subgenera *G.* (*Paranephrotus*) and *G.* (*Limnonephrotus*) are strongly clustered and the subgenus *G.* (*Metanephrotus*) (represented by *G. branchicus*) is found intermediately, grouping rather weakly with both *G.* (*Mesonephrotus*) genotypes. The subgenera *G.* (*Limnonephrotus*) and *G.* (*Paranephrotus*) clustered as sister species (Fig. 2).

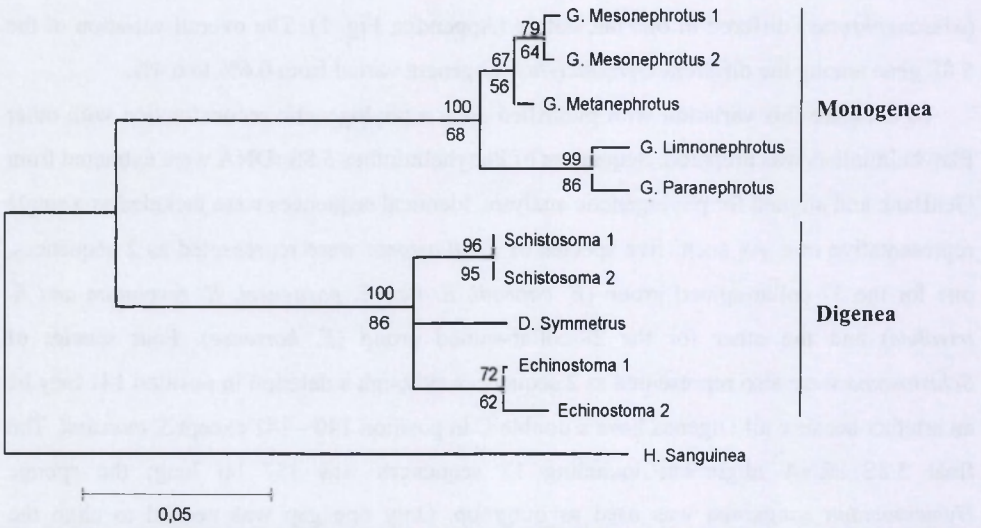

Fig. 2. Maximum likelihood phylogram constructed with 5.8S rDNA sequences of 11 taxa belonging to the Platyhelminthes, using the SYM + Γ_4 model with gamma shape parameter = 0.99, Ln *L* =-598.53, bootstrap support (percentage of 500 replicates, branch and bound algorithm) shown below branches. Above branches bootstrap support (percentage of 1000 replicates) of parsimony analysis based on 42 parsimony informative characters, length=65; CI=0.892; RI=0.950 (PAUP*, Swofford, 2001). *Echinostoma* 1 - 37-collar-spined *E. caproni*, *E. liei*, *E. paraensei*, *E. revolutum*, *E. trivolvis*; *Echinostoma* 2 - 28-collar-spined *E. hortense*; *Schistosoma* 1 - *S. haematobium*, *S. intercalatum*, *S. mattheei*; *Schistosoma* 2 - *S. mansoni*; *G.* (*Mesonephrotus*) 1 (*G. arcuatus*); *G.* (*Mesonephrotus*) 2 (*G.* sp. 1); *G.* (*Metanephrotus*) (*G. branchicus*); *G.* (*Limnonephrotus*) (*G. gasterostei*, *G. pungitii*, *G. salaris*, *G. truttae*); *G.* (*Paranephrotus*) (*G. micropsi*, *G. rugiensis*).

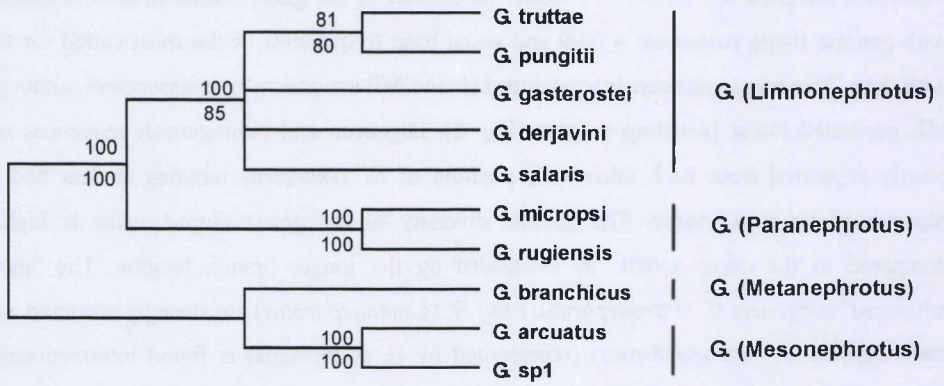

Fig. 3. Unrooted parsimony consensus tree of the 5.8S and ITS2 dataset, based on 190 parsimony informative characters with bootstrap support (percentage of 1000 replicates, branch and bound algorithm) shown above branches; length=1017; CI=0.789; RI=0.832. Bootstrap support (percentage of 100 replicates, branch and bound algorithm) for the maximum likelihood method is given below branches (TVM + Γ_4, gamma=0.48, Ln *L*=-2511.6). *G. arcuatus* (*G.* (*Mesonephrotus*) 1); *G.* sp.1 (*G.* (*Mesonephrotus*) 2); *G. branchicus* (*G.* (*Metanephrotus*)); *G. gasterostei*, *G. pungitii*, *G. salaris*, *G. truttae* (*G.* (*Limnonephrotus*)); *G. micropsi*, *G. rugiensis* (*G.* (*Paranephrotus*)).

3.5. A shallow division based on ITS1 and ITS2 rDNA

To resolve further the division of the *Gyrodactylus* subgenera, we used the 5.8S gene in combination with the more variable ITS2 rDNA. Although the ITS2 is quite variable among subgenera, the alignment can be checked by eye considering the presence of known monophyla (here subgenera) and the position of structural elements in the secondary structure (Schulenberg, Englisch and Wägele, 1999). The folding pattern consists of 4 main domains for all species studied and is in agreement with the structure proposed by Cunningham, Aliesky and Collins (2000). However, the domains B and C were the least stable as they contained the most deletions and insertions. Domain A and D were very consistent and remained almost invariant in shape, length and primary sequence.

Comparison of the different models of evolution revealed that the TVM + Γ_4 model (submodel of the general-time-reversible model) with gamma shape parameter = 0.48 as the most suited for the 5.8S and ITS2 data. The molecular clock was not enforced since PUZZLE showed rejection of the molecular clock hypothesis. PUZZLE showed a strongly deviating base composition for the subgenera *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*). Therefore, LogDet/paralinear distances were calculated and the tree constructed with neighbor-joining generated the same topology as the ML tree. The unrooted parsimony consensus tree is presented in Fig. 3. The different subgenera are clearly separated, and the overall tree topology is consistent regardless of the tree building method used. The division in the *G.* (*Limnonephrotus*) subgenus, however, remains unresolved. The unweighted and transversion parsimony, the Logdet and ML analysis all supported the clustering of *G. truttae* and *G. pungitii* (>81% BP, Fig. 3), in contrast to the weighted parsimony where only *G. gasterostei* clustered apart (66% BP, not shown). When treating gaps as a fifth character, the parsimony informative sites increased from 188 to 229 without changing the topology. Differences in pairwise LogDet distances between the subgenera are very high (up to 36%). However, plotting transitions and transversions against divergence of the 5.8S and ITS2 sequences (DAMBE, 4.0.24) showed no sign of saturation.

Aiming to increase the resolution, the five species of *G.* (*Limnonephrotus*) were aligned separately and used for phylogenetic analysis. ITS1, 5.8S rDNA and ITS2 alignments were 683 bp, 156 bp and 430bp long, respectively. Most gaps were within the more variable ITS1 region. All gaps longer than one nucleotide, all unreliable fragments and ambiguities were excluded from phylogenetic analysis. Final lengths of the fragments used were 473 bp and 428 bp long for ITS1 and ITS2, respectively. All the variation of the regions is given in

Appendix, Fig. 2, and 3. Neighbour-joining, maximum parsimony and maximum likelihood trees were generated for separate and combined spacers. It turned out that both spacers and the various tree building methods generated different topologies. When looking at the distribution of the various types of base changes (Table 5), it turns out that ITS1 and ITS2 have a clearly different frequency. The ti/tv ratio is 0.67 and 1.44 for ITS1 and ITS2, respectively. However, a *t*-test between the observed saturation index and the expected value assuming full saturation (DAMBE 4.0.24) shows that the ITS1 sequences are not saturated. The GC% is for both spacers from 41% to 48%, the estimated alpha amounts to 0.21 and 0.01 for ITS1 and ITS2, respectively.

Fig. 4 shows the ML tree based on ITS2, with gamma distributed rates (alpha=0.01) using the GTR model. In the weighted parsimony (ti/tv = 1:5), only *G. salaris* and *G. derjavini* cluster together with 67% bootstrap value, while the ML and distance based topologies are more resolved by grouping subsequently *G. pungitii* and *G. truttae*. The topology generated by the ITS1 conflicts with Fig. 4 in the sense that *G. salaris* and *G. gasterostei* cluster together.

Table 5. Calculated values of the 6 possible base changes by PAUP* (Swofford, 2001).

	AG	TC	AT	AC	TG	CG	Ts	Tv	Ts/Tv
ITS1	5.46	3.23	7.51	3.38	1	1.26	8.69	13.15	0.67
ITS2	3.47	1.11	1.41	0.55	1	0.28	4.58	3.19	1.44

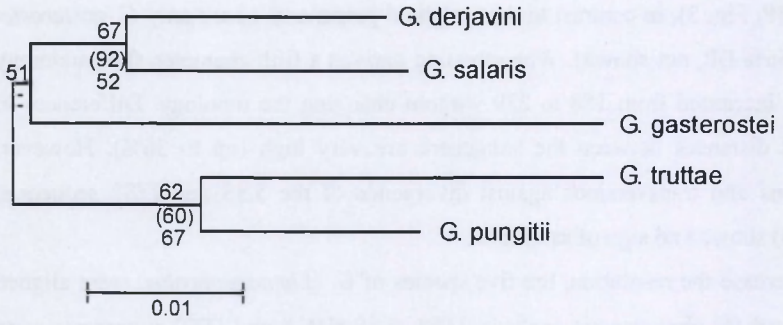

Fig. 4. Unrooted maximim likelihood tree for ITS2 data of the 5 *G.* (*Limnonephrotus*) species. Bootstrap support above branches for the ML analysis (Ln L = -1189.6), under the branches in brackets for the Paralinear/Logdet distance based method, without brackets for the MP analysis (length=76; CI=0,74; RI=0,44, PAUP*, Swofford, 2001).

4. Discussion

This paper adds new insights to a molecular phylogeny of *Gyrodactylus* as presented by Cable et al. (1999). Representatives from four out of six subgenera, as defined by Malmberg (1970), are included in the genetic analysis based on the ITS rDNA region. The 5.8S gene was found to be the most conservative part of the ITS region, but still phylogenetically informative among subgenera. We demonstrated that among ten species, each *Gyrodactylus* subgenus possesses a unique sequence of the 5.8S gene. Therefore this region can be used as a tool for a first division of *Gyrodactylus* into subgenera. To go deeper into the phylogeny of *Gyrodactylus*, more variable regions like ITS2 can be used. The combination of 5.8S and ITS2 provided a valuable tool to separate to species level, although the *G.* (*Limnonephrotus*) subgenus remains partly unresolved. ITS1 is the most variable region consisting of many repeats and insertions/deletions. Therefore, phylogenetic analysis based on ITS1 should be restricted to the subgenus level.

Although it is expected that a short and conserved sequence like the 5.8S rDNA gene yields low phylogenetic information (Joffe et al., 1995; Page and Holmes, 1998), the coding regions of the rRNA transcription unit have been extensively used to investigate phylogenetic relationships from phylum to genus level. According to Hershkovitz and Lewis (1996), 5.8S contains considerable phylogenetic information, particularly with respect to deep basal branches. The 5.8S sequence variation found in the genus *Gyrodactylus* (up to 6.4%) is higher than intrageneric differences reported for tropical cnidarians (up to 2.6%) and even higher than the variation found between nematode families and superfamilies (up to 5.2%) (Chen, Willis and Miller, 1996; Chilton, Gasser and Beveridge, 1997; Zhu, Gasser and Chilton, 1998). This is also visualized in the maximum likelihood phylogram based on 5.8S sequences of 11 Platyhelminthes (Fig. 2) where branches leading to the *Gyrodactylus* subgenera are relatively longer than those separating digenean genera. ML analysis clearly separated the Monogenea and the Digenea. With respect to the gyrodactylid clade we can not confirm the phylogeny proposed by Malmberg (1998), which is based on the protonephridial system. According to this scenario the subgenus *G.* (*Mesonephrotus*) may have given rise to the *G.* (*Metanephrotus*) system, which in turn gave rise to the most advanced subgenera *G.* (*Limnonephrotus*) and *G.* (*Paranephrotus*). When ITS2 is added to the analysis, both *G.* (*Mesonephrotus*) and *G.* (*Metanephrotus*), and *G.* (*Paranephrotus*) and *G.* (*Limnonephrotus*) cluster as sister groups. The various tree-building methods generate the same topology, but the division of the *G.* (*Limnonephrotus*) subgenus remains partly unresolved. At the moment,

more closely related outgroups are needed before a final conclusion can be made on the exact polarization of *Gyrodactylus*. It would be interesting to use *Macrogyrodactylus polypteri* as outgroup as it is suggested to be ancestral but closely related to *Gyrodactylus* (Malmberg, 1970; 1998).

The overall variation of ITS within the genus is very high, especially the ITS1 region of the *G.* (*Limnonephrotus*) group that consists of an array of repeats near the 5' end. This is also reported for ITS1 sequences of Digenea (Schulenberg, Englisch and Wägele, 1999; van Herwerden, Blair and Agatsuma, 1998; 1999). The ti/tv ratio lies below 1, which could indicate that the ITS1 sequences are saturated, but this is rejected by a *t*-test between the observed saturation index and the expected value assuming full saturation (DAMBE 4.0.24). The 3' end is less influenced by insertion/deletion repeats, and can be used to infer relationships within a subgenus. The ITS2 sequences however, have a ti/tv ratio of about 1.31 and the overall variation is thus lower. This region is more easily aligned, and can even be used across subgenera. The big difference in among-site rate heterogeneity, base composition and frequency of the various types of base change could indicate that ITS1 and ITS2 are subjected to different substitution pressures (Carranza, Baguna and Riutort, 1999). Another important characteristic of the ITS region is the evolutionary rate across sites. A likelihood ratio test (Modeltest) showed that the likelihood of the tree with gamma rates is significantly better than the likelihood of the tree without gamma rates. Consequently, for performing a reliable analysis ITS1 and 2 should be analysed separately and the gamma rates have to be taken into account.

The overall variation within *Gyrodactylus* is almost impossible to evaluate due to the highly unreliable alignment when all species are pooled. Nevertheless, variation as estimated for the subgenera *G.* (*Limnonephrotus*) (23%), *G.* (*Mesonephrotus*) (13%) and *G.* (*Paranephrotus*) (17%), already reaches the upper limits reported for the most related genera, which was already suggested by the differentiation at the 5.8S level (see above). ITS variation in related groups is presented in Table 6. These findings support the idea that *Gyrodactylus* species are much older and genetically more differentiated than might be deduced from their morphological similarity. An important trait of gyrodactylids is progenesis, which might have had a tendency towards uniformisation of the body plan and associated organs. An alternative explanation is that the substitution rate is unusually high due to the special combination of clonal and sexual selection with cross insemination. However, it is difficult to substantiate such an explanation. From the observed level of variation, it can be expected that the present genus *Gyrodactylus* contains species from a

much higher taxonomic level than species grouped in the *Schistosoma* or *Echinostoma* genera. Conventional taxonomy groups taxa, which are not equivalent in age, diversity, disparity, or any other consistent property of biology or evolutionary histories. In other words, any taxonomic rank such as for example 'genus' may not be equivalent across taxa and therefore hamper comparative evolutionary studies (Avise and Johns, 1999).

Table 6. Variation in ITS region.

	ITS1	ITS2	ITS
Cnidaria[1]	5-15%	-	-
Cooperia[2]	-	2-5%	-
Echinococcus[3]	1-18%	-	-
Echinostoma[4]	-	-	5-19%
Echinostomatidae[5]	11%	-	-
Fasciola[6]	-	2-13%	-
Mesometridae[7]	7-19%	3-15%	-
Nematodirus[8]	2-17%	-	-
Schistosoma[9]	-	5-11%	

1. Chen, Willis and Miller, (1996); 2. Newton et al., (1998); 3. Bowles, Blair and McManus, (1995); 4. Morgan and Blair, (1995); 5. Grabda-Kazubska et al., (1998); 6. Adlard et al., (1993); 7. Jousson et al., (1998); 8. Audebert, Durette-Dessett and Chilton, (2000); 9. Bowles et al., (1993)

Although very low, intraspecific variation is observed in the ITS region of *G.* sp. 1, *G. gasterostei* and *G. pungitii.* This has also been reported for *G. arcuatus* and *G. branchicus* (Zię tara et al., 2000) but, in contrast, Cable et al. (1999) reported no differences in ITS2 sequences of *G. kobayashii* from the U.K. and Australia.

However, no intra-individual variation in length is observed in the ITS region of the *Gyrodactylus* species studied here. It is reported for ITS1 of tropical cnidarians (Chen, Willis and Miller, 1996), *Paragonimus westermani* (Digenea) (van Herwerden, Blair and Agatsuma, 1999), 2 species of *Trichostrongylus* and *Nematodirus battus* (Nematoda) (Hoste et al, 1995). The intraindividual variation of the latter was sometimes greater than the interspecific variation.

Since primary sequence similarity appeared to be associated with secondary structure conservation, it is suggested that similarity is due to identity by descent and not chance (Schulenberg, Englisch and Wägele, 1999). All species studied share a folding pattern in which four main domains can be identified. The structure of the ITS2 sequence of *G. salaris* is identical with the one presented by Cunningham, Aliesky and Collins (2000).

When focusing on the *G.* (*Limnonephrotus*) group, different results were generated according to the region and tree-building method used. It seems that both spacers are

subjected to different selection pressures. The unresolved tree constructed on the ITS2 data may be ascribed to a radiation event. This radiation can be the outcome of speciation by host switching, which reflects an ecological rather than phylogenetic host specificity since the hosts occur in the same habitat rather than being closely related. According to Harris (1985) this group consist largely of sibling species, still undergoing speciation. This study confirms the observation of Cable et al. (1999) that molecular studies based on the ITS region cannot separate *G. salaris* from the other four *G.* (*Limnonephrotus*) species (representatives of *G. wageneri* species-group) as suggested by Malmberg (1993).

If a molecular clock is applied to the ITS2 data, evolving at 0.3 - 0.7%/Myr (Despres, 1992), the speciation event of the *G.* (*Limnonephrotus*) species took place about 10 Myr BP. This is after the hosts speciated. However, if the same rate is applied to all species, *G. rugiensis* and *G. arcuatus* (41%, uncorrected p-distance) for example would have speciated around 80 Myr BP (assuming a rate of 0.5% Myr BP). Here we arrive at a point where the molecular clock should be treated with caution. When dealing with all species together, the molecular clock hypothesis is rejected, but it is accepted when dealing within each subgenus. More analysis is needed before hypothesizing about differences in evolutionary rate between subgenera.

Cable et al. (1999) presented a first phylogeny of *Gyrodactylus* based on ITS1 and combined 5.8S rDNA with ITS2 sequences. They concluded that the 5.8S rRNA gene alone was phylogenetically uninformative; eleven species were separated into two groups based on combined 5.8S and ITS2 sequences. Here, their division is supported where *G.* (*Limnonephrotus*) and *G.* (*Paranephrotus*) have long ITS1 sequences (599 to 654 bp) while *G.* (*Mesonephrotus*) and *G.* (*Metanephrotus*) have clearly shorter sequences (364 to 392 bp and 372 bp, respectively). The differences between our conclusions and those of Cable et al. (1999) seem related to the quality of the 5.8S rDNA sequence. Cable et al. (1999) mentioned that their 5.8S rDNA and ITS2 sequences were subject to sequencing errors, which resulted in the inclusion of ambiguous or unidentified bases. This might be the most straightforward explanation why all published 5.8S rDNA sequences of *G. derjavini* differed by 30% although they should be identical (Table 4). It is obvious that when using short and highly conserved regions with a low number of variable sites, the sequences have to be of the highest quality.

In conclusion, we suggest a hierarchical approach to elucidating the phylogeny of the genus *Gyrodactylus* based on the ITS region. The conservative 5.8S gene is proven to be phylogenetically informative and it may even be used as an aid to detect the (sub)generic

position of difficult species or perhaps even as a tool to define a (sub)genus. As such, there is a concordance between the 5.8S gene and the excretory system used by Malmberg (1970) as a diagnostic character in designating the subgenus status. The molecular phylogeny for ten species is at first sight in agreement with the morphological phylogeny presented by Malmberg (1998). According to Milinkovitch et al. (2000), covariation between *a priori* morphological/physiological designations and a minimum of one molecular character is a sufficient condition for biological species recognition.

However, when moving to species level, there is a discrepancy between the morphological and molecular variation. Whereas morphological variation, expressed in shape and size of the attachment apparatus, is very low, molecular variation, expressed by variation in the ITS1 and ITS2 regions, is very high. This can be attributed to the fast evolving nature of the ITS region, or to the fact that this genus is constituted of groups of a higher taxonomic level then previously recognised. By including different tests and comparing different tree building methods, we tried to overcome the pitfalls of phylogenetic analysis. However, exploring another genetic marker and including more species should shed more light on this intriguing issue.

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Appendix

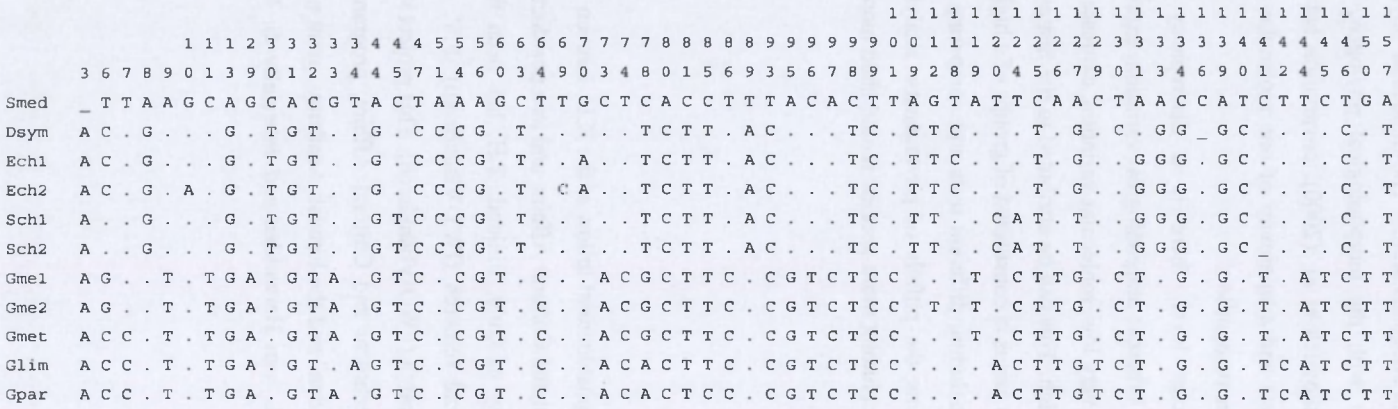

Fig. A1. Variable sites in 5.8S rDNA of 12 Platyhelminthes taxa. Numbering starts from the first nucleotide of the 5.8S gene. (.) indicates identity to Smed sequence. Abbreviations are as follow: Smed - *Schmidtea mediterranea*; Dsym - *Dolichosaccus symetrus*; Ech1 - 37-collar-spined *Echinostoma caproni*, *E. liei*, *E. paraensei*, *E. revolutum*, *E. trivolvis*; Ech2 - 28-collar-spined *E. hortense*; Sch1 - *Schistosoma haematobium*, *S. intercalatum*, *S. mattheei*; Sch2 - *S. mansoni*; Mes1 - *G.* (*Mesonephrotus*) (*G. arcuatus*); Mes2 - *G.* (*Mesonephrotus*) (*G.* sp. 1); Met - *G.* (*Metanephrotus*) (*G. branchicus*); Lim - *G.* (*Limnonephrotus*) (*G. gasterostei*, *G. pungitii*, *G. salaris*, *G. truttae*); Par – Paranephrotus (*G. micropsi*, *G. rugiensis*).

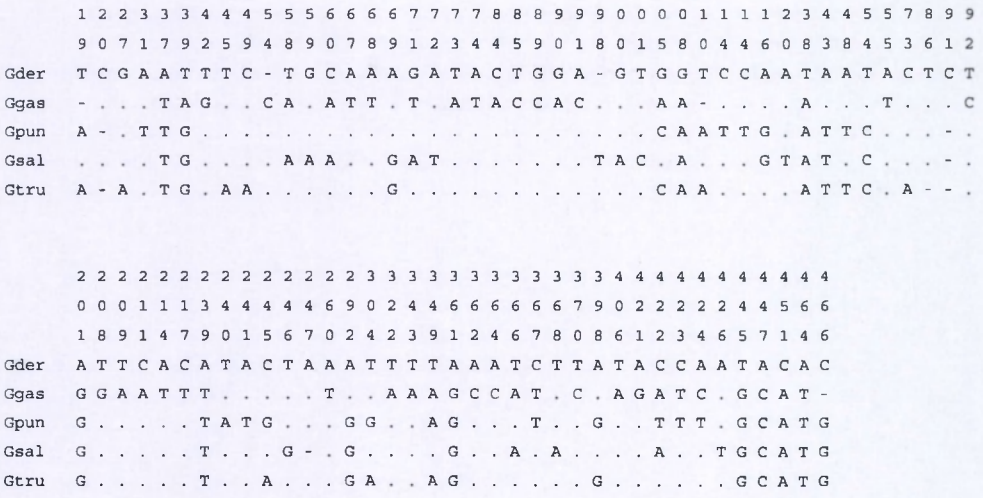

Fig. 2. Variable sites of the ITS1 fragment within the subgenus *G.* (*Limnonephrotus*). Numbers correspond to the first nucleotide of the aligned ITS1 sequence. A dot indicates identity to Gder sequence. Abbreviations are as follow: Gder - *G. derjavini*, Ggas - *G. gasterostei*, G. pun - *G. pungitii*, Gsal - *G. salaris* and Gtru - *G. truttae*.

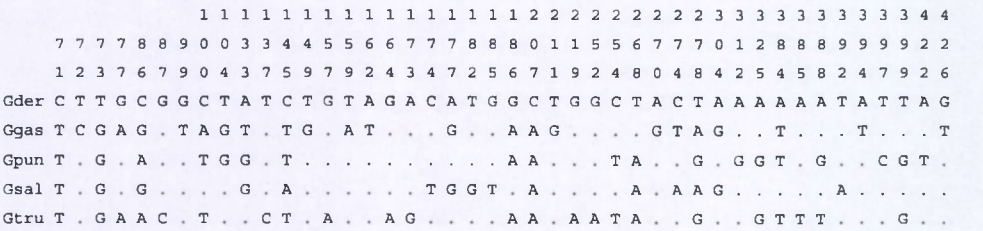

Fig. 3. Variable sites of ITS2 in subgenus *G.* (*Limnonephrotus*).
Numbers correspond to the first nucleotide of the aligned ITS2. (.) indicates identity to Gder sequence. Abbreviations are as follow: Gder - *G. derjavini*, Ggas - *G. gasterostei*, G. pun - *G. pungitii*, Gsal - *G. salaris* and Gtru - *G. truttae*.

CHAPTER 3

IDENTIFICATION OF A HOST-ASSOCIATED SPECIES COMPLEX USING MOLECULAR AND MORPHOMETRIC ANALYSES, WITH THE DESCRIPTION OF *GYRODACTYLUS RUGIENSOIDES* N. SP. (GYRODACTYLIDAE, MONOGENEA)

Tine Huyse and Filip A.M. Volckaert

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 ITS 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. rugiensisoides* 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.

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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 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 en 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 (1974): *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).

Table 1: *Gyrodactylus* species, host, locality and date of sampling of the specimens used for morphometric and molecular analysis. N = number of species measured, G = number of specimens sequenced in this study.

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 31.1 ppm	20/**
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	-/**

*fish were kept in the laboratory at a water temperature of about 18°C; ** see 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 3.06 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) 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-fitting model and optimised through repeated estimation. We conducted the exhaustive search method and the trees were statistically tested using 1000 bootstrap replicates. 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 x 40 x for the anchors, 10 x 100 x 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 x 90 x oil; drawings of the

marginal hook features were done using 16 x 90 x oil using the equipment from Malmberg (1970).

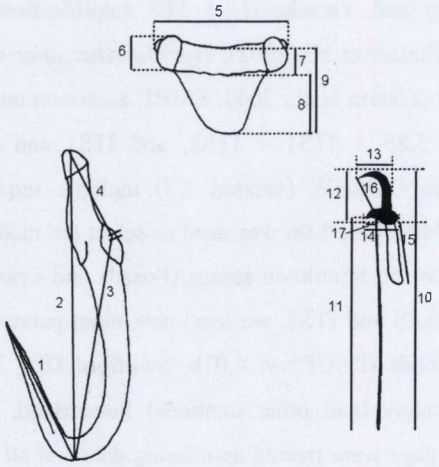

Fig. 1: Measurements of the opisthaptoral hard parts of *Gyrodactylus* spp. *Hamulus*: (1) LAP, length of anchor point; (2) LA, total length of anchor; (3) LAS, length of anchor shaft; (4) LAR, length of anchor root. *Ventral bar*: (5) LVB, length of ventral bar; (6) BWVB, basal width of ventral bar; (7) MWVB, median width of ventral bar; (8) VBM, length of ventral bar membrane; (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; (17) TOE, marginal hook toe length.

Statistical 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 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 = 0.9276, RI = 0.905. 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 the G. rugiensis*-like species*

Comparison between the second and eighth marginal hook

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.

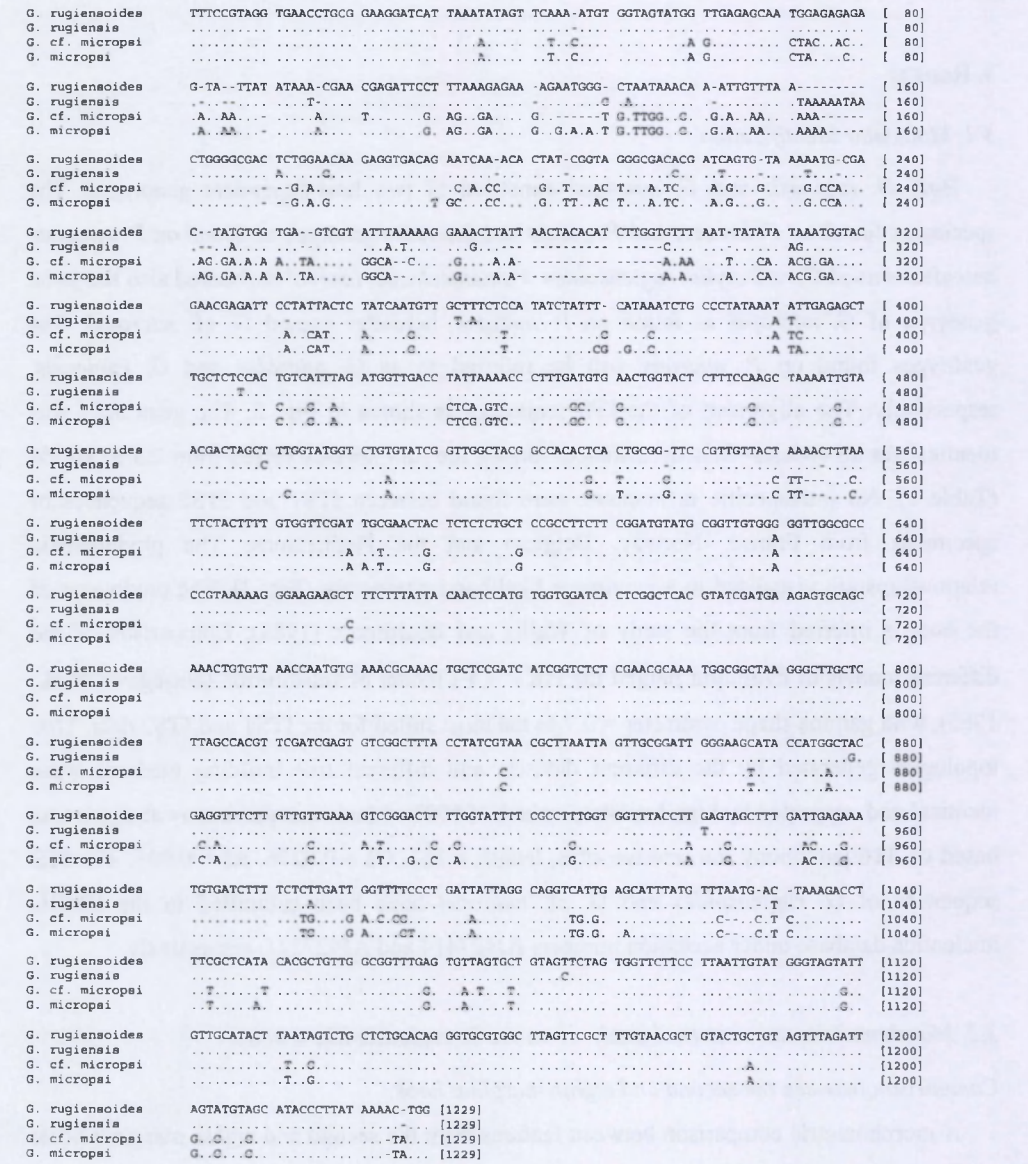

Fig. 2. Alignment of internal transcribed spacers (ITS) 1 and 2 and 5.8S gene sequences from *G. micropsi*, *G. rugiensis* (EMBL accession number AF328868 and AF328870), *G. rugiensoides* n. sp. and *G.* cf. *micropsi*. Dots (.) indicate nucleotides identical to *G. rugiensoides*; dashes (-) indicate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

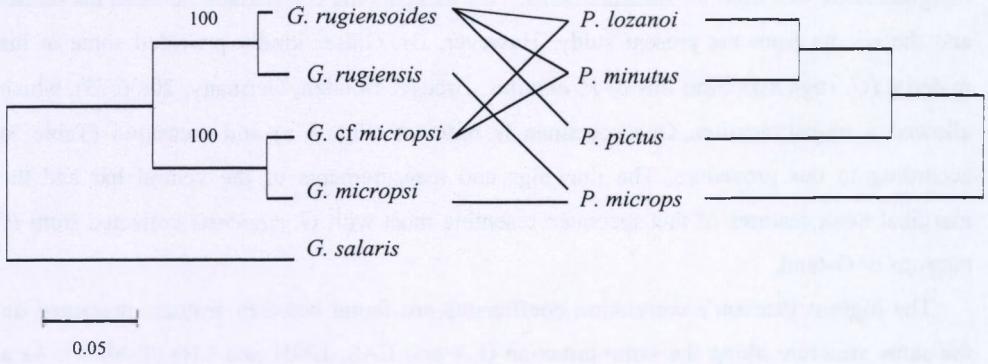

Fig. 3. Comparison of host and parasite phylogeny. The maximum likelihood phylogram of the parasites is constructed with the 5.8S and ITS2 sequences using the HKY + Γ_4 model of substitution with gamma shape parameter = 0.7; Ln L = -3207.3. The bootstrap values are identical for all treebuilding methods.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

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	-

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 in 1974 (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 (1974) 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 (1974) 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. 4 a) and measured (Table 3) according to our procedure. The drawings and 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 length of the anchor point, anchor shaft and the total length of anchor (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.

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: the median width of the ventral bar (BWBM), 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 total length of the ventral bar membrane (TLVMB) and to a lesser extent the median and basal width of the ventral bar (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 length of the ventral bar (LVB) and the length of the anchor shaft and anchor point (LAS, 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.

Table 3: Size range of characters of the opisthaptoral hard parts of *G. rugiensis* on *P. minutus* and *P. microps*, measured by Gläser (1974) and measured in this study on a single drawing of material provided by Gläser. Mean, range and C.V. (C.V. = 100 x 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. All measurements are in µm. N = number of parasite specimens measured. For abbreviations see Fig. 1.

Gyr. species		*G. rugiensis*	*G. rugiensis*	*G. rugiensoides*	*G. rugiensoides*	*All parasites*
Host species		*P. mic/P. min*	*P. microps*	*P. min/P. loz*	*P. pictus*	*All hosts*
N		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

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: *Pomatoschistus 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; Table 1 and 3.

Number drawn: 5.

Deposited specimens: two slides: Fig. 4 b 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: PCR amplified internal transcribed spacers (ITS) 1 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 *G. wageneri*-group, subgenus *G.* (*Limnonephrotus*) Malmberg, 1964. Anchors and anchor points longer than those of *G. micropsi* Gläser, 1974. 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 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: *Pomatoschistus 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.

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 collected on *P. minutus* and 20 on *P. pictus*; Table 1 and 3.

Number drawn: 7; Fig. 4 c, 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, 1974. Ventral bar with small processes, not always visible.

Median width of ventral bar wider than in *G. rugiensis* Gläser, 1974. Ventral bar membrane triangular and longer than in *G. 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 length of sickle (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 3µm. 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*.

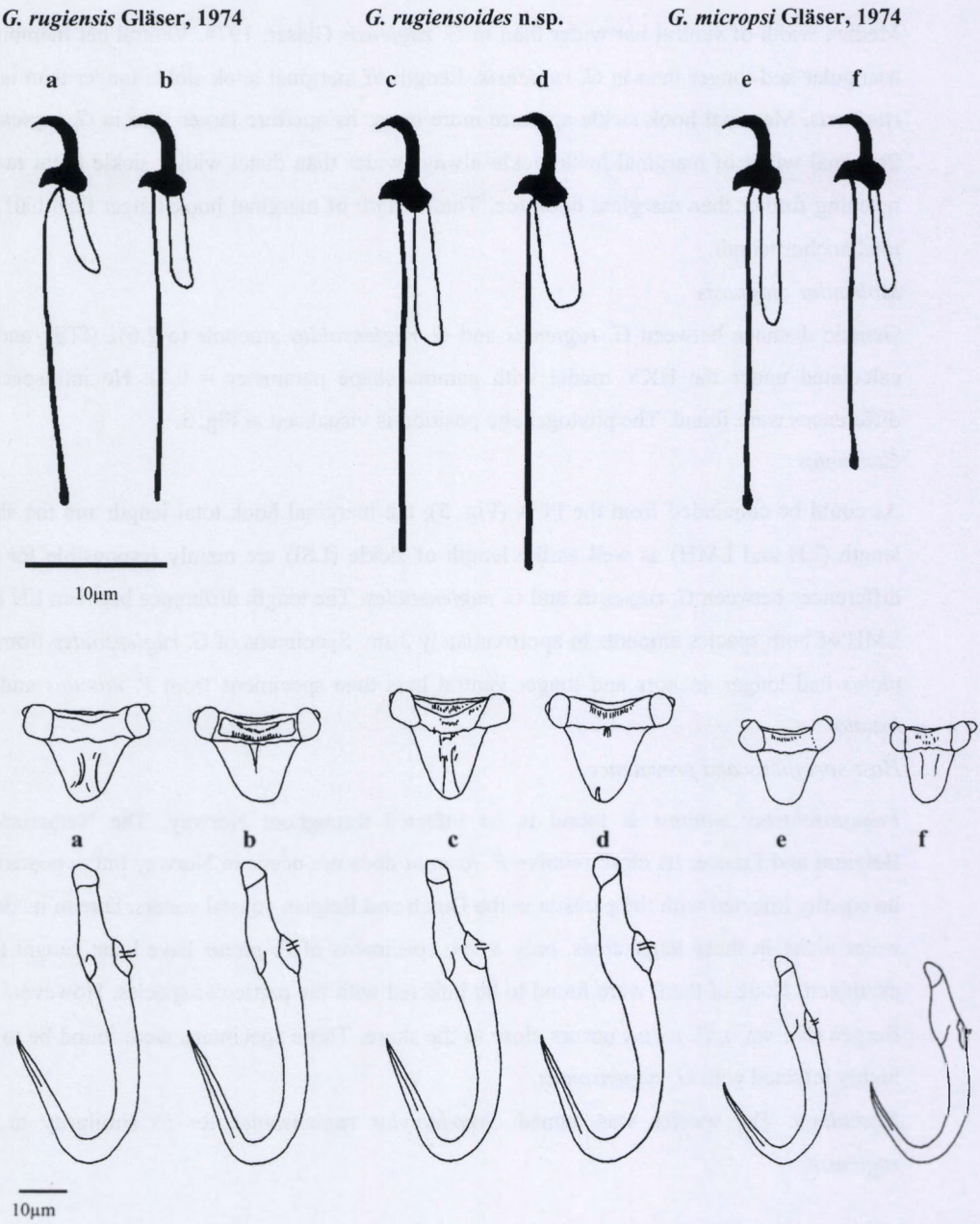

Fig. 4. Marginal hooks, ventral bars and anchors of *Gyrodactylus* species parasitising species of *Pomatoschistus*. **a** and **b**: *G. rugiensis* Gläser, 1974: **a.** specimen from Gläser's collection, fins of *P. microps* (Breeger Bodden, Germany, 20/06/73); **b.** specimen from the fins of *P. microps* (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, 1974: **e.** specimen from Gläser's collection, fins of *P. minutus* (Breeger Bodden, Germany, 27/06/73); **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.

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). Correlations in bold are significant at $p < 0.05$). Correlations > 0.70 are underlined. For abbreviations see Fig. 1.

	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

Table 5: Analysis of variance testing for differences in morphological traits of *G. rugiensis* and *G. rugiensoides* n. sp. Specimens are grouped according to genotype and respective host species. Correlations in bold are significant at $p < 0.05$. For abbreviations see Fig. 1.

Groups	*P. mic-P. pic/P. min*	*P. mic-P. min*	*P. min-P. pic*	*P. mic-P. pic*
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

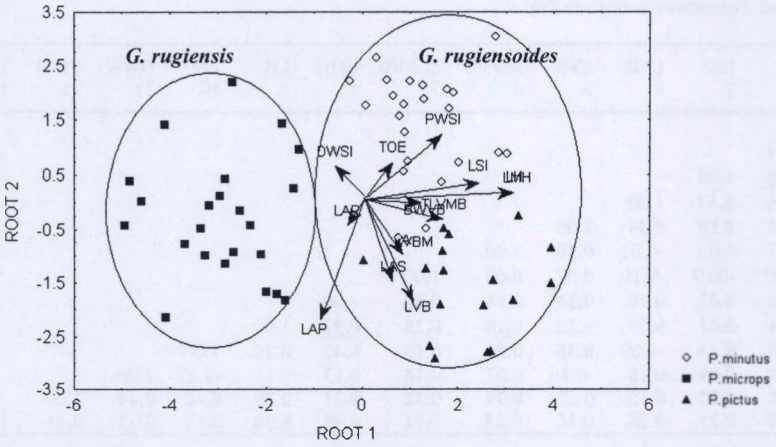

Fig. 5. Plot of standard discriminant analysis (Wilk's Lambda: 0.09, $p < 0.0001$) on morphological measurements of 72 *Gyrodactylus* species occurring on *P. minutus, P. pictus* and *P. microps*. For abbreviations see Fig. 1.

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. 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.

A) Nearest neighbours

1) By genotype

Predicted class	True class	
	G. rugiensis	***G. rugiensoides***
G. rugiensis	21	2
G. rugiensoides	0	41

estimated misclassification rate =3.1 %

2) By host

Predicted class	True class		
	P. mic.	***P. min.***	***P. pic.***
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%

Predicted class	True class		
	P. mic.	***P. min.***	***P. pic.***
P. microps	21	2	0
P. minutus	1	19	4
P. pictus	1	3	13

estimated misclassification rate = 17.2%

4. Discussion

Gyrodactylus rugiensis Gläser, 1974 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, 1974 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 ITS 1 and 2 region, respectively; no intraspecific 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 2.7-56% and 1.5-38.7% for ITS1 and ITS2 respectively (Kimura distances from Matejusova et al., 2001). In a study on polystomatid monogeneans the ITS1 sequence variation ranges from 0.6-23.3% (Tajima-Nei distances, Bentz et al., 2001). Species differences found in the present study varied from 2.5-16.5% (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 exceeds 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. 4 e) resembles very much the drawing from material collected from Doel (Fig. 4 f). 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 features 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*.

In summary: species diversity and host-specificity of *Gyrodactylus* species infecting the *Pomatoschistus* species has 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. The occurrence and importance of host-switching may be related to the dependence of *Gyrodactylus* species on host-to-host transfer by contagion (Kearn, 1994). Finally, all morphometric and molecular analyses used in the present study support the description of *Gyrodactylus rugiensoides* as a new species.

Acknowledgements

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CHAPTER 4

DISCRIMINATION OF FOUR NEW SPECIES OF GYRODACTYLUS (MONOGENEA, PLATYHELMINTHES) PARASITIZING GOBIID FISH: COMBINING DNA AND MORPHOLOGICAL ANALYSES

Tine Huyse, Göran Malmberg and Filip A.M. Volckaert

Abstract: Four closely related *Gyrodactylus* species parasitising the gills and fins of four closely related gobiid species were described: *G. branchialis* sp.n., *G. gondae* sp.n, *G. flavescensis* sp.n and *G. arcuatoides* sp.n. All four species were found to be highly host-specific. Complementary morphological, morphometric and molecular data analysis have been carried out. The ssrRNA V4 region and the complete ITS rDNA region has been sequenced. The morphological differences were small but consistent; the size of their marginal hook sickles is among the smallest (less than 2.5 μm) described in *Gyrodactylus*. The morphological resemblance with *G. arcuatus* from three-spined stickleback was striking but genetically they were clearly distinct. *Gyrodactylus gondae*, *G. gondae* and *G. flavescensis* belong to the *G. arcuatus*-species group. From a morphological point of view *G. branchialis* clearly is an undescribed speces not belonging to the *G. arcuatus*-group, although the V4 and ITS sequence analysis indicate a close relationship to the species-group.

This chapter has been submitted to *Zoologica Scripta*.

1. Introduction

Gyrodactylids are common fish parasites in fresh and salt water. They are viviparous, host- and organ-specific ectoparasitic worms and many species have a high reproductive capacity. In the uterus of a worm, new specimens at different developmental stages can be found. The marginal hook sickles of the posterior attachment organ (the haptor) is the most species discriminating character (Malmberg 1970). During development, the marginal hook sickles will be fully developed before the anchors and the ventral bar, two other important species characters. This implies that the precise shape of fully developed marginal hook sickles of a large embryo in the uterus can be compared with those of the maternal worm. Hitherto, no differences between marginal hook sickles in the adult and the embryo are described (eg. Malmberg 1970). In adult worms, however, variations, especially seasonal variations (eg. Malmberg 1970, Mo, 1991) of anchors, ventral bars and even marginal hooks are present.

By now, molecular techniques are widely accepted as an important tool in tackling taxonomic and systematic questions. The nuclear ribosomal RNA genes and their internal transcribed spacers (ITS1 and ITS2) consist of variable and conservative regions that provide an ideal target to compare both closely and distantly related species (Hillis et al., 1996). Due to the availability of conserved primer sequences this marker has almost exclusively been used for discriminating species in the genus *Gyrodactylus* (Cunningham et al., 1995, 2001; Matejusova et al., 2001; Huyse and Volckaert, 2002; Zietara and Lumme, in press).

This paper describes four similar and closely related *Gyrodactylus* species found on four goby species belonging to the genera *Pomatoschistus* and *Gobiusculus*. The closely related *Pomatoschistus* species occur in sympatry and they are the most abundant benthic species in the North Sea (Fonds, 1971). A combined molecular, morphological and statistical approach is used, with special attention for the marginal hook sickle as diagnostic character.

2. Material and Methods

2.1. Hosts and Gyrodactylus specimens

The material for the present study was collected along the Eastern Atlantic seaboard. Data regarding host species, site on host, localities, salinity, prevalence and abundance of *Gyrodactylus* specimens analyzed are listed under *Descriptions of species* and in Table 1. Fish were transported alive in local water to the laboratory and killed according to Malmberg (1970) before investigation. Under a stereomicroscope, *Gyrodactylus* specimens were

individually removed from the fish by means of preparation needles. Whenever possible, the body was separated from the haptor and fixed between slide and cover glass in ammonium picrate glycerin according to Malmberg (1970) for morphological analysis. The body, in turn was transferred into 5 µl of milli-Q water and stored at -20°C for further DNA analysis.

Table 1. Host species, site on host (G = gill; F = fin), localities, water temperature, salinity, prevalence (P) and abundance (A) and number of sequences (nS) of *Gyrodactylus* specimens analysed in this study.

Gyrodactylus spp.	Host spp.	Locality	Date, water temperature, salinity	N hosts	P*	A*	Site	nS
G. gondae	*Pomatoschistus minutus*	Texel, The Netherlands 53° N, 4° 48' E	15/06/99, 12°C 31. 0 ppm	11	91	330	G/F	2
	P. lozanoi			6	3/6	40	G/F	1
G. gondae	*P. minutus*	Texel, The Netherlands	25/11/00, 12°C	23	61%	372	G/F	3
	P. lozanoi	53° N, 4° 48' E	31.0 ppm	7	5/7	40	G/F	2
G. gondae	*P. lozanoi*	Belgian continental shelf 51° 35' N, 2° 18' E	26/10/99, 12°C 35.0 ppm	31	19%	14		2
G. gondae	*P. minutus*	Trondheim, Norway 63° 32' N, 10° 26 E	13/06/00, 12°C 32.0 ppm	3	2/3	14	F	2
G. gondae	*P. minutus*	Bergen, Norway 60°16' N, 5°10' E	21/05/01, 9-10°C 33. 0 ppm	10	60%	18	F	3
G. arcuatoides	*P. pictus*	Bergen, Norway 60°16' N, 5°10' E	22/06/00, 9-10°C 33. 0 ppm	16	81%	98	G/F	3
G. arcuatoides	*P. pictus*	Belgian continental shelf 51° 35' N, 2° 18' E	26/10/99, 12°C 35.0 ppm	5	2/5	3	G	2
G. arcuatoides	*P. pictus*	Bergen, Norway 60°16' N, 5°10' E	21/05/01, 8-9°C 33. 0 ppm	10	70%	29	G/F	2
G. flavescensis	*Gobiusculus flavescens*	Bergen, Norway 60°16' N, 5°10' E	22/06/00, 9-10°C 33. 0 ppm	6	6/6	70	F/G	3
G. flavescensis	*G. flavescens*	Bergen, Norway 60°16' N, 5°10' E	21/05/01, 8-9°C 33. 0 ppm	12	83%	78	F/G	1
G. flavescensis	*G. flavescens*	Trondheim, Norway 63° 32' N, 10° 26 E	13/06/00, 12°C 32.0 ppm	3	2/3	8	F/G	3
G. branchialis	*P. microps*	Ostend, Belgium 51°14' N, 2°57' E	18/08/99, 16-18°*C 31.1 ppm	25	84%	248	G	4
G. branchialis	*P. microps*	Ambleteuse, France 50° N, 1° 36' E	23/09/99, 15°C 16-30 ppm	15	47%	75	G	2
G. branchialis	*P. microps*	Texel, The Netherlands 53° N, 4° 48' E	15/06/99, 12°C 31. 0 ppm	8	50%	9	G	2
G. branchialis	*P. microps*	Yerseke, The Netherlands 51° 30' N, 4° 4' E	06/11/99, 16.7°C 30.1 ppm	8	88%	53	G	1
G. arcuatus	*P. microps*	Bergen, Norway 60°19' N, 5°14' E	21/05/01, 8-9°C 17. 0 ppm	16	25%	8	F	3
G. arcuatus	*G. arcuatus*	Edesö, Sweden 59° 22'N, 18° 27' E	11/06/01, 10°C, 5.0 ppm	4	3/4	12	F/G	1

P* = prevalence, calculated as the ratio of the number of infected fish specimens to the total number of fish (if N ≥ 10) examined; A* = abundance, calculated as the total number of *Gyrodactylus* specimens per fish population. G/F = more than 50% on gills, but also found on fins; F/G = more than 50% on fins, but also found on gills.

2.2. Molecular analysis

DNA extraction, ITS amplification and sequencing of individual parasites were performed as described by Zietara et al. (2002). Sequences were aligned with the Clustal X multiple sequence alignment program (version 1.81, Thompson et al., 1997); they have been submitted to the EMBL database under accession numbers X. 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). *Gyrodactylus rarus* (Acc. No x) was used as outgroup since it belongs to another subgenus (Malmberg, 1970). 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 and 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 1000 bootstrap samples for the minimum-evolution tree.

2.3. Morphological analysis

The microscopical analyses of *Gyrodactylus* specimens were performed at the Laboratory of Aquatic Ecology, Catholic University Leuven, Belgium and the Department of Zoology, Stockholm University, Sweden. In Belgium measurements were done using a Zeiss HBO50 microscope (oil immersion, 10x ocular for ventral bars and anchors). Images were analysed with the program SigmaScan Pro 5. In Stockholm the microscopical analyses were performed, using oil immersion (90x objective), phase contrast and drawing prism equipment (Malmberg 1970) improved with Leica DC 300 Digital Camera and Archiving System. The images of the opisthaptoral hard parts of all specimens were stored and printed for further analysis on an illuminated desk by a printer equipped with a LazerPrint system (Reality Imaging System, Munich, Germany). The analysed results from image materials were compared to drawings, made by means of a drawing prism (Malmberg 1970). These drawings were made at the same scale, using a 10x ocular for ventral bars and anchors or a 16x ocular for marginal hooks. Measurements of marginal hook sickles were performed by means of image analysis (Leica Q500*IW* with a Hamamatsu 3 CCD camera, C5810), the sickle area by

detection and the other measurements by interactive measuring on the computer screen. Holotype and the paratype specimens of the *Gyrodactylus* species in Huyse´s collection were compared to drawings of *G. arcuatus* specimens in Malmberg´s collection. In total, 10 *G. arcuatus* specimens (SMNH. Acc.No. 48440) were studied, 10 digitally recorded, three were drawn by means of a drawing prism and 12 marginal hooks were measured. For data on the four new species, see *Descriptions of species*.

For Scanning Electron Microscopy (SEM) purposes, 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.

For the statistical analyses STATISTICA 6.0 was used. 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. Observations with a coefficient of variation (C.V. value = 100 x the square root of the variance divided by the mean) exceeding 12% were excluded to avoid measurement errors. Observations with missing variables were substituted by means. Factor analysis and discriminant analyses were used to assess the contribution of each variable in the separation of the different species.

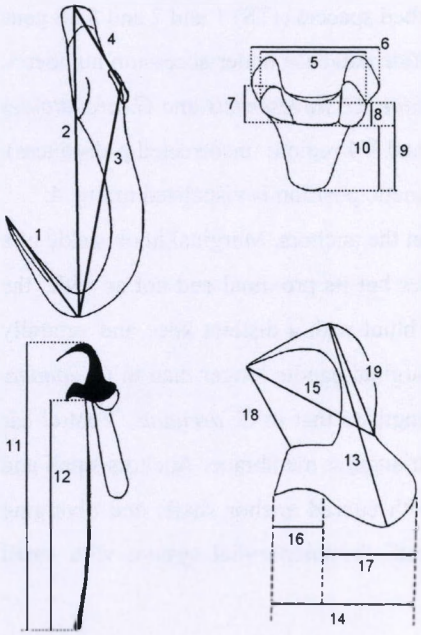

Fig. 1. Method of measuring opisthaptoral hard parts of *Gyrodactylus* species in this paper. – **Anchor:** - 1. Length of anchor point. – 2. Total length of anchor. – 3. Length of anchor shaft. – 4. Length of anchor root. – **Ventral bar:** - 5. Length of ventral bar. – 6. Maximal distance between ventral bar processes. – 7. Basal width of ventral bar. – 8. Median width of ventral bar. – 9. Median width of ventral bar + length of ventral bar membrane. – 10. Length of ventral bar membrane. – **Marginal hook:** - 11. Total length of marginal hook. – 12. Length of marginal hook handle. – **Marginal hook sickle:** - 13. Total length of marginal hook sickle. – 14. Proximal width of sickle. – 15. Distal width of sickle. - 16. Length of toe. – 17. Length of heel. – 18. Aperture between apex of sickle point and the most distal part of the toe. – 19. Length of sickle shaft. (The area of a marginal hook sickle was measured by detection at the image analysis).

2.4. Descriptions of species

Family GYRODACTYLIDAE

Genus *Gyrodactylus* Nordmann, 1832

Subgenus *G.* (*Mesonephrotus*) Malmberg, 1964

***G. branchialis* sp.n.** (Figs 3, 5E, 6E, 7E)

Recorded as *G.* sp. by Geets and Ollevier (1999).

Holotype: BELGIUM, Ostend, (Swedish Museum of Natural History, SMNH, Acc. No. 5834); Table 1.

Paratypes: FRANCE, Ambleteuse, THE NETHERLANDS, Texel, (Swedish Museum of Natural History, SMNH, Acc. No. 5835-5845); Table 1.

Type-host: *Pomatoschistus microps* Krøyer, 1838.

Site: Gills/Gill filaments.

Specimens studied: Totally 11; digitally recorded 11: drawn 4; measured 11 (marginal hooks 24); DNA-analysed 8. For specimens used for molecular analysis and morphological analyses, see also Table 1.

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

Diagnosis

Molecular diagnosis. PCR amplified internal transcribed spacers (ITS) 1 and 2 and 5.8S gene sequences have been submitted to the EMBL nucleotide database under accession number x. Genetic distance between *G. branchialis* and *G. gondae*, *G. flavescensis* and *G. arcuatoides* amounts to 0.9%, 1.6% and 1.6% respectively (ITS and V4 region; uncorrected p-distances). No intraspecific differences were found. The phylogenetic position is visualised in Fig. 4.

Morphological diagnosis. Marginal hooks longer than the anchors. Marginal hook sickle of a similar length as in *G. flavesensis* and *G. arcuatoides* but its proximal end not as wide; the heel longer and pointing more anteriorly, the toe is blunt with a distinct knee and ventrally pointed. The sickle point extends beyond the toe. Marginal handle longer than in *G. gondae*, *G. flavescensis* and *G. arcuatoides*, but of similar length as that in *G. arcuatus*. Ventral bar small with small processes pointing laterally, and a triangular membrane. Anchors small and of another type as that in the *G. arcuatus*-group with curved anchor shafts and diverging anchor roots. Dorsal bar may have a median notch. Protonephridial system with small bladders.

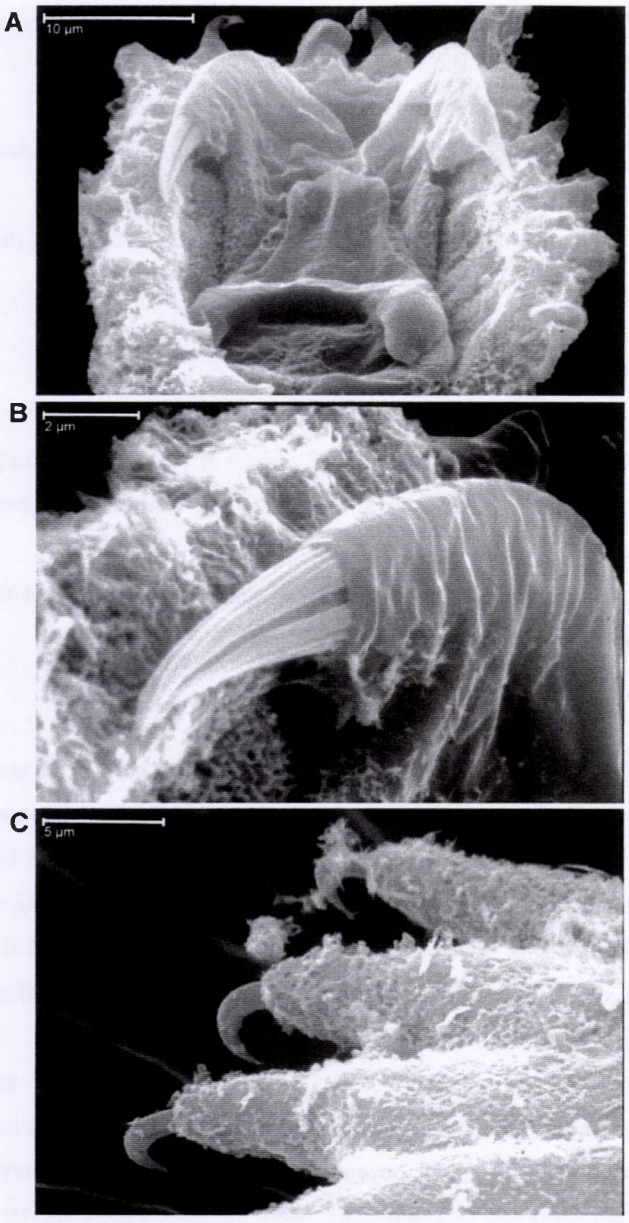

Fig. 2. SEM micrographs of *Gyrodactylus gondae*: (A) ventral view of the opisthaptor showing the ventral bar and the posterior part of two anchors. The ventral bar, the ventral bar membrane and the anchors, except for the outermost part of the anchor points are covered by the anchor membrane. One of the ventral bar processes and a furrow in one of the anchor points (left) are visible. (B) One of the anchor points in Fig. 2, showing the outermost part of the anchor point protruding from the anchor membrane. The lateral furrow of one side of the anchor point is seen. (C) Dorsal view of three of the 16 fingers of the haptor. From each finger the marginal hook sickle point is protruding. In the sickle point (middle) a lateral furrow is visible. Scale bars: - A. 10 µm. – B. 2 µm. – C. 5 µm.

G. arcuatus-group Malmberg, 1970

***G. gondae* sp. n.** (Figs 2, 3, 5B, 6B1,B2, 7B)

Recorded as *G.* sp. 1 by Geets and Ollevier (1999) and Zietara et al. (2002).

Holotype: THE NETHERLANDS, Texel, (Swedish Museum of Natural History, SMNH, Acc. No. 5782); Table 1.

Paratypes: BELGIUM, continental shelf, NORWAY, Trondheim and Bergen (SMNH Acc. No. 5783-57-97); Table 1.

Type-host: *Pomatoschistus minutus* Pallas, 1770.

Other host: *P. lozanoi* de Buen, 1923.

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

Specimens studied: Totally 22; digitally recorded 22; drawn 3; measured 22 (marginal hooks 38); DNA-analysed 2. For specimens used for molecular analysis and morphological analyses, see also Table 1.

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

Diagnosis

Molecular diagnosis. PCR amplified internal transcribed spacers (ITS) 1 and 2 and 5.8S gene sequences were previously obtained of specimens from the Belgian Continental Shelf (Zietara et al., 2002, EMBL accession number AF328866 previously named *G.* sp. North Sea). Additional specimens sequenced see Table 1. Genetic distance between *G. gondae* and *G. branchialis*, *G. flavescensis* and *G. arcuatoides* amounts to 1.3%, 1.1% and 0.9% respectively (ITS and V4 region; uncorrected p-distances). No intraspecific differences were found. The phylogenetic position is visualised in Fig. 4.

Morphological diagnosis. Marginal hook sickle shaft shorter than in *G. arcuatus*, *G. flavescensis* and *G. arcuatoides*, and its proximal part (toe) less prominent than in these three species. Its distal part (point) extending beyond the toe. Dorsal proximal part (heel) less prominent than in *G. arcuatus* and *G. branchialis*. Marginal hook handle shorter than in the latter two species. Ventral bar shape similar to that in *G. arcuatus* with laterally pointing processes, not so broad as in *G. flavescensis* and *G. arcuatoides*. Anchor roots not slightly diverging laterally from the median line as in *G. arcuatus* but slightly curved to the median line as in *G. flavescensis* and *G. arcuatoides*. Anchor point longer than in *G. arcuatus*. Dorsal bar may have a median notch. Protonephridial system with small bladders.

Note: The anchor point has a ribbed structure and on each lateral side there is a furrow (Fig. 2B). On each lateral side of the marginal hook sickle point there is a furrow (Fig. 2C).

***G. flavescensis* sp.n.** (Figs 3, 5C, 6C, 7C)

Holotype: NORWAY, Bergen, (Swedish Museum of Natural History, SMNH, Acc. No5798); Table 1.

Paratypes: Same data as for holotype, (SMNH Acc. No. 5799-5809).

Type-host: *Gobiusculus flavescens* Fabricius, 1779.

Site: Gill arches, gill filaments, in high infections also on pelvic and pectoral fins.

Specimens studied: Totally 13; digitally recorded 13: drawn 5; measured 13 (marginal hooks 21); DNA-analysed 9. For specimens used for molecular analysis and morphological analyses, see also Table 1.

Etymology: The name *G. flavescensis* refers to the Latin name of the host.

Diagnosis

Molecular diagnosis. PCR amplified internal transcribed specers (ITS) 1 and 2 and 5.8S gene sequences have been submitted to the EMBL nucleotide database under accession number x. Genetic distance between *G. flavescensis* and *G. gondae*, *G. branchialis* and *G. arcuatoides* amounts to 1.1%, 1.8% and 1.6% respectively (ITS and V4 region; uncorrected p-distances). No intraspecific differences were found. The phylogenetic position is visualised in Fig. 4.

Morphological diagnosis. Marginal hook sickle shaft longer and its proximal part (toe) more prominent than in *G. gondae*, and with a distinct knee, more prominent than in *G. gondae* and *G. arcuatoides*. Its distal part (point) extending beyond the toe. Dorsal proximal part (heel) less prominent than in *G. arcuatus* and *G. branchialis*. Marginal hook handle shorter than in the latter two species. Ventral bar broad with broad processes of a similar shape as in *G. arcuatoides*. The processes pointing more anteriorly than in *G. arcuatus* and *G. gondae*. Anchors more robust than in *G. gondae* and *G. arcuatoides*. Anchor roots slightly curved to the median line as in the latter two species. Protonephridial system with small bladders.

***G.arcuatoides* sp.n.** (Figs 3, 5D, 6D, 7D)

Recorded as *G.* sp. 2 by Geets and Ollevier (1999).

Holotype: NORWAY, Bergen, (Swedish Museum of Natural History, SMNH, Acc. No 5810); Table 1.

Paratypes: Same data as for holotype, (SMNH Acc. No 5811-5833).

Type-host: *Pomatoschistus pictus* Malm, 1865.

Site: Gill arches, gill filaments, in high infections also on pelvic and pectoral fins.

Specimens studied: Totally 25; digitally recorded 25: drawn 9; measured 25 (marginal hooks 36); DNA-analysed 4. For specimens used for molecular analysis and morphological analyses, see also Table 1.

Etymology: The species is given the name *G. arcuatoides* because its opisthaptoral hard parts remind of those of *G. arcuatus*.

Diagnosis

Molecular diagnosis. PCR amplified internal transcribed spacers (ITS) 1 and 2 and 5.8S gene sequences have been submitted to the EMBL nucleotide database under accession number x. Genetic distance between *G. arcuatoides* and *G. gondae*, *G. flavescensis* and *G. branchialis* amounts to 0.9%, 1.6% and 0.7% respectively (ITS and V4 region; uncorrected p-distances). Specimens collected in the North Sea (Belgium) and in Bergen (Norway) showed two substitutions in the ITS region; the V4 region was identical. The phylogenetic position is visualised in Fig. 4.

Morphological diagnosis. Marginal hook sickle shaft longer and its proximal part (toe) more prominent than in *G. gondae*. Its distal part (point) extending beyond the toe. Dorsal proximal part (heel) less prominent than in *G. arcuatus* and *G. branchialis*. Marginal hook handle shorter than in the latter two species. Ventral bar smaller than that of *G. flavescensis* but with processes of a similar shape and pointing more anteriorly than in *G. arcuatus* and *G. gondae*. Anchors similar to those in *G. gondae* with anchor roots slightly curved to the median line as in the latter species and in *G. flavescensis*. Dorsal bar may have a median notch. Protonephridial system with small bladders.

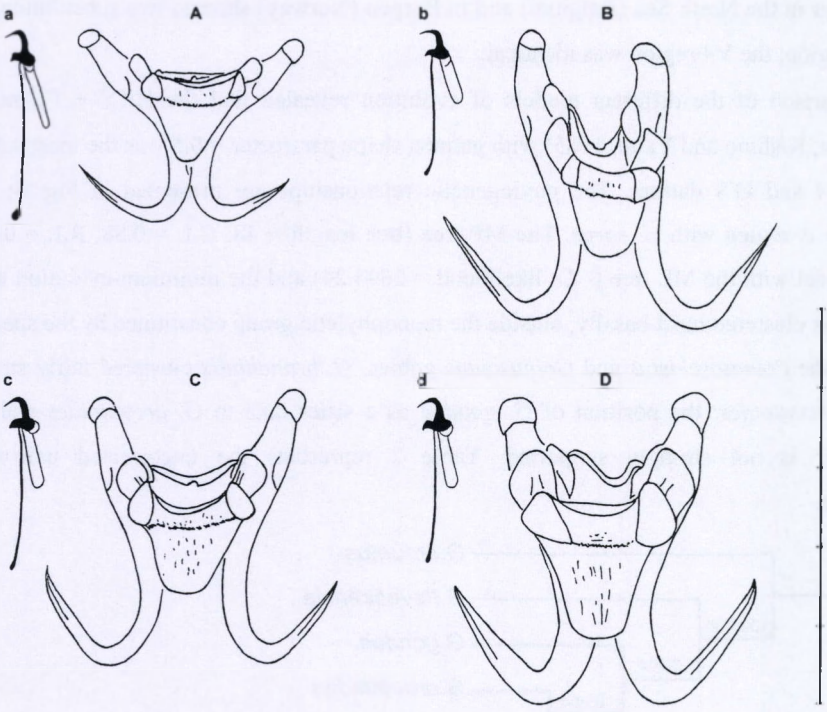

Fig. 3. Opisthaptoral hard parts (lower case: marginal hook; upper case: anchors, dorsal and ventral bars) of the holotypes of four *Gyrodactylus* species: (A, a) *G. branchialis* sp.n. from Ostend, Belgium 18/08/99; (B, b) *G. gondae* sp.n. from Texel, The Netherlands 25/11/00; (C, c) *G. flavescensis* sp.n. from Bergen, Norway 21/05/01; (D, d) *G. arcuatoides* sp.n. from Bergen, Norway 21/5/01. Scale bar = 50 µm.

3. Results

3.1. 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). The 5.8S gene was identical for all species, with the exception of *G. gondae* that showed one unique substitution. Since this gene is not informative at the subgenus level (Zietara et al., 2002), it has been excluded for 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 those species ranges from 0.7-1.8% (ITS: 0.5 – 1.6%). *Gyrodactylus arcuatus* sampled on *P. microps* from Bergen showed two transitions, one transversion and one deletion event in the complete ITS region when compared to *G. arcuatus* from *Gasterosteus aculeatus* from Stockholm (V4 region not compared). Specimens collected from *G.*

arcuatoides in the North Sea (Belgium) and in Bergen (Norway) showed two substitutions in the ITS region; the V4 region was identical.

Comparison of the different models of evolution revealed that the HKY + Γ_4 model (Hasegawa, Kishino and Yano, 1985) with gamma shape parameter = 0.5 was the most suited for the V4 and ITS dataset. The phylogenetic relationships are presented in Fig. 4; 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, outside the monophyletic group constituted by the species found on the *Pomatoschistus* and *Gobiusculus* gobies. *G. branchialis* clustered fairly strong with *G. arcuatoides*; the position of *G. gondae* as a sister taxa to *G. arcuatoides* and *G. branchialis* is not strongly supported. Table 2 represents the uncorrected pair-wise differences.

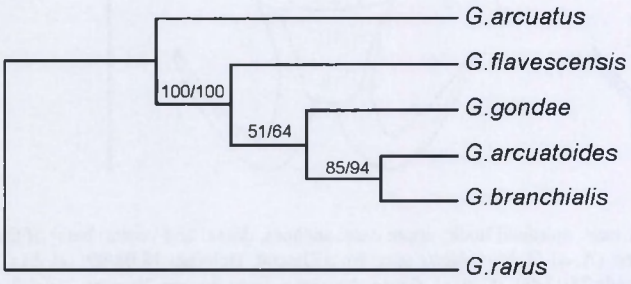

Fig. 4. Maximum likelihood cladogram constructed on the ssrRNA V4 region and complete ITS region (1124 bp) of six *Gyrodactylus* species. Bootstrap support (1000 replicates) is presented for the maximum parsimony/minimum evolution analyses.

Table 2. Uncorrected pair-wise distances calculated from the sequences of the ITS and V4 region of the *Gyrodactylus* spp. 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* spp are shown in the lower left triangle.

	G. arcuatoides	*G. flavescensis*	*G. branchialis*	*G. gondae*	*G. arcuatus*
G. arcuatoides	0.00	1.6	0.7	0.9	11.0
G. flavescensis	6.48	0.00	1.8	1.1	11.2
G. branchialis	23.49	42.16	0.00	1.3	11.0
G. gondae	10.36	7.21	59.71	0.00	10.9
G. arcuatus	7.55	16.91	22.71	25.40	0.00

3.2. Microscopical results

The differences between. *G. arcuatus*, *G. gondae*, *G. flavescensis* and *G. arcuatoides* (*G. arcuatus*-group) are small. Furthermore, the size of their marginal hook sickles is among the smallest (less than 2.5 µm) described in *Gyrodactylus* (eg. Malmberg, 1970). In order to reveal such small differences and to exclude the presence of intraspecific variations, a large number of specimens of each species had to be analysed. This problem was solved by using a digital camara connected to a rapid archiving system. Comparative digital photographs of the marginal hooks of the new species and of *G. arcuatus* are shown in Fig. 5. Comparative drawings of the marginal hooks, ventral bars and anchors of these species are presented in Figs 6 and 7. Our method of measuring the anchors, ventral bar and the marginal hook sickles is presented in Fig. 1. Measurements of the opisthaptoral hard parts of the four species are presented in Table 3 (see appendix).

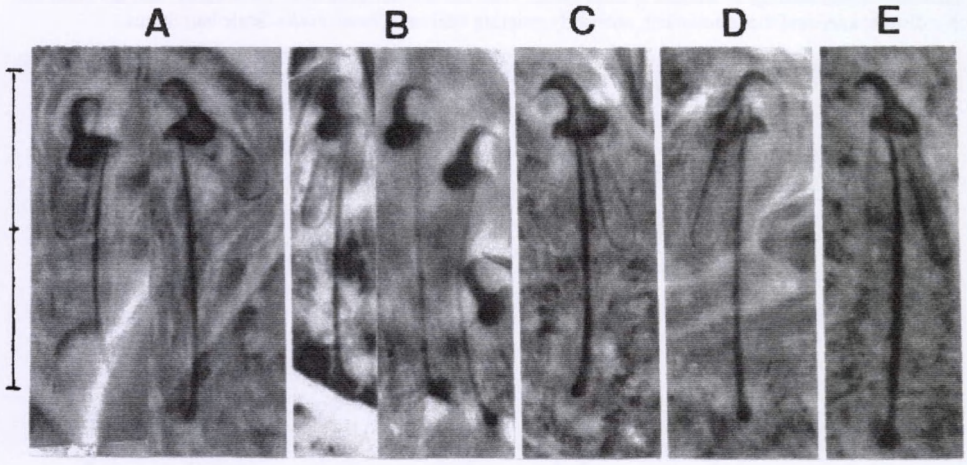

Fig 5. Comparative micrographs (phase contrast microscope) of marginal hooks of five *Gyrodactylus* species. **A**: *G. arcuatus* from a specimen from *Pomatoschistus microps* Bergen, 21/05/01 Norway. **B**: *G. gondae* , the holotype specimen, Fig. 3. **C**: *G. flavescensis*, the holotype specimen, Fig. 3. **D**: *G. arcuatoides*, the holotype specimen, Fig. 3. **E**: *G. branchialis*, the holotype specimen, Fig. 3. Scale bar 2µm.

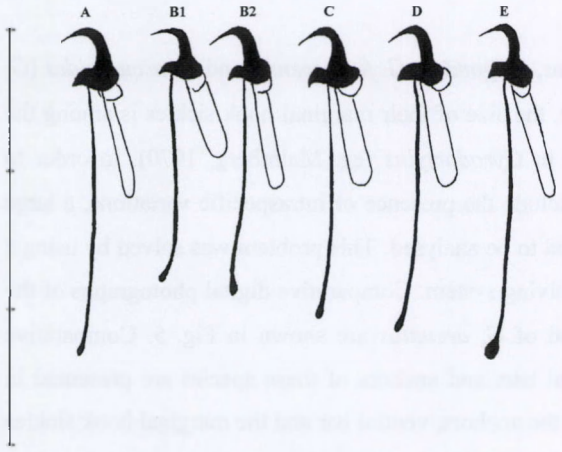

Fig. 6. Comparative drawings of marginal hooks of **A**: *G. arcuatus*, the same specimen as in Fig. 5 A; **B1** and **B2**: *G. gondae*, the holotype specimen, Fig. 3; **C.** *G. flavescensis*, the holotype specimen, Fig. 3; **D**. *G. arcuatoides*, the holotype specimens, Fig. 3; **E.** *G. branchialis*, the holotype specimen, Fig. 3. The species A – D belong to the *G. arcuatus*-group and have marginal hook sickles of a similar shape, different to that of *G. branchialis*, which belongs to another species group. Note the distinct knee in *G. flavescensis* and the blunt toe with a distinct knee and the prominnent, anteriorly pointing heel in *G. branchialis*. Scale bar: 30 μm.

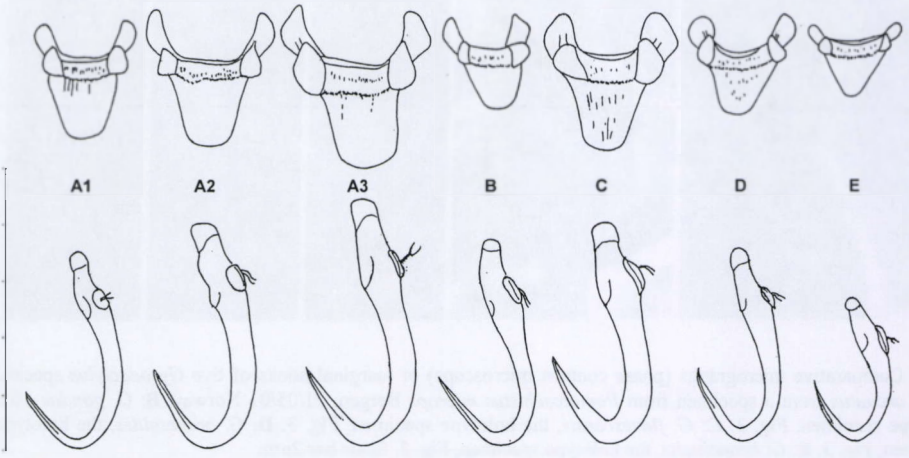

Fig. 7. Comparative drawings of ventral bars and anchors of specimens of the *G. arcuatus*-group (**A-D**) and a specimen of *G.branchialis* (**E**). **A1-A3**: *G. arcuatus* from *Gasterosteus aculeatus*, brackish water, Stockholm Arcipelago, Sweden: **A1** and **A2** (after Malmberg, 1964; Fig. 17) **A1** from a 1.35 cm long specimen, Nämdö; **A2** from a 6 cm long specimen, Nämdö; A3 from brackish water, Edesö, Stockholm Archipelago, Sweden. **B**: *G. gondae*, the same specimen as in Fig. 3 and 6 B1, B2. **C**: *G. flavescensis*, the same specimen as in Fig. 3 and 6 C; D. *G. arcuatoides*, the same specimen as in Fig. 3 and 6 D. **E**: *G.branchialis*, the same specimen as in Fig. 3 and 6 E. Note the small size of ventral bar and anchor and the different shape of the anchor in *G. branchialis* compared to the the shape of ventral bars and anchors in members of the *G. arcuatus*-group. Scale bar: 50 μm.

The slides for the present study were checked for specimens with a cirrus (Fig. 8), specimens with a cirrus and an embryo and specimens with only an embryo in the uterus (Table 4). The slides represent different populations.

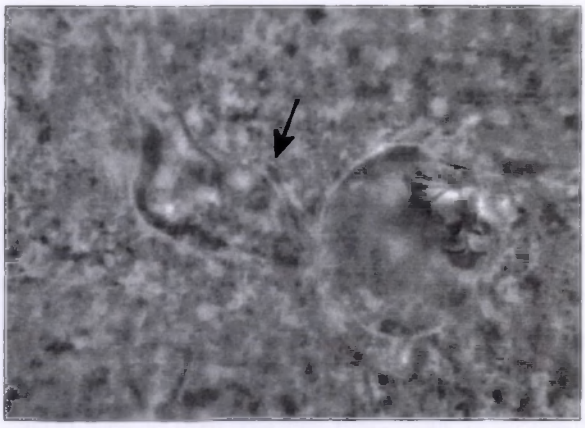

Fig. 8. Cirrus and vesicula seminalis (arrow) with sperms in a specimen of *G. gondae* (Pin.2 vht, ga, Bergen 23/6-00) from Bergen, Norway (23. 06. 2000).

Table 4. Slide specimens with a cirrus, a cirrus and an embryo or only an embryo.

Gyrodactylus spp.	Number studied	cirrus	cirrus + embryo	embryo
G. branchialis	12	0	0	3
G. gondae	22	5	8	6
G. flavescensis	13	1	0	7
G. arcuatoides	22	3	3	1

3.3. Morphometric and statistical analyses

For each specimen two marginal hook sickles were measured. The C.V. values of the marginal hook features were fairly low, with minor exceptions for the marginal hook toe length and the length of the marginal hook filament loop, which were excluded from further analyses. Thus in total, seven out of the nine measured marginal hook features have been used in the statistical analyses (see Fig. 1).

A factor analysis included these 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 marginal hook sickle and the length of the marginal hook heel, with factor loadings exceeding 0.70. The second factor is mainly determined by the length of the

marginal hook sickle and the distance of the marginal hook sickle aperture. The backward and forward discriminant analysis revealed that mainly 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 were responsible for the separation among species. This was confirmed by Tukey's honest significant difference test for unequal sample sizes, which found significant interspecific differences for these features. Fig. 9 shows a plot of the discriminant analysis. Specimens of *G. gondae* were sampled in Bergen in spring and in Texel during the winter. Also two populations of *G. arcuatoides* were sampled (see Table 1). The analyses were run both with and without the inclusion of these populations representing seasonal variation. The population of *G. gondae* from Texel was included as an example of seasonal and geographic variation.

The classification matrix misclassified *G. flavescens* once as *G. arcuatoides*, and it misclassified *G. arcuatoides* once as *G. flavescens* and once as *G. gondae*. Based on the F-values and the squared Mahalanobis distances, *G. branchialis* appeared most distinct, being most distantly related to *G. gondae*. The smallest distances (all significant at $p < 0.01$) were found between *G. gondae* and *G. flavescensis*, and *G. arcuatus* and *G. acrcuatoides* respectively (see Table 2).

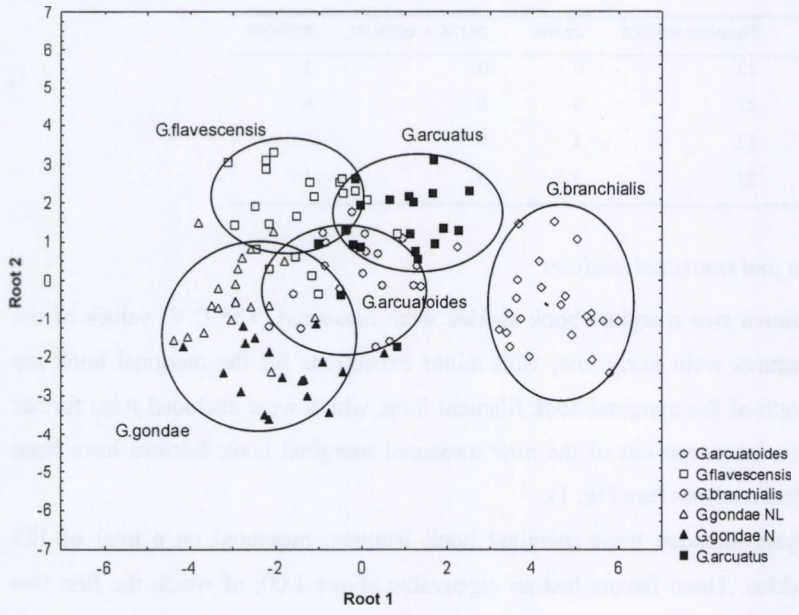

Fig. 9. Plot of standard discriminant analysis (root 1 vs. root 2) on morphological measurements on 123 marginal hook sickles of the *Gyrodactylus* spp. For *G. gondae*, specimens of The Netherlands (NL) and Norway (N) are included to visualize the geographic and seasonal variation.

The anchor and ventral bar features were measured for approximately 10-23 specimens per species. These numbers are rather low for proper statistical analyses, but the aim of this study was to reveal whether a trend was found similar to Geets et al. (1999). Most features had a C.V. value below 12%. However, the ventral bar membrane was hardly visible in most specimens when using a common light microscope, corresponding with high C.V. values for all species. Therefore, this feature is unlikely to produce useful information. Also, the median width of the ventral bar was relatively variable in *G. flavescensis* and *G. gondae*, which is reflected by their fairly high C.V. values. All measured features (see Fig. 1) have been used in the statistical analyses, except for the features of the ventral bar membrane. The standard and forward discriminant analyses showed that all anchor features and the median width of the ventral bar contributed significantly to the observed differences. Backward discriminant analyses selected the length of the anchor point as most contributing factor. The species were never misclassified amongst each other. Based on the F-values and the squared Mahalanobis distances, *G. branchialis* appeared most distinct; the highest distance was found in comparison with *G. gondae*. The smallest distances (all significant at $p < 0.01$) were found between *G. gondae* and *G. flavescensis* followed by the distance between *G. gondae* and *G. arcuatoides*.

4. Discussion

4.1. Morphological analysis

Although *G. arcuatus* is species-specific to the three-spined stickleback, *Gasterosteus aculeatus*, this species may be found on a number of other fish species during spring (Malmberg 1970). Thus when specimens of *Gyrodactylus*, very similar to *G. arcuatus* were found on *Pomatoschistus minutus* and *P. lozanoi*, the specimens were initially supposed to belong to *G. arcuatus*. The life-cycle and habitat of the two *Pomatoschistus* species compared to that of *Gasterosteus aculeatus,* however, indicated a different *Gyrodactylus* species. Further biological/parasitological analyses and morphometric analyses, however, proved the *Gyrodactylus* specimens to belong to an undescribed species, initially named *G.* cf. *arcuatus* or *G.* sp.1 (Geets 1998). Furthermore, preliminary data on the genetic variability of *G.* sp1 from *P. minutus* and *P. lozanoi* confirmed the presence of one and the same *Gyrodactylus* species (Geets et. al., 1999). Renewed morphological analyses by means of image analysis, revealed clear differences between specimens of *G. arcuatus* and *G.* sp. 1,

regarding the marginal hook sickles (Figure 6), ventral bars and anchors (Fig. 7). In this paper, *G.* sp. 1 is morphologically and genetically described as *G. gondae* sp. n.

Two more species presented in Geets et al. (1999) has here been characterized genetically, namely *G.* sp. 2 and *G.* sp., and described as *G. arcuatoides* sp. n. and *G. branchialis* sp. n. respectively. It could be supposed that differences in the size of the marginal hooks, ventral bars and anchors in *G. gondae* and *G. arcuatoides* might depend on intraspecific variations, similar to what has been described for these opisthaptoral hard parts in *G. arcuatus* (see Malmberg 1964; Fig. 7). The differences in the shape of marginal hooks, anchors and the ventral bar processes, however, indicate two different species (Fig. 3). This is also valid to *G. flavescensis*, found on *Gobiusculus flavescens*.

Small and/or reduced opisthaptoral hard parts characterize many gill living *Gyrodactylus* species (Malmberg, 1970). *G. branchialis* is a gill species and its ventral bar and anchors are comparatively small, but not reduced (Fig. 3). This may imply that *G. branchialis* is more recently adapted to a gill environment than species with reduced opisthaptoral parts.

The shape of the marginal hooks, the ventral bars and the anchors are of the same type in *G. arcuatus*, *G. gondae*, *G. flavescensis* and *G. arcuatoides*. Thus they belong to the same species group, i.e. the *G. arcuatus*-group (Malmberg 1970). This, however, is not valid to *G. branchialis*. Especially the shape of the marginal hook sickles with a blunt toe and the curved anchors clearly show that it belongs to another species group. From a genetical point of view, however, *G. branchialis* seems to be closely related to *G. arcuatoides*. In turn, genetically *G. arcuatus* and *G .gondae* clearly are two distant species, but morphologically they are easily confused.

The special egg cleavage in *Gyrodactylus* can cause that a large number of specimens in a population originate from the same fertilized egg. Such a population represents a clone. In the gyrodactylid life cycle, however, also sexual reproduction is present. At a certain stage, a fully developed male apparatus is present, with a testis connected to a cirrus (penis) via a vas deferens and a vesicula seminalis, often filled with sperm cells. Such specimens indicate sexually activity. Copulations and sperm injection by means of the cirrus is observed and described. Live sperm cells in the receptaculus seminis (behind the uterus and often with a large egg, which later on will be moved into the uterus) are most likely the result of copulations between specimens and indicate a potential genetic exchange between specimens. Checking of slides of the present material (Table 4) revealed a cirrus in five out of 22 *G. gondae* specimens, in one out of 13 *G. flavescensis* specimens and in three out of 22 *G. arcuatoides* specimens. A cirrus and a vesicula seminalis with sperm was also present in *G.*

arcuatus specimens studied. Thus most likely at least in the life cycle of these species, sexual reproduction is included. The expressed host specificity of the species, however, most likely imposes an effective barrier to copulation between the species.

4.2. Molecular analyses

The four new species found on *Gobiusculus* and *Pomatoschistus* spp. clustered strongly together as a monophyletic group. They were clearly separated from *G. arcuatus*, which differed about 13%. The genetic differentiation (0.7 – 1.8%, uncorrected p-distances constructed on the complete dataset) is very low compared to differentiation in *Gyrodactylus* reported in literature (2.7-56% and 1.5-38.7% for ITS1 and ITS2, Kimura distances from Matejusova et al., 2001), but it is about of the same magnitude as described by Huyse and Volckaert (2002) for *G. rugiensis* and *G. rugiensoides* (1.8 and 1.5%, respectively). The latter two species are mainly found on the fins of *P. microps* and *P. minutus*, *P. lozanoi* and *P. pictus*, respectively. The present study and the study by Huyse and Volckaert (2002) are the first papers describing the molecular variation of *Gyrodactylus* species found on closely related fish hosts belonging to the same genus[1].

It is known that the ITS region can vary greatly among species but is accepted as a species diagnostic marker for *Gyrodactylus* spp. since it shows very low intraspecific and geographic variation (Zietara and Lumme, in press). In the present study, only *G. arcuatus* and *G. arcuatoides* showed low geographic variation (in the ITS region) between specimens collected in the Belgian and Norwegian part of the North Sea. The highest geographic variation described in literature, has been found for *G. arcuatus* (Zietara et al. 2000). Freshwater, brackish, and marine isolates from Overpelt (Belgium), Gdansk (Poland), Doel (Belgium) and Aberdeen (Scotland) showed 8 variable sites in the complete ITS region (3 transitions, two transversions and 3 heterozygous sites). In contrast, the ITS sequences of *G. anguillae* collected from *Anguilla anguilla* of Spain and Australia, *A. australis*, *A. reinhardtii* and *A. rostrata* all appeared identical (Hayward et al. 2001).

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 a distinct species. Recently, Zietara and Lumme (in press) suggested a threshold of 1% variation in the ITS region. However, it is obvious that a single rule cannot be applied on all species of *Gyrodactylus* since the

[1] : *G. flavescens* is placed in another genus, however it is shown that it clusters strongly within *Pomatoschistus* based on ITS1 rDNA and 12S and 16S mtDNA (pers. data).

evolutionary rate can differ even amongst closely related species (e.g. Huyse et al., 2002). Additional morphological information has to be taken into consideration as well. The choice of marker is another important issue. For example, although *G. thymalli* is described as a distinct species, based on morphological, ethological and pathological grounds (Sterud et al., 2002), no variation could be found throughout the ITS region when compared with *G. salaris*. Recently however, genetic differentiation between both species has been found in the COI mtDNA region (3.4%; Meinila et al., 2002). According to Milinkovitch et al. (2000), co-variation between *a priori* morphological designations and a minimum of one molecular character should provide a valid basis for biological species recognition.

4.3. Morphometric analyses

An elaborate morphometric analysis was carried out by Geets et al. (1999), on 17 hook characters of *G. arcuatus*, *G. gondae* (or *G.*sp1, above) and *G. arcuatoides* (or *G.* sp. 2, above). Specimens were collected in different seasons and a total of 268 specimens were analysed. The authors could show both seasonal and species-specific variation by means of multivariate analyses. In this study, two more species, *G. flavescensis* and *G. branchialis* were included in the analysis, with special attention to the marginal hook features as a discriminating tool (see above). Based on seven out 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. Similar results were obtained by multivariate analyses on anchor and ventral bar features, although the discriminating power was lower. In agreement with Geets et al. (1999), the anchor features were more informative than the ventral bar features. High variation and low visibility of the ventral bar features when using light microscopy might explain their low contribution in species discrimination.

The morphometric resemblance between the species was not always reflected in the genetic distances. This is illustrated by the contrasting distances produced by morphometric measurements on the one hand, and the genetic distances on the other hand (Table 2). For example, *G. arcuatus* and *G. arcuatoides* are very similar to each other with respect to the morphometry of the marginal hook features, but genetically they differ in 11% (ITS and V4 region). In contrast, *G. branchialis* is most distinct in its morphology compared to the other

here-described species, but it differs only 0.7% with e.g. *G. arcuatoides*. Thus molecular and phenotypic evolution were not always associated.

The here-described *Gyrodactylus* species found on the gills of *Pomatoschistus* and *Gobiusculus* spp. might be a result of host-switching events from *Gasterosteus aculeatus*, the three-spined stickleback onto the three gobiids, causing the evolution of *G. gondae*, *G. arcuatoides* and *G. flavescensis*, all members of the *G. arcuatus*-group. Initially host-switching between the gobiids may have been involved, followed by co-evolution between invaded *G. arcuatus* specimens and their "new" gobiid host. Sampling and comparison of *Gyrodactylus* spp. found on other sympatric fish species might help to clarify the evolutionary history of the present *Gyrodactylus* species.

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Appendix

Table 3. Mean, range and C.V. (C.V. = 100 x the square root of the variance divided by the mean) of characters of the opisthaptoral hard parts of *G. branchialis* sp.n., *G. gondae* sp.n, *G. flavescensis* sp.n and *G. arcuatoides* sp.n. All measurements are in µm. N = number of parasite specimens measured.

Gyrodactylus species		*G. branchialis*	*G. gondae*	*G. flavescensis*	*G. arcuatoides*
Host Species		*P.microps*	*P. lozanoi/P. minutus*	*G. flavescens*	*P. pictus*
N		12	13	12	11
Anchors					
Length of anchor point	mean	12.40 (11.08-13,51)	17.72 (16.45-19.78)	18.83 (17.40-20.59)	15.66 (14.26-18.25)
	C.V.	6.54	5.97	5.54	8.54
Total length of anchor	mean	24.56 (22.94-25.78)	36.71 (33.63-41.44)	37.53 (34.09-39.27)	33.83 (31.31-40.12)
	C.V.	3.78	7.30	3.78	7.02
Length of anchor shaft	mean	18.80 (16.93-20.89)	28.13 (25.98-31.50)	29.57 (27.57-31.64)	25.74 (23.45-31.08)
	C.V.	6.23	6.72	4.24	8.44
Length of anchor root	mean	8.83 (6.42-10.33)	9.90 (8.61-11.71)	10.27 (9.01-11.05)	10.23 (8.74-11.62)
	C.V.	11.48	9.64	6.04	8.69
Ventral bar					
Length of ventral bar + processes	mean	16.57 (15.09-18.34)	18.67 (16.90-21.85)	19.71 (17.94-21.90)	19.76 (18.25-21.49)
	C.V.	5.99	7.51	7.61	5.89
Length of ventral bar	mean	12.77 (11.97-14.28)	15.58 (14.16-18.13)	16.61 (14.20-18.38)	16.20 (14.82-17.62)
	C.V.	5.29	8.53	9.83	6.30
Basal width of ventral bar	mean	4.01 (3.36-4.39)	5.49 (4.79 6.42)	5.77 (5.49-6.39)	4.91 (4.36-5.51)
	C.V.	7.99	10.62	6.40	8.66
Median width of ventral bar	mean	2.98 (2.53-3.54)	3.79 (3.18-4.54)	4.83 (3.92-5.89)	4.08 (3.66-4.74)
	C.V.	9.52	11.27	13.13	9.87
Length of ventral bar membrane	Mean	9.30 (7.68-10.38)	13.20 (10.96-14.85)	15.00 (13.93-16.05)	13.13 (12.19-14.78)
	C.V.	11.23	11.91	5.41	6.12
Total length of ventral bar membrane	Mean	6.53 (5.41-7.51)	9.57 (7.93-10.82)	10.08 (8.69-11.28)	8.90 (8.26-10.90)
	C.V.	11.88	11.99	8.41	9.54
Marginal hook					
Total length of marginal hook	mean	24.21 (22.56-25.39)	19.63 (18.67-20.36)	21.28 (18.59-23.16)	21.53 (20.48-22.41)
	C.V.	4.16	2.35	6.42	3.12
Length of marginal hook handle	mean	20.24 (18.56-21.53)	15.97 (14.63-17.91)	17.26 (15.09-18.77)	17.44 (16.61-18.24)
	C.V.	5.26	3.05	6.65	3.31
Length of marginal hook sickle	mean	4.50 (4.10- 5.20)	4.19 (3.83-4.47)	4.59 (4.09-5.00)	4.50 (4.24-4.76)
	C.V.	6.72	3.92	5.36	3.77
Proximal width of marginal hook sickle	mean	2.82 (2.36-3.15)	3.44 (3.14-3.87)	3.51 (3.03-4.20)	3.33 (2.99-3.66)

	C.V.	8.08	6.31	7.26	6.12
Distal width of marginal hook sickle	mean	3.38 (3.02-3.85)	3.52 (3.17-3.84)	3.83 (3.41-4.18)	3.49 (3.08-3.91)
	C.V.	6.33	5.32	5.36	6.73
Marginal hook toe length	mean	0.94 (0.73-1.18)	1.27 (0.91-1.64)	1.21 (0.85-1.44)	1.28 (1.10-1.64)
	C.V.	11.83	13.48	12.51	13.25
Length of marginal hook heel	mean	1.80 (1.38-2.18)	2.19 (1.83-2.50)	2.37 (1.95-2.77)	2.10 (1.73-2.35)
	C.V.	11.32	8.30	9.14	7.54
Marginal hook sickle aperture distance	mean	2.22 (2.03-2.59)	1.96 (1.66-2.27)	2.05 (1.74-2.76)	2.16 (1.87-2.56)
	C.V.	6.40	8.62	10.72	7.83
Length of marginal hook filament loop	mean	7.05 (5.08-9.70)	7.45 (6.32-9.68)	7.86 (6.26-9.76)	7.30 (5.99-8.40)
	C.V.	17.92	11.02	10.72	9.96
Area of marginal hook	mean	6.50 (4.87-7.78)	7.85 (7.06-9.50)	8.72 (6.79-11.68)	7.72 (6.27-9.13)
	C.V.	11.70	8.03	11.91	9.99

CHAPTER 5

FIRST REPORT ON THE OCCURRENCE OF *GYRODACTYLUS* (MONOGENEA, PLATYHELMINTHES) IN THE MEDITERRANEAN SEA: MORPHOMETRIC AND MOLECULAR INVESTIGATIONS

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Abstract: Until now, investigation of gyrodactylid ectoparasites has been mainly restricted to temperate northern regions, where, in Norwegian rivers, the pathogenic *G. salaris* causes major losses among salmon parr. Here we describe the *Gyrodactylus* fauna of gobies (the genera *Pomatoschistus* and *Knipowitschia*) from the Mediterranean Sea. Infection intensities sometimes exceeded 200 *Gyrodactylus* specimens per host specimen. A morphometric comparison between this fauna with populations collected in the North Sea showed that they were strikingly similar. Moreover, almost no geographical differentiation was found throughout the complete ITS region: one species showed three substitutions whereas the other three appeared identical. Hence, *Gyrodactylus* spp separated by a shoreline of approximate 7000 kilometres are essentially the same.

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1. Introduction

The genus *Gyrodactylus* is one of the most species rich groups within the Monogenea (Brooks and McLennan, 1993). The estimated species number, more than 400 species, is based on a partial examination of their natural distribution area (Harris, 1993). Consequently, expanding the sampling range would imply a further increase of this number. To gain more insight in the processes triggering this enormous diversity, studies on *Gyrodactylus* throughout its complete natural habitat are needed. An example of such an "unexplored" region seems to be the Mediterranean Sea. So far, we found no records of *Gyrodactylus* infecting marine fish species. This study shows the first results of morphological and molecular investigations on the Mediterranean *Gyrodactylus* fauna on gobies from the genus *Pomatoschistus* Gill, 1864 and *Knipowitschia* Iljin, 1927. These fish species are among the most abundant along the Eastern Atlantic and Mediterranean coasts of Europe, playing a key role in the marine ecosystem (Wallis and Beardmore, 1984; Miller, 1986). Besides the traditional species discrimination based on the opisthaptoral hard parts, additional information is obtained by sequencing the complete ITS region.

2. Material and Methods

2.1. Collection of material

Sampling took place in the fall of 1998 and 1999. *Pomatoschistus microps, P. minutus* and *P. marmoratus* were collected in Sète lagoon, in Etang de l'Arnel and Vaccarès lagoon complex (France). Specimens of *P. marmoratus* and *K. panizzae* were also collected in Venice lagoon near Treporti and in the Po-Delta near Scardovari (Italy). Fish were brought alive to the laboratory and immediately screened for *Gyrodactylus*-infection using a stereomicroscope. The prevalence of each *Gyrodactylus* species was calculated as the ratio of the number of infected fish specimens to the total number of fish examined. The abundance was calculated as the total number of *Gyrodactylus* specimens per fish population (see Table 1). Whenever possible, the opisthaptor was separated from the body enabling simultaneous morphological and molecular analyses. The body was placed in 5 µl of milli-Q water and stored at -20°C, while the opisthaptor was fixed in ammonium picrate glycerin as described by Malmberg (1970), to examine the haptoral sclerites by phase contrast microscopy. Drawings were made with a drawing tube mounted on an Olympus microscope (oil immersion, 10x ocular).

2.2. Molecular analysis and phylogeny reconstruction

DNA extraction, ITS amplification and sequencing of individual parasites were performed as described by Zietara et al. (2002). Sequences were aligned with the Clustal X multiple sequence alignment program (version 1.81, Thompson et al., 1997). The obtained sequences have been submitted to the EMBL database under accession number X. Additional sequences were obtained from Genbank: *G. arcuatus* (AF328865), *G. branchicus* (AF156669), *G. pungitii* (AF328869) and *G. salaris* (AF328871). Regions with an ambiguous alignment were excluded from further analyses. Modeltest 3.06 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 5.8S and ITS2, we used maximum parsimony (MP), maximum likelihood (ML) and distance-based methods (PAUP* v. 4.01b, Swofford, 2001). 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 but different transition:transversion (ti/tv) ratios were applied (10:5 for 5.8S and 1:5 for ITS2). The ML analysis was performed using the parameters estimated under the best-fit model. The heuristic search method was applied and we bootstrapped (n=1000) with the tree-bisection-reconnection algorithm in force. With the minimum-evolution distance method, the distance matrix was calculated using the paralinear/LogDet distances.

2.3. Morphometric analysis

A total of 40 specimens from three *Gyrodactylus* spp. collected in the Mediterranean Sea were analysed. Seventeen hook characteristics were selected for morphometric analysis (Table 2). Measurements were done using a Zeiss HBO50 microscope (oil immersion, 10x ocular) and images were analysed with the program SigmaScan Pro 5. The morphological characters were described by their means and their range (Table 2). The obtained results were compared with morphometric data of 43 specimens of *G. rugiensis, G. gondae* and *G.* cf. *harengi* from the North Sea, which were obtained in previous studies (Huyse and Volckaert, 2002; Huyse et al., submitted). For all 83 specimens, the total marginal hook length was plotted against the total anchor length.

3. Results

3.1. Prevalence and host-specificity

In total, 344 fish specimens were examined among which 296 from France (the Mediterranean Sea) and 64 from Venice (the Adriatic Sea). Four different species of the genus *Gyrodactylus* were encountered. Table 1 summarizes the number of fish specimens examined and the number of specimens of each *Gyrodactylus* species sequenced. The prevalence ranged from 18 to 100% and the abundance ranged from 0.2 to > 66.7 *Gyrodactylus* specimens/fish. The highest abundance and prevalence was found in Venice lagoon for the fin parasite *G.* cf. *harengi*. In this area some very high infection levels (n > 200) were found. The lowest prevalence was found for *G. rugiensis* and *G. rugiensoides*. In the Vaccarès lagoon complex *G. rugiensis* and *G. branchialis*, normally living on fins and gills respectively, of *P. microps*, occasionally occurred on *P. minutus*, while in Venice lagoon a few individuals of *K. panizzae* were infected with *G.* cf. *harengi* which is a fin parasite of *P. marmoratus* and *P. microps* (Table 1). *Gyrodactylus branchialis* and *G.* cf. *harengi* seem to be euryhaline since they occur in localities with considerable differences in salinity (from around 10 ppm in the Vaccarès lagoon complex to 33 ppm in Venice lagoon).

3.2. Molecular and phylogenetic analyses

On the gills and fins of *P. microps* three *Gyrodactylus* species were found. The ITS region of *G. rugiensis* from the fins of *P. microps* collected in France was identical with the sequences obtained from specimens of the North Sea (AF328870, Zietara et al., 2002). Also the ITS region of the two other species was identical to the species found on gills and fins of *P. microps* collected in the North Sea, Belgium (Huyse et al., submitted). They will be hereafter referred to as *G. branchialis* and *G.* cf. *harengi* respectively, in accordance to Huyse et al. (in prep.). Both species were also found on the gills and fins of *P. marmoratus* collected in France and Venice; they differed in two substitutions in the ITS region. No difference in ITS region was found between *G.* cf. *harengi* found on *K. panizzae* and *P. marmoratus*. *Gyrodactylus rugiensoides* was only found on *P. minutus* caught in Sète. This species was originally described by Huyse and Volckaert (2002), infecting *P. minutus, P. lozanoi* and *P. pictus* in the eastern shores of the North Atlantic. The ITS region of both populations showed three substitutions.

Table 1. *Gyrodactylus* species found on *Pomatoschistus* and *Knipowitschia* gobies sampled in coastal areas of Belgium, France and Italy. nF = number of examined fish, nG = number of *Gyrodactylus* specimens, P= prevalence, nS = number of parasites sequenced (ITS1, 2 and 5.8S).

Gyrodactylus spp.	Host spp.	Locality	Date, water temperature, salinity	nF	P	nG	nS
G. rugiensoides	*P. minutus*	Etang de l'Arnel, France 43°33' N, 3°56' E	12/11/99 13°C , 32.7 ppm	15	33.3%	13	3
G. rugiensis	*P. microps*	Vaccarès lagoon 43° 30' N, 4° 3' E	16/11/99 10.7°C, 10 ppm	28	35.7%	31	8
G. rugiensis	*P. minutus*	Vaccarès lagoon 43° 30' N, 4° 3' E	16/11/99 10.7°C, 10 ppm	28	7.1%	3	2
G. rugiensis	*P. microps*	Ostend, Belgium 51°14' N, 2°57' E	18/08/99, 16-18°*C 31.1 ppm	25	36.0%	233	
G. cf. *harengi*	*K. panizzae*	Venice lagoon, Italy 45° 27' N, 12° 26' E	10/10/99 13.9°C, 33 ppm	16	18.6%	5	2
G. cf. *harengi*	*P. marmoratus*	Venice lagoon, Italy 45° 27' N, 12° 26' E	10/10/99 13.9°C, 33 ppm	12	100%	900	7
G. cf. *harengi*	*P. marmoratus*	Vaccarès lagoon 43° 30' N, 4° 3' E	16/11/99 10.7°C, 10 ppm	2	2/2	11	3
		Sète, France 43°23' N, 3°41' E	12/11/99 13°C , 32.7 ppm	7	5/7	92	
G. cf. *harengi*	*P. microps*	Ostend, Belgium 51°14' N, 2°57' E	18/08/99, 16-18°*C 31.1 ppm	25	96.0%	950	9
G. branchialis	*P. marmoratus*	Vaccarès lagoon 43° 30' N, 4° 3' E	16/11/99 10.7°C, 10 ppm	2	2/2	8	3
		Sète, France 43°23' N, 3°41' E	12/11/99 13°C , 32.7 ppm	7	5/7	87	
G. branchialis	*P. marmoratus*	Venice lagoon, Italy 45° 27' N, 12° 26' E	10/10/99 13.9°C, 33 ppm	12	75.0%	132	9
G. branchialis	*P. microps*	Vaccarès lagoon 43° 30' N, 4° 3' E	16/11/99 10.7°C, 10 ppm	15	46.6%	75	5
G. branchialis	*P. microps*	Ostend, Belgium 51°14' N, 2°57' E	18/08/99, 16-18°*C 31.1 ppm	25	84.0%	248	

The size of the total amplified ITS region varied from 919 bp for *G. branchialis* to 1199 bp for *G. rugiensoides*. This pronounced length difference was mainly due to different lengths of ITS1, which is characterized by large insertions and deletions. Since this hampers the alignment considerably, ITS1 was omitted from further analyses. Comparison of the different models of evolution revealed that the HKY + Γ_4 model (Hasegawa, Kishino and Yano, 1985) with gamma shape parameter = 0.4 was the most suited for the 5.8S and ITS2 dataset. The phylogenetic relationships are presented as a midpoint rooted cladogram (Fig. 2). The MP tree (tree length = 880; C.I. = 0.88; R.I. = 0.91) was identical with the ML tree (- Ln likelihood = 2253.4) and the minimum-evolution tree. *Gyrodactylus branchialis* and *G.* cf. *harengi* clustered strongly with *G. arcuatus*, subgenus *G.* (*Mesonephrotus*), while *G. rugiensis* and *G. rugiensoides*, belonging to the subgenus *G.* (*Paranephrotus*), clustered as a sister group to *G. truttae* and *G. salaris*, two representatives of the subgenus *G.* (*Limnonephrotus*).

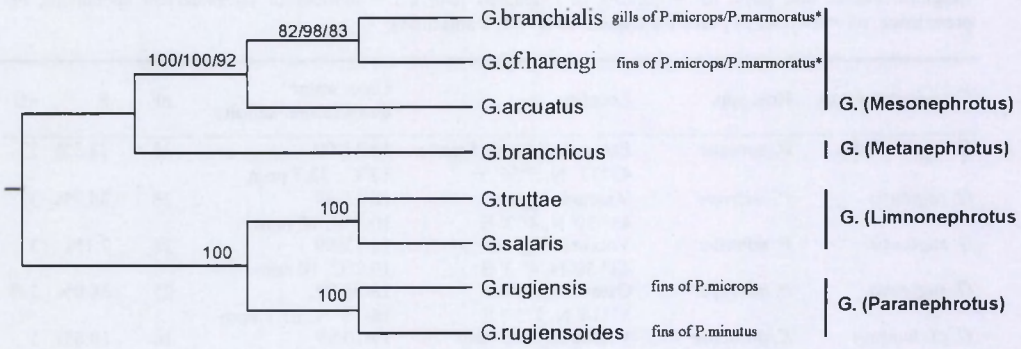

Fig. 2. Parsimony consensus tree of the 5.8S and ITS2 dataset, based on 202 parsimony informative characters (midpoint rooted). Bootstrap support of the MP/NJ/ML are indicated above branches, only one value is given when all values are identical. * = differing with Gyrodactylus specimens on P. microps by two mutations in the whole ITS region.

3.3. Morphometric analyses

The *Gyrodactylus* spp. found in the Mediterranean are represented by means of their haptor and marginal hooks in Fig. 1. The morphometric data are summarized in Table 2 (see appendix). Fig. 3 shows a plot of the total length of the marginal hook against the total anchor length, for *G. branchialis*, *G.* cf. *harengi* and *G. rugiensis* collected in the Mediterranean Sea and the Atlantic Ocean.

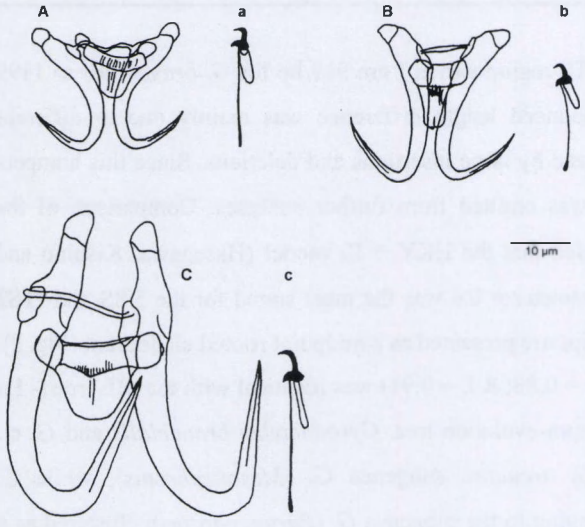

Fig. 1. Anchors (capital letters) and marginal hooks (small letters) of:
A, a: *G. branchialis* from the gills of *P. marmoratus,* from Venice lagoon, Italy, 4/10/99
B, b: *G.* cf. *harengi* from the fins of *P. marmoratus,* Venice lagoon, Italy, 4/10/99
C, c: *G. rugiensis* from the fins of *P. microps,* Vaccarès lagoon complex, France, 15/11/99

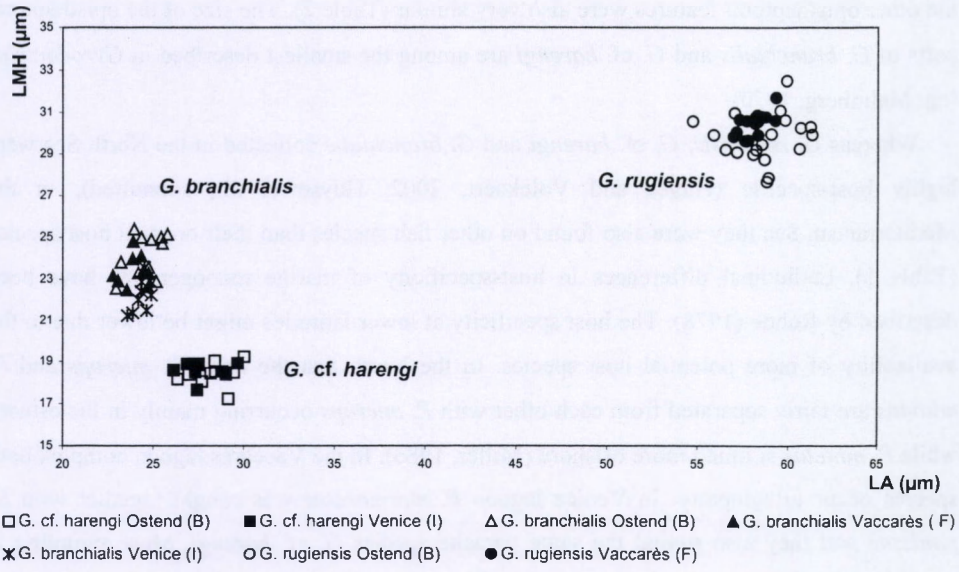

Fig. 3. Plot of the total marginal hook length (LMH) *versus* the total length of the anchor point (LA) of three *Gyrodactylus* species collected in the North Sea and Mediterranean Sea. Species name and sampling sites are presented in the legend.

4. Discussion

This study is the first report on *Gyrodactylus* species in the Mediterranean and Adriatic Seas. *Gyrodactylus rugiensis* and *G. rugiensoides* have been previously described on gobies from the Baltic and Eastern Atlantic Ocean (Gläser, 1974; Huyse and Volckaert, 2002). *Gyrodactylus branchialis* and *G.* cf. *harengi* collected from the North Sea has been genetically characterized by Huyse et al. (submitted); the morphological description of both species is currently in progress (Huyse et al., in prep.). Except for *G. rugiensoides*, the complete ITS rDNA region was identical for all specimens collected in the Eastern Atlantic Ocean and in the Mediterranean Sea. Also the morphological similarity was striking. Plotting the marginal hook total length against the anchor total length could readily separate all species, but it was almost impossible to separate the populations collected from the Eastern Atlantic Ocean and Mediterranean Sea. The specimens from *G. branchialis* collected in the Adriatic showed slightly lower values for the length of the marginal hook sickle, but no such trend was found for either *G. rugiensis* or *G.* cf. *harengi*. The relationship between the marginal hook total length and the anchor total length was used to separate the extremely similar *Gyrodactylus* species of the *G. wageneri* group (Harris, 1985). The mean and range of

the other opisthaptoral features were also very similar (Table 2). The size of the opisthaptoral parts of *G. branchialis* and *G.* cf. *harengi* are among the smallest described in *Gyrodactylus* (eg. Malmberg, 1970).

Whereas *G. rugiensis, G.* cf. *harengi* and *G. branchialis* collected in the North Sea were highly host-specific (Huyse and Volckaert, 2002; Huyse et al., submitted), in the Mediterranean Sea they were also found on other fish species than their original host species (Table 1). Latitudinal differences in host-specificity of marine monogeneans have been described by Rohde (1978). The host specificity at lower latitudes might be lower due to the availability of more potential host species. In the North Sea the hosts *P. microps* and *P. minutus* are fairly separated from each other with *P. microps* occurring mainly in the estuary while *P. minutus* is found more offshore (Miller, 1986). In the Vaccarès lagoon complex both species occur in sympatry. In Venice lagoon *P. marmoratus* was caught together with *K. panizzae* and they also shared the same parasite species *G.* cf. *harengi*. More sampling is needed to test whether this could be attributed to accidental host-switching or whether it is an actual host species. The *Gyrodactylus* species found on both *P. microps* and *P. marmoratus* were genetically almost identical. Since both hosts speciated only recently (Wallis and Beardmore, 1984), it can be hypothesized that this resulted in a reduced gene flow between both parasite populations, initiating speciation, ongoing at the very moment. Since *P. microps* and *P. marmoratus* are very abundant and since they share the same habitats, e.g. in the Vaccarès lagoon complex, host-switching might still be possible.

Harris (1993) suggested a positive correlation between the amount of intraspecific morphological variation and the degree of sexual reproduction. A negative correlation between the amount of intraspecific morphological variation and water temperature has also been reported in literature (Harris, 1993; Appleby, 1996; Geets et al., 1999; Dmitrieva and Gerasev, 2002). The specimens compared in the present study were collected from different seasons (10.7 – 18°C) and different localities (salinity ranging from 10 – 33 ppm). The lack of morphological and molecular variation is all the more surprising considering the fact that the host *P. microps* shows considerable population differentiation in the cytb mtDNA between Eastern Atlantic, Mediterranean and Adriatic populations (Gysels, pers. comm.). This might suggest that the ITS rDNA region in *Gyrodactylus* spp. is not sensitive below species level. In the literature, low intraspecific and geographic variation was found by Zietara et al. (2000; 2002) for *G. arcuatus, G. branchicus, G.* sp. 1 and *G. pungitii*, but no intraspecific variation could be found in ITS2 sequences of *G. kobayashii* from the U.K. and Australia (Cable et al. 1999), nor for *G. anguillae* collected on *A. anguilla* from Spain and

Australia and on *A. australis*, *A. reinhardtii* and *A. rostrata* (Hayward et al. 2001). Mitochondrial DNA markers are therefore expected to provide more information on the population-level differences within these species.

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Appendix

Table 2. Mean and range of characters of the opisthaptoral hard parts of *G. branchialis*, *G.* cf. *harengi* and *G. rugiensis* collected in the coastal areas of Belgium, France and Italy. All measurements are in µm. N specimens = number of parasite specimens measured.

Gyrodactylus species		*G. branchialis*	*G. branchialis*	*G. branchialis*	*G.*cf. *harengi*	*G.*cf. *harengi*	*G. rugiensis*	*G. rugiensis*
Host Species		*P.microps*	*P. microps*	*P. marmoratus*	*P. microps*	*P. marmoratus*	*P. microps*	*P. microps*
Sampling site		Ostend Belgium	Sète, France	Venice, Italy	Ostend, Belgium	Venice, Italy	Ostend, Belgium	Sète, France
N specimens		10	9	10	10	9	23	12
Anchors								
Length of anchor point	Mean	12.40	12.43	11.82	14.59	14.85	30.2	29.25
	Range	(11.08-13.51)	(11.86-13.50)	(11.66-12.64)	(13.52-15.49)	(13.41-16.73)	(28.6-32.0)	(28.32-31.73)
Total length of anchor	Mean	24.56	23.93	24.35	28.22	27.97	58.4	58.07
	Range	(22.94-25.78)	(22.78-24.75)	(23.57-24.88)	(26.35-30.02)	(26.12-29.67)	(54.7-61.2)	(56.95-59.27)
Length of anchor shaft	Mean	18.80	18.80	18.67	23.96	23.19	42.5	42.29
	Range	(16.93-20.89)	(17.76-19.83)	(17.78-19.76)	(22.27-24.94)	(20.96-24.60)	(39.0-44.4)	(41.12-43.66)
Length of anchor root	Mean	8.83	8.05	8.06	7.39	7.71	19.7	19.47
	Range	(6.42-10.33)	(6.41-8.67)	(7.50-8.82)	(6.14-8.06)	(6.37-10.13)	(18.1-21.1)	(18.31-20.36)
Ventral bar								
Length of ventral bar + processes	Mean	16.57	14.06	15.01	11.29	11.37		
	Range	(15.09-18.34)	(8.58-17.23)	(14.22-16.47)	(10.93-11.97)	(10.88-12.01)		
Length of ventral bar	Mean	12.77	12.37	11.04	10.39	10.58	25.9	24.85
	Range	(11.97-14.28)	(10.31-13.61)	(10.60-11.29)	(9.03-11.01)	(9.23-11.20)	(23.1-28.5)	(23.07-25.91)
Basal width of ventral bar	Mean	4.01	4.76	5.23	5.51	5.46	7.3	7.19
	Range	(3.36-4.39)	(3.96-5.81)	(4.53-5.67)	(5.47-5.55)	(5.33-5.60)	(6.1-8.3)	(6.30-8.30)
Median width of ventral bar	Mean	2.98	3.42	3.76	5.84	5.77	5.5	5.10
	Range	(2.53-3.54)	(2.69-4.41)	(3.44-4.27)	(4.68-7.00)	(4.82-6.11)	(3.4-6.8)	(4.08-6.06)
Length of ventral bar membrane	Mean	9.60	10.03	9.75	12.45	12.29	12.1	12.31
	Range	(7.68-10.38)	(9.31-11.55)	(9.65-9.79)	(11.50-13.41)	(11.67-12.93)	(9.4-15.0)	(9.9-12.66)
Total length of ventral bar membrane	Mean	6.53	6.81	6.67	6.63	6.70	17.6	17.83
	Range	(5.41-7.51)	(6.42-7.68)	(6.57-6.75)	(6.38-6.89)	(6.45-7.10)	(15.0-20.2)	(16.1-19.3)
Marginal hook								
Total length of marginal hook	Mean	24.21	23.42	22.23	18.43	18.61	29.4	30.47
	Range	(22.56-25.39)	(22.48-24.80)	(21.22-23.56)	(17.23-19.23)	(17.64-18.92)	(26.7-30.6)	(29.66-31.70)
Length of marginal hook handle	Mean	20.24	19.81	18.48	14.97	15.14	23.5	24.34
	Range	(18.56-21.53)	(18.59-20.83)	(17.90-19.47)	(13.75-15.54)	(14.32-16.09)	(21.4-24.8)	(23.12-27.12)

Length of marginal hook sickle	Mean	4.50	4.43	4.35	3.82	3.78	6.5	5.84
	Range	(4.10- 5.20)	(3.92-4.97)	(4.02-4.72)	(3.55-4.36)	(3.31-4.03)	(5.7-6.9)	(5.43-6.32)
Proximal width of marginal hook sickle	Mean	2.82	2.94	2.81	2.82	2.74	3.7	3.95
	Range	(2.36-3.15)	(2.32-3.28)	(2.45-3.34)	(2.52-3.09)	(2.45-2.96)	(3.1-4.3)	(3.68-4.88)
Distal width of marginal hook sickle	Mean	3.38	3.20	2.97	2.19	2.16	3.8	2.86
	Range	(3.02-3.85)	(2.97-3.78)	(2.87-3.67)	(1.82-2.93)	(1.79-2.60)	(3.4-4.2)	(2.43-3.65)
Marginal hook sickle aperture distance	Mean	2.22	2.31	2.14	2.67	2.85	5.0	
	Range	(2.03-2.59)	(2.01-2.61)	(1.92-2.46)	(2.18-3.71)	(2.26-3.31)	(4.5-5.5)	
Length of marginal hook filament loop	Mean	7.05	6.46	6.70	5.70	5.99	8.4	9.35
	Range	(5.08-9.70)	(5.80-7.33)	(5.87-7.62)	(4.69-6.51)	(5.46-6.87)	(5.1-10.4)	(8.05-10.48)

CHAPTER 6

TOWARDS AN UNDERSTANDING OF SPECIATION IN *GYRODACTYLUS* (MONOGENEA, PLATYHELMINTHES)

Tine Huyse, Vanessa Audenaert and Filip A.M. Volckaert

Abstract: Fine-scale associations of the parasite *Gyrodactylus* within a single host genus were examined by sequencing the V4 region of the ssrRNA and the complete ITS rDNA. Fifteen species were collected from gobies of the genus *Pomatoschistus* and sympatric fish species across the distribution range of the hosts. Complimented with sequences from GenBank, a total of 23 *Gyrodactylus* species representing all subgenera were used in phylogenetic analyses. Whereas the overall topology was highly consistent, it was difficult to resolve the relationships within subgenera due to net- and star-like phylogenetic noise. This might be due to the molecular characteristics (e.g. rate heterogeneity) of the ITS and V4 region, or it might be linked with the peculiar reproduction mode of these viviparous flatworms. Paraphyly of the total *Gyrodactylus* fauna of the gobies indicates that at least two independent colonization events were involved, giving rise to two separate groups (A and B), belonging to *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*), respectively. The most recent association (Group A) probably originated from a host-switching event from *G. arcuatus*, which parasitizes three-spined stickleback, onto *Pomatoschistus* gobies. These species are highly host-specific and form a monophyletic group, two possible 'signatures' of co-speciation. Host-specificity was lower in Group B. The colonizing capacity of these species is illustrated by a host jump to a fish species belonging to another fish order (Anguilliformes). Hence, allopatric speciation seems to be the dominant mode of speciation in this host-parasite system, with a possible instance of sympatric speciation.

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1. Introduction

Parasites are particularly interesting for studying speciation processes. The relative contribution of the different speciation modes can be assessed using species-level phylogenies with inclusion of the geographical distribution of sister taxa (Barraclough and Nee, 2001). However, in the case of free-living animals, the range of species can change quite rapidly, such that the observed range might not reflect the actual speciation mode. The niche of a parasite is, by its lifestyle, relatively fixed, providing a more straightforward framework to study. Since parasites are dependent on their hosts, speciation in the latter is likely to induce speciation in the parasite, resulting in mirror-image phylogenies (Page, 1994; Hafner and Page, 1995; Poulin, 1998). However, parasites should not always be regarded as 'passive' members of the association; some taxa can also move independently of their hosts and actively switch hosts (Brooks and McLennan, 1993). Whereas co-speciation can be regarded as allopatric vicariant speciation, host-switching can be regarded as peripatric or peripheral isolate speciation. A third phenomenon, sympatric speciation, is gaining recognition as an alternative speciation mode, operating under well-specified circumstances (Dieckmann and Doebeli, 1999; Tregenza and Butlin, 1999; Via, 2001). Parasite groups belonging to the Monogenea meet many of these conditions (Poulin, 2002). Despite various interesting characteristics that render them an ideal study target, monogeneans have been rarely studied within this context.

The Monogenea is one of the largest groups of Platyhelminthes, characterized by a high species diversity and a high host specificity (Gusev, 1995; Kearn, 1994; Poulin, 1998). The direct life cycle leads to auto-infection of the host, which means that they experience only one adaptive barrier in their life (Gusev, 1995). *Gyrodactylus* is the only monogenean genus that displays the extreme of this developmental trend, namely hyperviviparity. Embryos develop within each other inside the mother's uterus and asexual reproduction alternates with sexual reproduction (Harris, 1993). This rapid reproduction in close relationship with the host, together with the high host specificity is thought to promote co-evolution between host and parasite (Connell, 1980; Humphery-Smith, 1989; Hafner and Page, 1995). At first sight, the absence of free-living larvae (and hence the absence of an adaptive barrier) might decrease the chance to encounter other host species, minimizing the opportunities for host-switching. However, it has been shown that gyrodactylids can survive for a while independent of their host and some kind of 'swimming behaviour' has also been described (Cable et al., 2002). This 'active' dispersion capacity of *Gyrodactylus* in combination with

the ability to produce a viable deme from only one individual might increase the chance for speciation by host-switching. Furthermore, auto-infection and the high level of host specificity might also enhance sympatric speciation. The succession of several generations on a single host specimen ensures the continuity of a population, but increases the chance of inbreeding (Brooks and McLennan, 1993). In spite of these life history traits known to allow sympatric speciation, there is limited evidence in the literature. Gusev (1995) presents numerous examples where congeneric doublets are likely to be the result of sympatric speciation, but without any molecular evidence. Molecular studies have been carried out on site-specific polystome monogeneans by Littlewood et al. (1997), but they did not find any evidence for speciation on the same host species. In conclusion, all speciation modes appear equally probable in this group, making *Gyrodactylus* an ideal candidate for speciation studies.

A tool to discriminate among these scenarios is provided by phylogenetic systematics (Brooks and McLennan, 1993). Phylogenetic trees provide an indirect record of the speciation events that have led to the present-day species (Barraclough and Nee, 2001). Furthermore, by mapping biological characteristics onto the tree, inferences can be made regarding the evolution of a suite of comparative data e.g. host specificity and ecological shifts (Schluter, 2001). It is of special interest to focus on fine-scale parasite associations, within a single host genus or within one group of sympatric host species. It is within this frame that speciation takes place. Furthermore, in this way all possible sister species and host transfer routes are expected to be included in the analysis.

This study focuses on *Gyrodactylus* spp. parasitizing gobies of the genus *Pomatoschistus* Gill, 1864. It is the dominant gobiid genus of the Atlantic and Mediterranean coasts of Europe comprising about ten species (Miller, 1986). Until now, not much attention has been paid to their role as a host for *Gyrodactylus*. Four *Gyrodactylus* species are known to parasitise *Pomatoschistus* spp.: *G. longidactylus* Geets, Malmberg and Ollevier, 1998 (on the gills of *P. lozanoi*), *G. rugiensis* Gläser, 1974a (on fins of *P. micropsi* and *P. minutus*), *G. micropsi* Gläser, 1974a (on fins and gills of *P. microps* and *P. minutus*), and *G. rugiensoides* Huyse and Volckaert, 2002 (on the fins of *P. minutus*, *P. lozanoi* and *P. pictus*). Yet several other still undescribed *Gyrodactylus* spp. are suspected to live on *Pomatoschistus* spp. Therefore, we first made an inventory of the *Gyrodactylus* fauna parasitizing the *Pomatoschistus* gobies and assessed their degree of host specificity. Secondly, a robust phylogeny of both hosts and parasites is required. In this paper we focus on the parasites, interrelationships between the present *Gyrodactylus* species were inferred by using the V4 region of the ssrRNA and the complete ITS rDNA region. These markers consist of variable

and conservative regions, which make them an ideal target to compare both closely as well as distantly related species (Hillis et al., 1996). This allowed us to test hypotheses that will provide more information on host-parasite evolution without the need of a host phylogeny: (1) *Gyrodactylus* spp. found on gobies of the genus *Pomatoschistus* are monophyletic; (2) congeners on the same host species or with the same niche are each other's closest relative; (3) *Gyrodactylus* spp. cluster according to their excretory system as defined by Malmberg (1970; 1998). The first scenario is expected under a mode of speciation through co-evolution. Following from this, the fauna of sympatric non-*Pomatoschistus* species will be compared to that of *Pomatoschistus* spp. and all available sequences will be included in the phylogenetic analyses. The second hypothesis will provide more information on the speciation mode within one host species (intrahost speciation *vs* co-speciation or speciation by host-switching). Finally, a comparison can be made between the molecular phylogeny and the phylogeny based on morphological criteria proposed by Malmberg (1970; 1998).

2. Material and Methods

2.1. Collection of material, morphological determination and DNA extraction

Samples were collected throughout the geographical range of *Pomatoschistus* spp., in the Mediterranean Sea and along the North-Eastern Atlantic continental shelf (Table 1). In addition, *Gyrodactylus* spp. were sampled from the two-spotted goby *Gobiusculus flavescens* Duncker 1928, European plaice, *Pleuronectes platessa* Linnaeus 1758, three-spined stickleback, *Gasterosteus aculeatus* Linnaeus 1758, nine-spined stickleback, *Pungitius pungitius* Linnaeus 1758 and sea stickleback, *Spinachia spinachia* Linnaeus 1758. Fish were brought live to the laboratory and immediately screened for *Gyrodactylus*-infection using a stereo-microscope. Whenever possible, the opisthaptor was separated from the body enabling simultaneous morphological and molecular analyses. The body was then placed in 5 µl of milli-Q water and stored at -20°C. The opisthaptor was fixed in ammonium picrate glycerin as described by Malmberg (1970), to examine the haptoral sclerites by phase contrast microscopy. DNA extractions were performed as described by Zietara et al. (2002).

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

Approximately 900-1200 bp of the rDNA complex, spanning the 3' end of the 18S subunit, the internal transcribed spacer 1 (ITS1), the 5.8S subunit, ITS2, and the 5' end of the 28S subunit were amplified for three to 15 specimens per species (Table 1). Amplification

and sequencing were performed as described by Zietara et al. (2002). A negative control was included in each PCR reaction. Sequences were verified by comparing each sequence with its complement, and in case of ambiguities, the sequencing reaction was repeated.

For the initial amplification of the V4 region (354 bp), the primers V4F and V4R designed by Cunningham et al. (1995) were used. Since the PCR products were not specific enough, new primers V4f2 (5'-GAGGGCAGTCTGGTGCC-3') and V4r2 (5'-CAGGCTTCAAGGCCTGC-3') were designed, located six and five bp respectively inwards from the original primers. The amplification reactions consisted of 1x PCR buffer (Eurogentec, Seraing, Belgium), 1.75 mM $MgCl_2$ (Eurogentec), 200 µM of each dNTP (Amersham Biosciences, Sweden), 1 µM of each primer (Eurogentec), 2 µl lysate, 1 unit *Taq* polymerase (Eurogentec) and milli-Q water. The mixtures of 20 µl were layered with mineral oil, heated for 4 min at 97°C and subjected to 35 cycles as follows: 95°C for 1 min, 60°C for 30s and 72°C for 30 s, followed by a final extension step at 72°C for 7 min. The PCR products were visualised using ethidium bromide on a 1.2% agarose gel and purified by means of GFX columns according to the manufacturer's instructions (Amersham Pharmacia). These products were used for cloning following the manufacturer's instructions (TA cloning system, Invitrogen). The PCR products of the cloned products were purified by means of GFX columns and directly sequenced in both directions. Sequencing was done following the protocol of SequiTherm EXCEL II (Epicentre Technologies). The reaction products were separated on a LICOR 4200 system and visualised on a 6% Long Ranger gel (FMI BioProducts). For each species, 2-3 individual specimens were cloned and 2 clones per specimen were sequenced.

2.3. Sequence alignment

The ITS region shows substantial variation between *Gyrodactylus* spp. from different subgenera (Zietara et al., 2002). Therefore different datasets have been prepared. Within single subgenera the 5.8S sequence is identical, so only the ITS1, ITS2 and V4 sequences were aligned using Clustal X v. 1.81 (Thompson et al., 1997). When including species belonging to different subgenera, the highly variable ITS1 region was skipped and only the V4, 5.8S and ITS2 sequences were aligned using the program SOAP (Löytynoja and Milinkovitch, 2002). SOAP generates alternative CLUSTAL W alignments by using all possible combinations of gap opening penalties, ranging from e.g. 4-10 and GEP gap extension penalties ranging from e.g. 3-7. The program then identifies the "unstable-hence-

unreliable" positions by comparing the different alignments. These particular regions are then excluded and the file is exported in NEXUS format. Afterwards, PAUP can re-include those characters and the impact of unstable sites on phylogeny reconstruction can be evaluated. Exclusion of the unstable characters in the V4, 5.8S and ITS2 alignment resulted in a 675 bp fragment. An alternative evaluation of the alignment was performed by dot plots implemented in the GeneWorks software (Intelligenetics, Oxford, UK). All regions with a similarity less than 70% were excluded. The resulting fragment was very similar to the fragment obtained by SOAP. To compare relative speciation dates, the following sequences from GenBank were included: *G. truttae* (AJ132260, AJ407913), *G. salaris* (AF328871, Z26942) and *G. teuchis* (AJ249349, AJ249350). Finally, to analyse the evolutionary relationships between *Gyrodactylus* spp. collected from *Pomatoschistus-Gobiusculus* spp. and sympatric host species, the following GenBank sequences were added: *G. arcuatus* (AF328865), *G. branchicus* (AF156669), *G. gasterostei* (AF328867), *G. pungitii* (AF328869), *G. anguillae* (AB063294), *G. nipponensis* (AB063295), *G. elegans* (AJ407920, AJ407870), *Gyrdicotylus gallieni* (AJ001843) and *Gyrodactyloides bychowskii* (AJ249348). Since the V4 region of those species was not available, this second dataset consisted of 5.8S and ITS2 sequences only. Again, SOAP was used to remove the most unstable regions resulting in a 330 bp fragment.

2.4. Phylogenetic analyses

First, a consensus tree was made from the topologies obtained by TREE-PUZZLE 5.0 (Schmidt et al., 2002), maximum parsimony (MP), maximum likelihood (ML) and Neighbor-Joining (NJ) using PAUP* v. 4.01b (Swofford., 2001). This consensus tree was then used as input tree in the PAUP* command block from ModelTest 3.06 (Posada and Crandall, 1998). The parameters and likelihood scores were estimated upon that tree, and then the program uses the likelihood scores (LK) to select the model of DNA evolution that best fits the data. The parameters estimated under this best-fit model were entered in the ML search and nearest-neighbour-interchange branch swapping was performed. The respective parameters were then optimised upon this tree through successive iteration. Trees were statistically tested by calculating *P* values for the ML tree. MP trees were inferred with the branch and bound algorithm (100 replicates). In these analyses gaps were treated both as fifth base and as missing data, all sites were equally weighted and different transition:transversion (ti/tv) ratios were applied; 10:5 for 5.8S and V4 region and 1:5 for ITS2. The minimum-evolution search

was conducted (1000 replicates of tree-bisection reconnection branch swapping) from a matrix of ML genetic distances calculated under the optimised model. The base composition for all sequences was compared using a 5% χ^2 test on the average composition (TREE-PUZZLE). The molecular-clock hypothesis was tested assuming the HKY model (Hasegawa, Kishino and Yano, 1985) and γ-distributed rates across sites, with the likelihood ratio test for the clock hypothesis implemented in TREE-PUZZLE.

Gyrodactyloides bychowskii was used as outgroup in the 5.8S and ITS2 dataset, but no sequence of the V4 region was available. Therefore we implemented midpoint rooting for the V4-5.8S-partial ITS2 dataset. A likelihood-ratio test (LRT) showed that this tree was not significantly worse than the unrooted tree. PAML v.3.1 (Yang, 2001) was further used for its implementation of the auto-discrete-gamma model that considers correlation of rates at adjacent sites. Conflicting phylogenetic signal was evaluated with the split decomposition method in the program SplitsTree 3-1 (Huson, 1998). The phylogenetic content of a sequence alignment can also be visualized by the likelihood mapping analysis implemented in TREE-PUZZLE. This method distinguishes between phylogenetic signal producing treelike topologies, and phylogenetic noise producing star- and/or netlike topologies. Bootscanning analysis was performed as implemented in the program Simplot 2.5 (Lole et al., 1999). It uses bootstrap analyses on a sliding window of sequential and overlapping segments of the sequence alignment; inconsistent bootstrap support for a clade across the genome could be an evidence of recombination. In order to test the presence of saturation, DAMBE v. 4.0.75 (Xia and Xie, 2001) was employed to compare the observed saturation index with the saturation index expected when assuming full saturation. A *t*-test with infinite degrees of freedom was used to assess statistical significance. Plotting transitions and transversions against divergence did not show a sign of saturation in any dataset.

3. Results

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

In total, 91 complete ITS1-5.8S-ITS2 sequences of 15 species from eight localities and 47 V4 sequences of 15 species were obtained (Accession Nos. x). Table 1 lists the species with information on their subgenus status, their respective host and site on the host, geographic locality and the species included from GenBank. We consider *Gobiusculus flavescens* together with *Pomatoschistus* spp. since molecular analysis clusters this species firmly within *Pomatoschistus* (pers. data). The thirteen parasite species found on *Pomatoschistus* spp.

clustered genetically in two groups (A and B, see Fig. 1) differing about 24.8 - 28.7%, based on the V4-5.8S-partial ITS2 gamma corrected distances. These groups are readily distinguished from each other since their sequences differ by about 200 bp in length. None of these species were found on non-*Pomatoschistus* fish species examined in this study. Within each group, genetic differentiation was much lower, ranging from 0.3 - 4.9%.

Some of the species of group A have been described by Geets et al. (1999), but none of them have been named. They showed, by means of multivariate analyses on morphometric data of 17 anchor features, that each group could be separated according to their respective host species. A combined morphometric and molecular sequencing analysis has been carried out to describe these species (Huyse et al., in prep.). They all belong to the subgenus *G.* (*Mesonephrotus*). Each species was recorded from only one host species, except for *G.* sp. 1 that was found on both *P. minutus* and *P. lozanoi.* Group B contains three undescribed species. One of them is only found on gills of *P. norvegicus*, hereafter referred to as *G.* cf. *longidactylus*, because of its morphological resemblance to *G. longidactylus* found on the gills of *P. lozanoi.* The other two are hereafter referred to as *G.* cf. *micropsi* 1 and *G.* cf. *micropsi* 2 respectively, in accordance with their morphological and genetical (ITS and V4 region) similarity to *G. micropsi.* They all belong to the subgenus *G.* (*Paranephrotus*). More than one species of Group B were found on the same host, and some hosts shared the same species (see Table 1). Only *G. micropsi, G. rugiensis* and *G.* cf. *longidactylus* were recorded from a single host species.

Table 1. Collection sites, subgenus, respective hosts and location on the host of *Gyrodactylus* species used for sequencing (V4, ITS1, 5.8S, ITS2). Sampling dates are for Belgium (Ostend, 18/8/1998 and the North Sea, 25/10/99), The Netherlands (Texel, 12/6/99, 5/12/99 and Yerseke, 29/9/99), France and Italy (10/98, 11/99 and 10/99 respectively), and Norway (06/2000 and 05/2000, Bergen and Trondheim). All *Gyrodactylus* sequences included in the analysis are shown with the respective accession number.

Species[1]	Subgenus	Host	Collection site	Country	Site on host	N°
Gyrodactylus sp1	*Mesonephrotus*	*P. minutus*	Ostend, North Sea	Belgium	G/F	AF328866
			Texel, Yerseke	The Netherlands	G/F	5
			Trondheim,	Norway	F	2
			Bergen			2
Gyrodactylus sp1	"	*P. lozanoi*	North Sea	Belgium	G/F	2
			Texel	The Netherlands	G/F	3
Gyrodactylus sp2	"	*P. pictus*	North Sea	Belgium	G	2
			Bergen	Norway	G/F	5
Gyrodactylus sp3	"	*P. norvegicus*	Bergen	Norway	G	3
Gyrodactylus sp.	"	*P. microps*	Ostend, North Sea	Belgium	G	4
			Texel, Yerseke	The Netherlands	G	2
			Ambleteuse	France	G	2
			Camargues, Sète	France	G	3/*
			Venice lagoon	Italy	G	4/*
Gyrodactylus sp.4	"	*P. microps*	Ostend, North Sea	Belgium	F	4

			Texel, Yerseke	The Netherlands	F	2
			Ambleteuse	France	F	3
			Camargues, Sète	France	F	3/*
			Venice lagoon	Italy	F	5/*
Gyrodactylus sp. 5	"	*G. flavescens*	Trondheim, Bergen	Norway	G/F	3 4
Gyrodactylus sp.6	"	*G. flavescens*	Trondheim, Bergen	Norway	F	1 1
G. rugiensis	*Paranephrotus*	*P. microps*	Ostend, North Sea	Belgium	F	AF328870
			Texel, Yerseke	The Netherlands	F	"
			Ambleteuse	France	F	"
			Camargues	France	F	2/*
			Ëdeso, Stockholm	Sweden	F	2
G. rugiensoides	"	*P. minutus*	Ostend, North Sea	Belgium	F	AJ427414
			Texel, Yerseke	The Netherlands	F	"
			Bergen	Norway	F	"
			Sète	France	G	3/*
G. rugiensoides	"	*P. lozanoi*	North Sea	Belgium	F	AJ427414
			Texel	The Netherlands	F	"
G. rugiensoides	"	*P. pictus*	Bergen	Norway	F	AJ427414
G. micropsi	"	*P. microps*	Doel; North Sea	Belgium	G	AF328868
G. cf *micropsi*	"	*P. minutus*	North Sea	Belgium	F	AJ427221
			Texel	The Netherlands	F	"
G. cf *micropsi*	"	*P. lozanoi*	Texel	The Netherlands	F	"
G. cf *micropsi* 1	"	*P. lozanoi*	Texel	The Netherlands	F	2
G. cf *micropsi* 2	"	*P. lozanoi*	Texel	The Netherlands	F	2
*G.*cf *longidactylus*	"	*P. norvegicus*	Bergen	Norway	G	5
G. arcuatus	"	*G. flavescens*	Bergen	Norway	F	1
G. arcuatus	"	*G. aculeatus*	Doel	Belgium	?	AF328865
G. arcuatus	"	*P. microps*	Bergen	Norway	F	3
G. arcuatus	"	*P. pungitii*	Ëdeso, Stockholm	Sweden	F	1
G. pungitii	*Limnonephrotus*	*P. pungitius*	Ëdeso, Stockholm	Sweden	F	1
G. rarus	*Metanephrotus*	*S. spinachia*	Trondheim	Norway	G	3
G. flesi	*Paranephrotus*	*P. platessa*	Trondheim	Norway	F	2
G. flesi		*P. platessa*	Bergen/North Sea	Norway/Belgium	F	1
G. truttae	*Limnonephrotus*	*S. truttae*	Vlára river	Czech Republic/ Scotland	F	AJ132260 AJ407913
G. salaris	"	*S. salar*	Fish farm	Finland	F	AF328871 Z26942
G. teuchis	"	*O. mykiss*	Britanny	France	F	AJ249349 AJ249350
G. gasterostei	"	*G. aculeatus*	Overpelt	Belgium	F	AF328867
G. pungitii	"	*P. pungitii*	Overpelt	Belgium	F	AF328869
G. branchicus	*Metanephrotus*	*G. aculeatus*	Doel	Belgium	G	AF156669
G. anguillae	*Neonephrotus*	*A. anguilla*		Spain/Australia	G	AB06329 4
G. nipponensis	*Neonephrotus?*	*A. japonica*	Lake Hamana	Japan	G	AB06329 5
G. elegans	*Gyrodactylus*	*B. bjoerkna*	Morava river	Czech Republic	F	AJ40792 AJ407870

° Accession number or number of ITS1,2 and 5.8S sequences are given (for the V4 region 2-3 specimens per species were sequenced, except in cases of geographic variation, additional specimens were sequenced). * Huyse et al., in prep.

A *Gyrodactylus-Pomatoschistus* association was found all over the distribution range of the host, but not all species were recorded from every location. *Pomatoschistus minutus* and *P. microps* have the widest distribution and were thus most frequently sampled. As a

consequence, their *Gyrodactylus* fauna had, with few exceptions, the widest geographic record. *Pomatoschistus minutus* specimens caught in Trondheim harboured only *G.* sp. 1 and no *G. rugiensoides*, while in the Mediterranean the opposite was found (Huyse et al., in prep.). From the Baltic only *P. microps* was caught. Its gill parasite *G.* sp. was not found there, while *G. rugiensis* was present on the skin and fins. Those specimens showed one substitution and two insertion/deletion events (1 and 3 bp) compared to the ITS region of *G. rugiensis* collected in the North Sea. No variation was found in the V4 region. *Pomatoschistus pictus* was mainly sampled in Bergen (Norway); only few specimens from the Belgian section of the North Sea were available. *Gyrodactylus* sp. 2 from both populations differed in two substitutions in the whole ITS region. The fin parasite *G. rugiensoides* was only found in Bergen, precluding any further comparison.

Apart from its original host *Gasterosteus aculeatus, G. arcuatus* was occasionally found on *P. microps* and *P. pungitius* from Stockholm (Sweden), and on *P. microps* and *G. flavescens* from Bergen (Norway). In addition to one site polymorphic for all specimens, another C/T substitution was found when comparing the ITS2 sequences of specimens from *G. aculeatus* and *P. pungitius* with those collected from *P. microps* and *G. flavescens*. In the ITS1 region of *G. arcuatus* collected from *G. aculeatus* and *P. pungitius*, one transition, one transversion and one insertion/deletion event (1 bp) was detected in comparison to the ITS1 region of *G. arcuatus* found on the common goby. *Gyrodactylus pungitii* from nine-spined stickleback sampled in Stockholm showed 1 transition in the ITS1 region compared to *G. pungitii* from three-spined stickleback sampled in Overpelt (Belgium, AF328869). *Gyrodactylus micropsi,* infecting the gills and fins of *P. microps*, was only found in the estuary at Doel and two times in the North Sea (Belgium). The species most widely encountered were *G. rugiensis*, *G. rugiensoides*, *G.* sp. and *G.* sp. 1.

3.2. Phylogeny reconstruction

The 5.8S gene and the V4 region were most conservative and p-distances between the gyrodactylids found on *Pomatoschistus* species ranged from 0 - 5.8% and 0 - 22.2% respectively. The latter region showed four insertion/deletion events of one bp and one of three bp long. The ITS region was much more variable, a difference of 200 bp was found between Group A and B. Due to the introduced gaps, both groups could be easily separated by eye based on the alignment alone. Whereas the dataset of Zietara et al. (2002) suffered from deviating base composition (p-value: 37-99%) the current dataset created with SOAP

had a base composition p-value of 70-92% and yielded 118 parsimony informative sites. Modeltest selected the HKY + Γ_4 model (Hasegawa, Kishino and Yano, 1985) with gamma shape parameter = 0.3. After optimisation, the general reversible model (REV, Rodriguez et al., 1990) was selected (LRT $p < 0.001$), with the parameters of the auto-discrete-gamma model (Yang 1995) estimated as follows: $\alpha = 0.3$; $\rho = 0.97$. The clock was not rejected. Figure 1 shows the ultrametric tree of the V4-5.8S-partial ITS2 dataset constructed with PAML. The low value for alpha indicates that there is strong rate heterogeneity in the dataset; the rates among adjacent sites appear to be highly correlated. By excluding the correlation parameter rho, the log likelihood score dropped from -1902.75 to -1982.42. The topology and bootstrap values are more or less in agreement with the trees generated by MP and NJ (bootstrap values shown in Fig. 1). Only the position of *G.* cf. *longidactylus* depended on the tree-building method used: NJ and ML grouped *G.* cf. *longidactylus* together with *G. micropsi, G.* cf. *micropsi* and *G.* cf. *micropsi* 1, while MP clustered it together with *G. rugiensis* and *G. rugiensoides.* When the unstable alignment positions were re-included in the analysis or when gaps were treated as fifth character, the number of parsimony informative sites increased to 136 and 126 respectively, although this did not affect the topology; the bootstrap values varied only slightly.

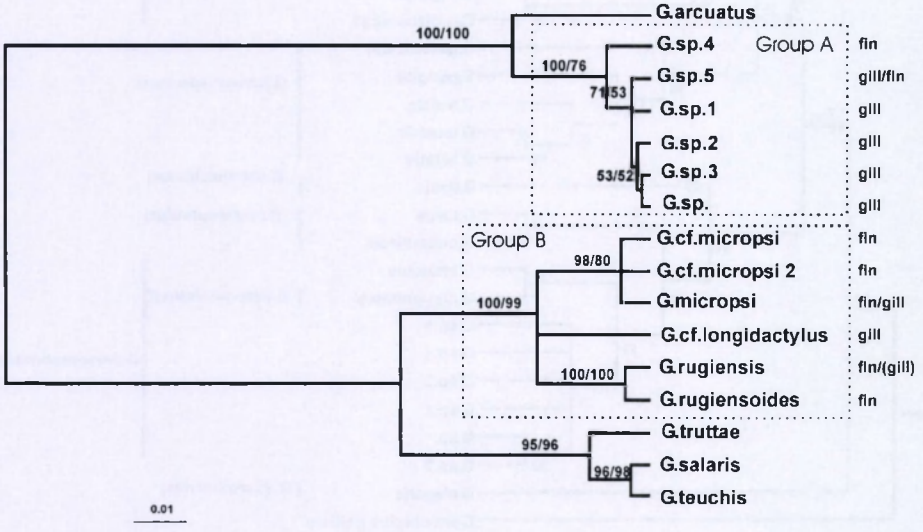


Fig. 1. Ultrametric tree of the V4-5.8S-partial ITS2 dataset including 16 *Gyrodactylus* species (675 bp; lnL = -1982.4; rmatrix = (2.1 7.6 4.3 1.4 7.7); $\alpha = 0.3$). Bootstrap values are shown for the MP/NJ analyses. (MP: 118 parsimony informative sites; tree length: 914; C.I. = 0.81; R.I. = 0.94). *Gyrodactylus* spp. found on *Pomatoschistus* hosts fall within two groups (A en B), which are marked with a dotted line; their site on the host is given.

The speciation events within both groups are relatively recent (Fig. 1). Members of Group A have about the same relative age as *G. salaris* - *G. teuchis* while diversification among Group B is more ancient. The analysis of the combined 5.8S and ITS2 region (330 bp, see Fig. 2) included *Gyrodactyloides bychowskii* as outgroup. The likelihood ratio clock test showed a significant increase in the log-likelihood of the non-clock tree and TREE-PUZZLE showed that the base-composition was not homogenous. In this case including or excluding 'unstable alignment positions' did affect phylogeny reconstruction, but only with respect to the clustering within each subgenus. The REV + Γ_4 model with gamma shape parameter = 0.7 was selected. Figure 2 shows the NJ tree but an identical topology was obtained by TREE-PUZZLE and MP (213 parsimony informative sites when gaps were treated as a fifth character; C.I. = 0.62; R.I. = 0.85); the position of *G. rugiensis* and *G. rugiensoides* could not be resolved by ML. Species from Group A represent a monophyletic group while *G. anguillae* from European eel clusters within Group B.

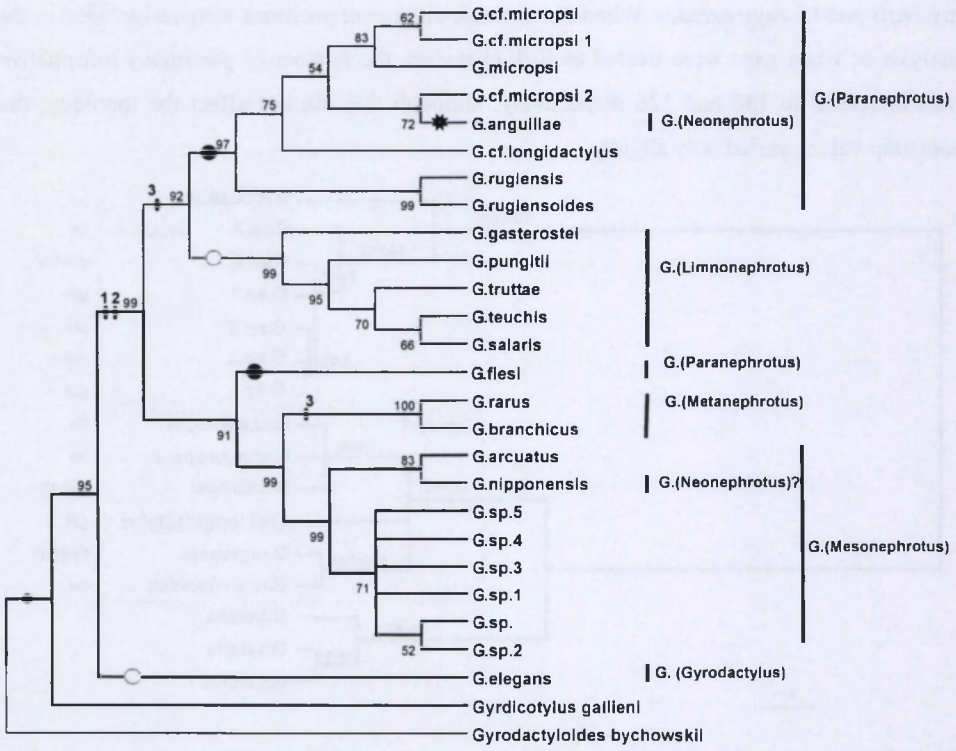
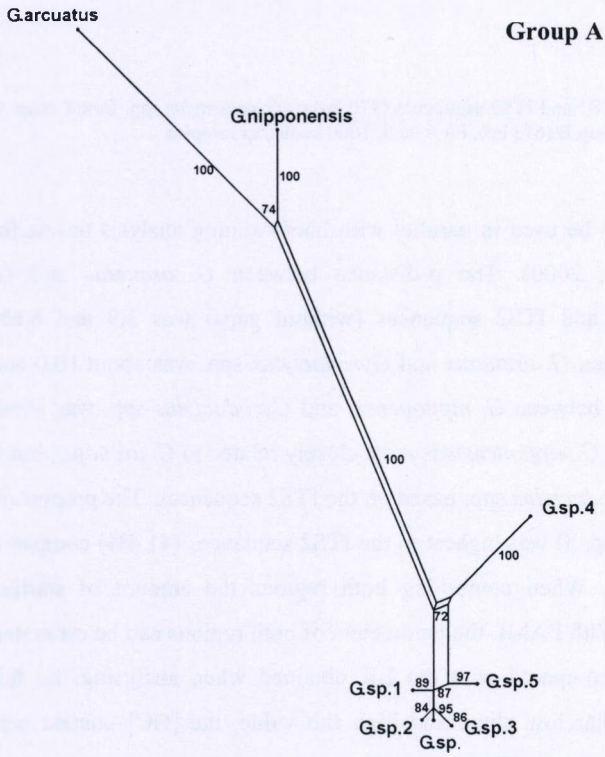


Fig. 2. Neigbour Joining tree based on a 330 bp fragment of partial 5.8S+ITS2 sequences of representatives of all six *Gyrodactylus* subgenera (only transversions were taken into account); diagnostic features of the excretory system (Malmberg, 1970) are mapped on the tree. (1) :Reduced number of flame bulbs; (2): Reduced number of lateral flame cells; (3): no lateral flame cells. The size of the circle refers to the size of the bladders: small, large, absent (open circle) or constantly pumping (star symbol).

3.3. Phylogenetic relationships within Gyrodactylus subgenera

The combined V4-ITS1-ITS2 sequences were 1112 bp long in *G.* (*Mesonephrotus*). Since ITS1 was too variable in *G.* (*Paranephrotus*), only the most conservative part was used, resulting in a total of 848 bp. Including *G. nipponensis* and *G. anguilla* (no V4 available) yielded datasets of 969 bp and 848 bp, respectively. Results of the split decomposition analysis are shown in Fig. 3. Both graphs show a considerable amount of phylogenetic conflict in the ITS data. Within *G.* (*Mesonephrotus*), the highest supported conflicting split is between *G. nipponensis*, *G. arcuatus* and *G.* spp. Bootscanning analysis (Fig. 4) suggested this may be due to a putative recombination event involving a 200 bp stretch at the 3'end of ITS1 in the *G. nipponensis* sequence. The bootstrapping threshold for assignment of parenthood was 96%. The informative sites analysis in Simplot showed that there were very few informative sites supporting the conflicting phylogenies.



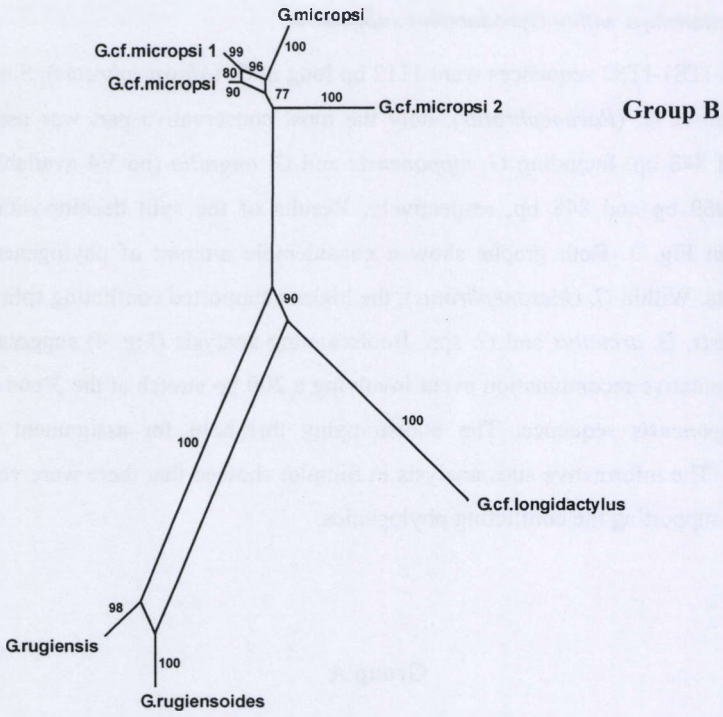


Fig. 3. Splits graph obtained from the ITS1 and ITS2 sequences (970 bp) of *Gyrodactylus* spp. from Group A, Fit = 95, 1000 bootstrap samples and Group B (673 bp), Fit = 96.3, 1000 bootstrap samples.

Pairwise distance analysis can be used in parallel with bootscanning analysis to test for recombination (Anderson et al., 2000). The p-distance between *G. arcuatus* and *G. nipponensis* based on the ITS1 and ITS2 sequences (without gaps) was 3.9 and 6.6% respectively; the difference between *G. arcuatus* and *Gyrodactylus* spp. was about 10.0 and 8.3% respectively; the difference between *G. nipponensis* and *Gyrodactylus* spp. was about 10.1 and 5.8%. So based on ITS1, *G. nipponensis* is most closely related to *G. arcuatus,* but it is more closely related to the *Gyrodactylus* spp. based on the ITS2 sequences. The proportion of starlike phylogenetic signal (Fig. 5) was highest in the ITS2 sequences (41.4%) compared to the ITS1 sequences (24.3%). When combining both regions the amount of starlike phylogenetic signal was 17.1%. With PAML the parameters of both regions can be estimated separately and this LK can be compared with the LK obtained when analysing the full dataset. Both regions had a similar low alpha and high rho value, the [GC] content was similar (26.4 and 28.6%), the rate for the ITS1 and ITS2 was 1 and 0.7 respectively, and the substitution matrix was (4.8 3.6 5.6 – 7.4) and (0.8 1.8 1.2 0.6 2.3) respectively. Despite these

differences, the decrease in LK was not significant when taking both regions together compared to a phylogenetic analysis based on the separate regions (LRT p = 0.07). The genetic differentiation in both regions was of the same order (0.4 – 3.6%), and slightly smaller than the genetic differentiation found in the V4 region. *Gobiusculus flavescensis* was infected with another species, of which only two specimens were found. The ITS1 region was identical to that of *G.* sp. 1 while the ITS2 region was identical to *G.* sp. 2. No mixing was possible since ITS1 and ITS2 were amplified at the same time, and sequence reactions were repeated twice. This species was excluded from the dataset since it would confound phylogenetic analyses.

The splits graph of the ITS1 sequences of Group B (Fig. 3) was similar when ITS2 and V4 were included, but the bootstrap support of the conflicting splits was lower. Including or excluding gaps also had an influence on the bootstrap values. The likelihood mapping analysis showed 8.6% of star-like phylogenetic signal for ITS1; 28.6% for ITS2 (Fig. 5) and 4.3% for ITS1 and ITS2 together. Whereas in Group A the sequences were evolving clock-like, the molecular clock hypothesis was rejected ($p < 0.001$). Also, large insertions and deletions of 19 bp were found in ITS1. The distances based on the whole ITS region were 10 times higher in comparison to the distances in Group A, while the V4 distances of both groups were about the same magnitude.

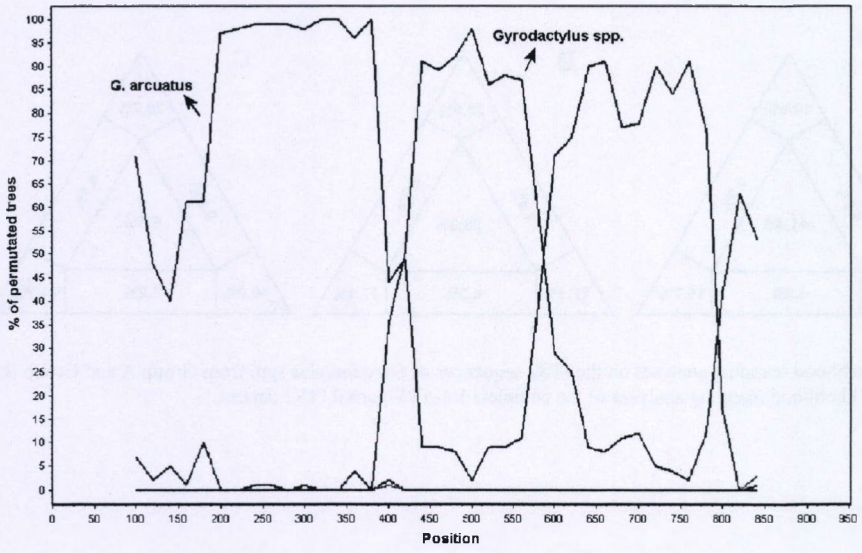


Fig. 4. Bootscan plot of the complete ITS sequences. *Gyrodactylus* spp. belonging to Group A were used as reference sequences; *G. nipponensis* was assigned as query sequence. Window size was 200 bp and the step size 20 bp.

3.4. Comparison with the morphological phylogeny

Diagnostic morphological characters as defined by Malmberg (1970) are mapped onto the phylogenetic tree constructed from the partial 5.8S and ITS2 dataset (Fig. 2). *Gyrodactylus branchicus* infecting the gill of *Gasterosteus aculeatus* appeared to be very closely related to *G. rarus* found on *Zoarces viviparus* in Trondheim (ITS2, p-distance of 1.7%). They belong to *G.* (*Metanephrotus*), which appeared monophyletic and formed a sister group to *G.* (*Mesonephrotus*) and *G. nipponensis*. *G.* (*Limnonephrotus*) was also monophyletic and appeared as a sister group to *G.* (*Paranephrotus*). This grouping of subgenera is consistent with the morphological phylogeny (Malmberg, 1970). There are however, two exceptions: (1) *G. nipponensis* and *G. anguillae* from *Anguilla japonica* and *A. anguilla* respectively, are regarded as members of the subgenus *G.* (*Neonephrotus*) (Ernst et al., 2000). Yet, they did not cluster, but were firmly joined with *G. (Mesonephrotus)* and *G.* (*Paranephrotus*) respectively. (2) *G. flesi* had a very distinct ITS sequence and clustered outside *G.* (*Paranephrotus*), to which it is currently assigned. The only other sequenced species belonging to *G.* (*Paranephrotus*) is *G. lotae*. However, only a partial ITS1 sequence is available and this fragment was more dissimilar to the present *G.* (*Paranephrotus*) spp. than *G. salaris* is. *Gyrodactylus elegans* branched off earlier than the other *Gyrodactylus* spp.; *Gyrdicotylus gallienni* clustered most basal.

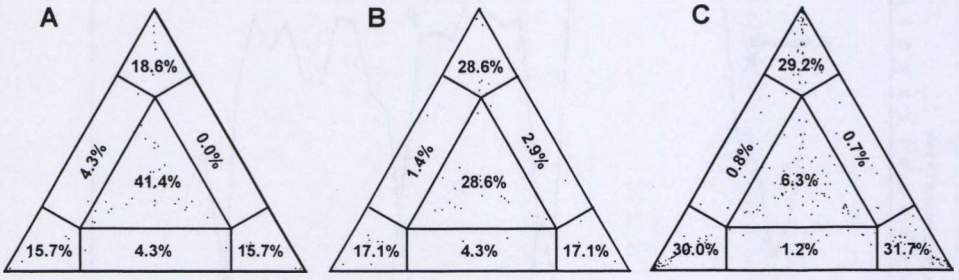


Fig. 5. Likelihood mapping analyses on the ITS2 sequences of *Gyrodactylus* spp. from Group A and Group B; C shows the likelihood mapping analyses of the complete V4-5.8S-partial ITS2 dataset.

4. Discussion

4.1. Inventory of the Gyrodactylus fauna on Pomatoschistus gobies

A total of 13 *Gyrodactylus* species were found on gobies of the genus *Pomatoschistus* and *Gobiusculus*. Some of them have been described (Gläser, 1974a; Geets et al., 1999; Huyse and Volckaert, 2002); the morphological description of the remaining species will follow (Huyse et al., in prep.). As generally expected for gyrodactylids, true generalists are underrepresented in the present study. Most species were highly host-specific, although some were found on two or three *Pomatoschistus* species. None of the other fish species examined in this study or in other studies (Gläser, 1974b; Geets, 1998; Zietara et al., 2000) were found to be infected with the present species, pointing to phylogenetic host specificity towards gobies of the genus *Pomatoschistus*.

The *Gyrodactylus* fauna could be separated into 2 groups; group A comprised of species belonging to the subgenus *G.* (*Mesonephrotus*) while the species of Group B belonged to the subgenus *G.* (*Paranephrotus*). Besides morphological characters that won't be discussed here, both groups differed in their genetic structure and in some ecological traits. Group A was mainly found on gills and was highly host-specific while Group B infected primarily the skin and fins of the host; one species infected up to three host species. Using the semi-quantitative classes of Desdevises et al. (in press), they might be regarded as specialist species (using only one host), intermediate specialist species (using two closely related hosts, e.g. *G.* sp. 1 and *G.* cf. *micropsi* 1 and 2) and intermediate generalist species (using two or more hosts but still in the same clade, e.g. *G. rugiensiodes*). In agreement with Desdevises et al., the present phylogenetic analyses showed that specialisation does not seem to be a derived condition and it does not lead to an evolutionary dead end: e.g. *G.* cf. *longidactylus* is a specialist branching off earlier than an intermediate specialist like *G.* cf. *micropsi*.

Monogeneans show a link between host body size and parasite specificity (Sasal et al., 1999; Simkova et al., 2001; Desdevises et al., in press). Larger hosts tend to live longer, providing a predictable and stable environment for parasites, allowing specialisation. The correlation could not be tested in the present system since each fish species was infected by both intermediate generalist and specialist parasite species. Moreover, gobies of the genus *Pomatoschistus* are small sized and short-lived (1-2 years), features that would make them a more unpredictable host. This might be somehow compensated by their exceptionally high abundance. Norton and Carpenter (1998) state that relative host abundance is the key to host specificity, although this feature was not statistically linked to specificity in the case of

monogenean *Lamellodiscus* species (Desdevises et al., in press). The link between host specificity and the number of potential hosts available (Poulin, 1992) is found for Group B but was absent in Group A. For example, *P. pictus* and *P. minutus,* occurring sympatrically in Norway, harbour a different gill parasite but they are infected by the same fin parasite. This suggests that in this particular system host specificity is shaped by phylogenetic influences (both gill parasites are sister species), and by ecological features of the host (habitat) and of the parasite itself (niche on the host). Furthermore, *G. rugiensis* and *G. rugiensoides* are sister species but while the first is a specialist species the latter is an intermediate generalist. The same is true for *G. micropsi* and *G.* cf. *micropsi.* The host species sharing the intermediate generalists also share habitat, while *P. microps*, which is harboring the specialist species, is more isolated by its niche (estuary). These observations show once more that specialization is not an irreversible condition and they confirm the impact of biological factors on host specificity.

A *Gyrodactylus-Pomatoschistus* association was found all over the distribution range of the host. The geographic variation was very low: one or two point mutations in ITS2 and in the ITS1 region seldom an insertion/deletion of 1-3 bp. The V4 region did not show geographical variation. *Gyrodactylus arcuatus* is quite frequently found on accidental hosts (Malmberg, 1970); in this study it was found, besides on its type host *G. aculeatus*, on *P. pungitius*, *P. microps* and *G. flavescens*. Very low intraspecific variation was found, although it is not clear whether this is due to geographic (Baltic Sea *vs.* Atlantic Ocean) rather than host related variation. In the literature, intraspecific and geographic variation was also found by Zietara et al. (2000; 2002) for *G. arcuatus, G. branchicus, G.* sp. 1 and *G. pungitii*. No intraspecific variation could be found in ITS2 sequences of *G. kobayashii* from the U.K. and Australia (Cable et al. 1999), nor for *G. anguillae* collected on *A. anguilla* from Spain and Australia and on *A. australis*, *A. reinhardtii* and *A. rostrata* (Hayward et al. 2001).

From the above paragraph we conclude that both the parasite as the host ecology provide the opportunity for host-specificity and host-specialization to develop. It has been suggested that the frequency of co-speciation tends to be higher if host specificity is also high, since host-specific parasites are usually phylogenetically conservative in their host choice (Rohde, 1993). This can be evaluated by means of phylogeny reconstruction: in case of co-speciation, the parasites involved should form a monophyletic group.

4.2. Are the gyrodactylids infecting gobies of the genus Pomatoschistus monophyletic?

Paraphyly of the *Gyrodactylus* spp. infecting the gobies suggests that at least two independent colonization events were involved. However, *within* the parasite groups A and B, co-evolution and co-speciation might have played an important role. Group A, mainly found on gills, is monophyletic and each host is infected by only one unique species, except for *P. minutus* and *P. lozanoi* who share *G.* sp. 1. Also, *P. microps* harbors two of those closely related species: *G.* sp. is exclusively found on gills and *G.* sp. 4 on fins. Co-existence of congeneric parasites on the same host species might be an indication of sympatric speciation by site shift. However, *G.* sp. is more closely related to *G.* sp. 1, 2 and 3 found on the gills of *P. minutus, P. lozanoi, P. pictus* and *P. norvegicus* respectively. Hence these parasites are more closely related to each other than they are to the parasites on the same host. Such a scenario can be explained by (1) strict co-speciation with their host or (2) host-switching followed by speciation. A combination of both scenarios is also possible, although it is very difficult to differentiate between them. Several statistical methods are available to test these ideas (Page, 1994; Huelsenbeck, 1997; 2000; Legendre et al., 2002), but most methods require a robust and resolved phylogeny for both hosts and parasites, preferably constructed from molecular data. In this study, evaluations are only based on the parasite phylogeny and the ecological background of the host.

A study of the *Gyrodactylus* fauna of sympatric fish species and an additional screening of GenBank showed that the most closely related species were *G. arcuatus* and *G. nipponensis.* Morphologically, *G. arcuatus* is remarkably similar to the present species (Geets et al., 1999) but genetically they differ 8.2% (ITS2, uncorrected p-distance). It is possible that Pleistocene conditions promoted host-switching from e.g. *G. arcuatus* of three-spined stickleback onto the various goby species; euryhaline *Pomatoschistus* gobies and stickleback might have shared the same refugium, e.g. in the Bay of Biscay (Nesbø et al., 2000). According to Bakke et al. (2002) host-switching in gyrodactylids has been facilitated by the mixing of fish strains following glaciation. For example, *G. salaris* and *G. teuchis*, infecting *Salmo salar* and *Onchorhinchus mykiss* respectively, are thought to have diverged within the North Sea ice lake and the Iberian salmon refugium, respectively, during the last ice age. Based on the linearized tree, the speciation events in Group A seem to have almost the same evolutionary age as the speciation of *G. salaris* and *G. teuchis.* However, Wallis and Beardmore (1984) state that the speciation of the *P. minutus* complex (*P. minutus*, *P. lozanoi* and *P. norvegicus*) should have occurred quite recently, suggesting that the Pleistocene

period was very important in the genealogical history of the host. During glaciation, populations were forced into separate refugia, initiating allopatric speciation. The alternating cycles of glaciation and deglaciation are believed to have speeded up the speciation process in all present day sister taxa (Avise and Walker, 1998). In this context, it could be that hosts and parasites co-speciated, triggered by the Pleistocene ice ages.

The close relationship between *G. nipponensis*, infecting the gills of Japanese eel *A. japonica*, and the present *G.* (*Mesonephrotus*) species was not suspected. Based on the ITS2 region this species was more closely related to *Gyrodactylus* sp. 1, sp. 2, ..., than *G. arcuatus* is. This is in contrast with the morphological characteristics and its taxonomic status (see below). Although it occurs on Japanese eel, *G. nipponensis* probably originated in Europe since it was reported there before the development of the international eel trade (Hayward et al., 2001).

By analogy with the literature on plant feeding insects (Bush et al., 1998; Emelianov, 2001; Graig et al., 2001; Via, 2001), *Gyrodactylus* spp. of Group A could also be regarded as 'host races'. Since the *Pomatoschistus* species involved are very abundant and occur in sympatry, they might belong to the same cruising range of actively dispersing gyrodactylids. As such, speciation by host-switching could be regarded as sympatric speciation. However, in the present study we adopted the definition of sympatric speciation of Brooks and McLennan (1993), implying speciation on the same host species (intra-host speciation). Such an example might be found in Group B: *G.* cf. *micropsi* and *G.* cf. *micropsi* 1 are each other's closest relatives and are found on the same host species (*P. lozanoi* and *P. minutus*). Hyperviviparity results in a very short generation time, allowing the parasite to evolve faster than its host. During asexual population growth, inbreeding might create different 'strains' of *Gyrodactylus* (Brooks and McLennan, 1993) but other, more complex mechanisms might be involved. A third congener, *G.* cf. *micropsi* 2, also found on skin and fins of *P. lozanoi*, is genetically very closely related to the former two species. However, *G. micropsi*, found on the gills and fins of another host *P. microps*, is more closely related to *G.* cf. *micropsi* and *G.* cf. *micropsi* 1 than *G.* cf. *micropsi* 2 is. Thus, the situation is more complicated and cannot be resolved with the present knowledge or without rigorous statistical analysis.

The two sister species *G. rugiensis* and *G. rugiensoides* are also found on different hosts: on *P. microps,* and on *P. pictus, P. lozanoi* and *P. minutus* respectively (Huyse and Volckaert, 2002). Such host-associated species complexes might suggest that co-speciation or speciation by host-switching shaped the observed pattern. If the genetic differentiation between the host-associated species complex is comparable to the differentiation between the

respective hosts, co-speciation is favored. However, given the lower host specificity displayed in Group B, host-switching should not be underestimated. The present study has also shown a close relationship between *G. anguillae* and Group B. Based on the 5.8S and ITS2 dataset *G. anguillae* appeared to be most closely related to *G.* cf *micropsi* 2, which might point to another relatively recent host-switching event, this time between *A. anguillae* and *Pomatoschistus* spp. The direction of the host transfer is more likely to be from *Pomatoschistus* to *Anguilla* since (1) there are more *Gyrodactylus* sister species on *Pomatoschistus* than on *Anguilla*, and (2) according to Malmberg (1970), the occurrence of a species with a specialized excretory system like *G. anguillae* on a primitive fish like *A. anguilla* points to a secondary infestation. When comparing sclerite morphology, similarities can be found in the shape of the haptor, ventral bar and marginal hook, although the sclerites in *G. micropsi* are larger. Malmberg (1970) found *G. anguillae* only on migrating elvers, which are relatively small and abundantly found in estuaries (like *P. microps*). In order to obtain a more complete picture of possible host transfer routes, it might be of interest to obtain sequences of the *Gyrodactylus* fauna of the Gadidae (*G. callariatis*, *G. pharyngicus*, *G. elegini*, all members of the subgenus *G.* (*Mesonephrotus*)) and the species infecting Cottidae, Pleuronectidae and Zoarcidae (*G. perlucidus* and *G. errabundus* belonging to the subgenus *G.* (*Paranephrotus*)).

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

As discussed above, only *G.* cf. *micropsi* and *G.* cf. *micropsi* 1 were each other's closest relative found on the same host. Each host species was infected by at least one *Gyrodactylus* species of Group A and one species of Group B. For example, *P. microps* was infected with *G.* sp. and *G.* sp. 4 of Group A, found on gills and fins respectively, while *G. rugiensis* belongs to Group B and was found on fins and skin. All three species are more related to species found on other host species than they are to each other. This points to an allopatric mode of species formation. At first sight it seems that the site of infection is constrained by phylogeny: Group A is mainly found on gills, while Group B mostly infects fin and skin. However, *G.* sp. 4 of Group A was exclusively found on fins, while *G.* cf. *longidactylus* mainly infected gills, in contrast to the other members of Group B. Hence, the niche of *Gyrodactylus* spp. can apparently switch in a relatively short evolutionary timescale.

4.4. Phylogenetic relationships within Gyrodactylus subgenera

Tree-like phylogenetic signal was very high in the V4-5.8S-partial ITS2 dataset comprising all species; the overall phylogeny was very robust and independent of the tree-building method. Phylogenetic relationships within subgenera were less clear. Both Split-decomposition and Likelihood mapping analysis showed the presence of star- and net-like phylogenetic signal in the dataset, with ITS2 producing more star-like phylogenetic signal than ITS1. Also, although Group B consists of very closely related species, the ITS and V4 regions were not behaving clock-like. This might point to the inadequacy of the ITS and V4 region to resolve interrelationships within the genus *Gyrodactylus*. However, bootscanning analyses showed a signal of a putative recombination event in the sequence of *G. nipponensis*, at the 3' end of ITS1. This was also reflected in the pairwise distance analysis where this species was most closely related to *G. arcuatus* when looking at the ITS1 sequences, while it was more closely related to the *Gyrodactylus* spp. of Group A when considering only ITS2 sequences. But, simulations show that rate differences may seriously affect the outcome of bootscanning and pairwise distance results and it might also create conflicting split graphs (Anderson et al., 2000; Worobey et al., 2002). As already reported above, the ITS regions displayed strong rate heterogeneity with a high correlation of rates among adjacent sites. This reflects the complex secondary structure of ribosomal RNA, characterized by stems and loops. If this would confound bootscanning analysis, the same recombination signature should have been found in all taxa, but this was not the case. Furthermore, according to Posada and Crandall (2001) several recombination events are needed before they can be detected and recombination methods do not seem to infer many false positives. Either way, only sampling of more species and more loci might help to discriminate between the possible causes and consequences.

The lowest pairwise genetic distances were found in Group A, ranging from 0.5 - 3.6% (complete ITS region). Sequence variation between *Gyrodactylus* species reported in the literature ranges from 2.7-56% and 1.5-38.7% for ITS1 and ITS2 respectively (Kimura distances from Matejusova et al., 2001). In a study on polystomatid monogeneans the ITS1 sequence variation ranged from 0.6-23.3% (Tajima-Nei distances, Bentz et al., 2001), while the ITS differences in the coral genus *Alcyonium* ranged from 0.3 - 39.0% (McFadden et al., 2001). In these latter species, shared polymorphisms in the ITS region were found, similar to what has been found in the present study. The authors presented two possible explanations: either the two species diverged very recently, or hybridization is responsible for the observed

pattern of shared polymorphism. ITS regions may prove useful for fine-scale comparisons, but in case of very recent (e.g. post-Pleistocene) divergences it is difficult to sort uniquely derived character states from random fixation of ancestral polymorphisms (Hillis et al., 1996). Species of Group A probably diverged very recently (see above), so this pattern might be the result of incomplete lineage sorting. However, recently sperm transfer has been observed between *G. arcuatus* and *G. gasterostei* both parasitizing three-spined stickleback (Scott et al., 2001). Nothing is known yet about the possible offspring resulting from such pairings, but it does show that hybridisation might occur.

4.5. Does the molecular phylogeny reflect the morphological phylogeny?

On the basis of six main types of protonephridial systems, Malmberg (1970; 1998) subdivided *Gyrodactylus* into six subgenera: *G.* (*Gyrodactylus*), *G.* (*Mesonephrotus*), *G.* (*Metanephrotus*), *G.* (*Paranephrotus*), *G.* (*Neonephrotus*) and *G.* (*Limnonephrotus*). A complex excretory system (*Gyrodactylus*) is considered as primitive, while the simplest systems (*G.* (*Limnonephrotus*)) are regarded as more advanced. As such, the excretory system of *G.* (*Mesonephrotus*) may have given rise to the system of *G. (Metanephrotus)* by a reduction of the lateral flame cells. This excretory system may have developed into that of *G.* (*Neonephrotus*) through the excretory bladders specializing for a constantly pumping function. It is suggested that it has also given rise to the subgenus *G.* (*Limnonephrotus*) through reduction of the excretory bladders. The absence of excretory bladders is thought to be a limnic adaptation since this character is shared with the freshwater subgenus *G.* (*Gyrodactylus*), while large bladders found in *G.* (*Paranephrotus*) might originally have been an adaptation to salt water. This subgenus probably developed from *G.* (*Mesonephrotus*) by a total reduction of the lateral flames and an enlargement of the excretory bladders (Malmberg, 1970). The system of the closely related genus *Gyrdicotylus* is in accordance with that in *G.* (*Mesonephrotus*). It has however, a lower number of lateral flames and a higher number of flame bulbs (Malmberg, 1998). The gyrodactylid genus *Gyrodactyloides* has no published record on its excretory system but according to Malmberg (pers. comm.) it has small excretory bladders and the system is most likely of the *G.* (*Metanephrotus*) type.

Five out of six subgenera were included in the present molecular analysis. Both *G.* (*Mesonephrotus*) and *G.* (*Metanephrotus*) appeared monophyletic and clustered strongly together. The genetic distance between them was similar to the distance found between *G.* (*Paranephrotus*) and *G.* (*Limnonephrotus*) that also appeared as sister groups. All members

of *G.* (*Paranephrotus*) formed a single monophyletic group, with the exception of *G. flesi.* Its aberrant clustering suggests that either it does not belong to *G.* (*Paranephrotus*) or that this subgenus is paraphyletic. The latter hypothesis might be supported by *G. lotae*, which is the only other member of *G.* (*Paranephrotus*) available from GenBank. Based on the partial ITS1 fragment (392 bp), the genetic difference with the other *G.* (*Paranephrotus*) species was higher than the difference between *G. salaris* (*G.* (*Limnonephrotus*)) and the present *G.* (*Paranephrotus*) species. The rare presence of lateral flames in the main canals in *G. lotae* is thought to be more primitive and the presence of rudimentary lateral flames in the excretory system together with short pharyngeal processes might indicate a relation to *G.* (*Mesonephrotus*) (Malmberg, 1970). This would suggest that the presence of large bladders (the most typical character of *G.* (*Paranephrotus*)) could also evolve paraphyletically. Malmberg (1970; 1998) described *G. anguillae* as the representative of the subgenus *G.* (*Neonephrotus*). Based on the similar sclerite morphology, Ernst et al. (2000) state that *G. nipponensis* belongs to the *G. anguillae*-species group (Malmberg, 1970). Molecular data suggest that the two species belong to *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*) respectively, thus making *G.* (*Neonephrotus*) polyphyletic or indicating that *G. nipponensis* is not a member of *G.* (*Neonephrotus*). However, the authors did not study the excretory system of *G. nipponensis*. Hook morphology alone is not sufficient for assessing the subgenus status (Malmberg, 1970). In either case, the present results do not support *G.* (*Neonephrotus*) as a distinct subgenus.

Confirmed by the present molecular analysis, a small excretory bladder appears to be the ancestral character state. The evolution of big bladders apparently happened more than once since *G.* (*Paranephrotus*) is likely to be paraphyletic, and bladders disappeared at least twice: in *G.* (*Gyrodactylus*) and in *G.* (*Limnoneprotus*). A complex excretory system characterized by many flame bulbs and lateral flame cells is confirmed to be primitive, with a decrease in number along the lineage leading from *G.* (*Gyrodactylus*) to the other subgenera. A further simplification of the excretory system by the loss of lateral flames evolved twice: in the lineage leading to *G.* (*Metanephrotus*) and the lineage leading to *G.* (*Limnonephrotus*) and *G.* (*Paranephrotus*). The length of the ITS fragment might be another, molecular character to map onto the tree. A short fragment appears to be ancestral, while a long fragment only occurs in the lineage leading to *G.* (*Limnonephrotus*) and *G.* (*Paranephrotus*). Apart from *G.* (*Gyrodactylus*), it is not possible to infer which subgenus is ancestral, nor is it possible to confirm that *G.* (*Mesonephrotus*) is the oldest subgenus. However at this level, more

conserved markers (e.g. ssrRNA) should be used to resolve this question, in combination with a complete dataset of *Gyrodactylus* species.

5. General conclusions

Gobies of the genus *Pomatoschistus* were colonized by at least two independent evolutionary lineages of *Gyrodactylus*, belonging to the subgenera *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*). Most likely the first group (A) evolved from a host-switch event of *G. arcuatus* from three-spined stickleback. If this host switch occurred before the speciation of the gobies, the host-parasite association might have evolved through co-speciation. In case of a more recent host-switching event, the present pattern might be the result of successive host-switching between the extant goby hosts. Paraphyly of Group B shows that host-switching even to other fish orders (*A. Anguilla*) occurred as well. The origin of this clade is still unknown, as no closely related species were found. Inclusion of other *G.* (*Paranphrotus*) species parasitizing e.g. Cottidae and Zoarcidae, will shed more light on this complex, but interesting system.

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CHAPTER 7

DISENTANGLING THE HISTORY OF THE "SAND GOBY" GROUP (GOBIIDAE, TELEOSTEI): MTDNA AND PALEOCLIMATIC HISTORY

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Abstract: The so-called 'sand gobies' are among the most abundant fish species of the Eastern Atlantic-Mediterranean, playing an important role in the aquatic ecosystem. They belong to four phenetic genera, *Pomatoschistus*, *Gobiusculus*, *Knipowitschia* and *Economidichthys*. Their small size and morphological similarity have given rise to considerable taxonomic confusion. Despite cladistic analyses on morphological and allozyme data, phylogenetic relationships remain unclear. Here we used both nuclear DNA (ITS1 locus) and mtDNA (12S and 16S fragments) as independent estimates of the phylogeny. Considerable ITS1 length differences, primarily due to the presence of several tandem repeats, were found between species and even within individuals. Therefore, phylogenetic analyses focused on fragments of the 12S and 16S mtDNA region that have been sequenced for 14 goby species. The four genera clustered as one monophyletic group as proposed on morphological grounds; with respect to the interrelationships however, some conflicts arose. *G. flavescens* and *K. punctatissima* clustered within the *Pomatoschistus* species, pointing to a paraphyletic origin of both genera or a flaw in the phenetic methodology used in goby classification. Furthermore, the differentiation between *P. minutus minutus* and *P. minutus elongatus* from the Adriatic is as high as the differences within the *P. minutus* complex. As such, it should be considered as a distinct species, by analogy with *P. norvegicus* and *P. lozanoi*. The resulting "star" phylogeny and the origin of the freshwater life-style in the sand gobies are most likely linked to the drastic alterations during and after the Messinian salinity crisis (end of the Miocene). The origin of the shallowest clades dated back to the Pleistocene epoch.

1. Introduction

The Gobiidae is one of the most diverse families of fish, occupying marine, brackish and freshwater habitats in the tropical and temperate seas of the world (Hoese, 1984; Miller, 1986). Among the eastern Atlantic-Mediterranean gobioid fishes, a so-called 'sand goby' group consisting of four phenetic genera can be recognized: *Pomatoschistus* Gill, 1864, *Gobiusculus* Duncker 1928, *Knipowitschia* Lljin 1927 and *Economidichthys* Bianco, Bullock, Miller and Roubal 1987 (McKay and Miller, 1997). They can be defined by lateral-line and meristic criteria (Miller, 1986; McKay and Miller, 1997). The sand gobies possess many interesting biological features such as courtship behavior, sneaking, cannibalism and sound production, making them an ideal subject of ecological, evolutionary and behavioral studies (Lugli and Torricelli., 1999; Lindstrom and Lugli, 2000; Jones et al., 2001a,b; Pampoulie et al., 2001; Mazzoldi et al., 2002). At the same time, some of their biological features seem to render them vulnerable to anthropogenic environmental change. Certain *Knipowitschia* and *Economidichthys* populations are especially vulnerable and careful management is warranted (Miller, 1990). A prerequisite for evolutionary studies and conservation management is a sound taxonomy and classification. In the past, many systematic difficulties have arisen, due to the small body size and superficial resemblance to each other (Webb, 1980). Because of the high species diversity and the paucity of taxonomically informative morphological characteristics, the gobies are viewed as one of the most difficult fish groups to classify and identify (Springer 1983; Winterbottom 1984). Several allozyme studies have been carried out (Wallis and Beardmore, 1983; 1984; McKay and Miller, 1991; 1997; Miller et al., 1994), resulting in conflicting phylogenies.

Pomatoschistus is the dominant gobiid genus of the Atlantic and Mediterranean coasts of Europe, comprising about 11 species (Miller, 1986). The species belonging to the *Pomatoschistus minutus* complex, namely *P. minutus, P. lozanoi* and *P. norvegicus*, are thought to have speciated only recently; although rarely, hybrids of the former two species have been reported (Fonds, 1973; Wallis en Beardmore, 1980). They occupy slightly different ecological niches, but since their breeding distributions overlap they must be regarded as truly sympatric. However, they spawn at different times and the nuptial coloration of *minutus*, *lozanoi* and *norvegicus* males is different, suggestion a role for reproductive and ethological isolation in preventing hybridisation (Webb, 1980). A subspecies of *P. minutus* is reported in the Mediterranean and Black Sea: *P. minutus elongatus* (Miller, 1986). Until now, only specimens from the Adriatic have been analysed by

means of allozymes, revealing a fairly high differentiation compared with Atlantic populations of *P. minutus* (Wallis and Beardmore, 1983).

The monotypic boreal genus *Gobiusculus* is represented by *G. flavescens,* a marine species with a midwater habitat. It is recorded from the eastern Atlantic excluding South-Eastern North Sea; Mediterranean records have to be confirmed (Miller, 1986). *Knipowitschia* contains a species flock of freshwater and euryhaline gobies occurring in the Black and Caspian basins, and two freshwater species are endemic to the Mediterranean. Its systematic status has been under much debate: it embraces a number of species otherwise placed in the separate genera *Orsinigobius* created by Gandolfi, Marconato and Torricelli (1985) and *Hyrcanogobius* Ljin 1930. The West Balkanian genus *Economidichthys* is thought to share common ancestry with *Knipowitschia*; it is characterized by a perianal organ that is unique among teleosts (Bianco et al., 1987). It comprises two endemic species, *E. pygmaeus* and *E. trichonis*, the latter being Europe's smallest freshwater fish (Economidis and Miller, 1990).

Besides *Gobiusculus flavescens, P. quagga* and *P. knerii* that have a midwater lifestyle, all sand gobies are benthic. Most of them are marine or euryhaline, with the exception of *P. canestrinii* (brackish and freshwater), *K. punctatissima* and *Economidichthys,* suggesting a polyphyletic origin of a freshwater life style. The only other freshwater gobiids from the Mediterranean region are *Padogobius martensii* and *P. nigricans.* Although initially it was suggested that the freshwater adaptation of all these species originated from the Messinian salinity crisis (Miller, 1990; see below), the high genetic distance between *Padogobius* spp. and the sand gobies based on allozyme and molecular sequencing analysis (McKay and Miller, 1997; Penzo et al., 1998) made the authors conclude that both groups acquired the freshwater lifestyle independently of each other. However, no consensus is reached yet, concerning the historical trigger of this adaptation.

Mitochondrial DNA has been proven useful for reconstruction phylogenetic relationships between gobiid species (Penzo et al., 1998). Here, fragments of the 12S and 16S rDNA region and the nuclear ITS1 region have been sequenced for 14 goby species (*Pomatoschistus, Knipowitschia, Economidichthys* and *Gobiusculus*) from the Atlantic, Mediterranean and Adriatic Sea. The first goal was to construct a robust phylogeny, that could be used as a basis to test (1) whether the nuclear tree corresponds with the mitochondrial tree, (2) the monophyly of the "sand goby group" and its respective genera, (3) the monophyly of the freshwater species (adaptation to freshwater happened only once) (4) whether the origin of the freshwater lifestyle is linked with hydrographic and paleoclimatic

events (e.g. the Messinian salinity crisis at the end of the Miocene), (5) whether the acquirement of a midwater lifestyle is constrained by phylogeny, and finally (6) to compare the results with the morphological phylogeny. The origin and evolution of this goby group are discussed in the light of the paleoclimatic and geological history of its habitat.

2. Material and Methods

2.1. Collection of material

Fourteen species of Gobiidae were collected along the North-Eastern Atlantic continental shelf, the Adriatic, and Mediterranean Sea. *Economidichthys pygmaeus* specimens were collected in freshwaters of Greece. Fish species, collection site, geographic distribution and habitat preference are shown in Table 1. The geographic distribution of the freshwater goby species is shown in Fig. 1. All specimens were preserved in 85% ethanol.

2.2. Amplification and sequencing of the ITS 1, 12S and 16S rDNA

DNA was extracted following the NucleoSpin Tissue protocol (BD Biosciences, clontech). The complete ITS1 region was analysed for 2 specimens per species. The primers MD1F: 5' CTT GAC TAT CTA GAG GAA GT 3' and 5.8SR: 5' AGC TTG GTG CGT TCT TCA TCG A 3' (Sajdak and Phillips, 1997) were used. The total reaction volume (25 µl) consisted of: 1x PCR buffer (Eurogentec, Seraing, Belgium), 0.5 mM $MgCl_2$ (Eurogentec), 200 µM of each dNTP (Amersham Biosciences, Sweden), 1 µM of each primer (Eurogentec), 1 µl template, 1 unit Taq polymerase (Eurogentec) and mQ-H_2O. The mixtures were layered with mineral oil, heated for 4 min at 97°C and subjected to 35 cycles as follows: 95°C for 30s, 55°C for 30s and 72°C for 45s and then cooled at 4°C. The PCR products were purified by means of GFX columns according to the manufacturer's instructions (Amersham Pharmacia). These products were used for cloning following manufacturer's instructions (TA cloning system, Invitrogen). The PCR products of the cloned products were purified by means of GFX columns and directly sequenced in both directions. Sequencing was done following the protocol of SequiTherm EXCEL II (Epicentre Technologies); 5% DMSO was added to overcome sequencing difficulties related to G/C rich templates. The reaction products were separated on a LICOR 4200 system and visualised on a 6% Long Ranger gel (FMI BioProducts). For each specimen two clones were sequenced.

Table 1. Goby species used in this study, collection site, number of specimens sequenced (12S and 16S) or accession number, habitat and natural distribution range.

Species	Collection site	Country	N sequences/ Accession nr	Habitat salinity	Distribution
Pomatoschistus minutus Pallas, 1770	Ostend/Trondheim	B/N	3/1	D/E	Eastern Atlantic, northern Mediterranean and Black Sea
	Etang de l'Arnel	Fr	2		
	Camargues	Fr	2		
	Venice lagoon	I	3		
P. lozanoi de Buen, 1923	North Sea	B	2	D/S	Eastern Atlantic (North sea to north-western Spain + Portugal
	Texel	NL	2		
P. pictus Malm, 1865	Bergen	N	5	D/S	Eastern Atlantic: Norway to Spain and Canary Is.
P. norvegicus Collet, 1902	Bergen	N	4	D/S	Eastern Atlantic (Lofotens to western English Channel) + Mediterranean
P. microps Kroye, 1838	Ostend, North Sea	B	3	D/E	Eastern Atlantic, Baltic Sea, north-western Mediterranean and Atlantic Morocco
P. marmoratus Risso, 1810	Venice lagoon	I	1	D/S,B	Mediterranean, Black Sea, Sea of Azov, Suez Canal, Iberian Peninsula
	Chioggio	I	1		
P. marmoratus sp. 1	Venice lagoon	I	2		
	Chioggio	I	2		
P. marmoratus sp. 2	Venice lagoon	I	2		
P. knerii Steindachner, 1861	Venice lagoon	I	1	M/S	Adriatic and Toscanic archipelago, Tyrrhenian Sea (?)
P. quagga Heckel, 1837			AF067277 AF067264	M/S	Western Mediterranean and Adriatic
Gobiusculus flavescens Fabricius, 1779	Trondheim, Bergen	N	3 2	M/S,B	Eastern Atlantic, from western Baltic to north-west Spain, Mediterranean (Sicily and the Adriatic)
Economidichthys pygmaeus Holly, 1929	Acheron river	G	3	D,F	rivers and streams of western Greece north Albania to be confirmed.
Knipowitschia panizzae Verga, 1841	Po-Delta	I	1	D,E	Adriatic and Tyrrhenian brackish waters; lake Trasimeno, Italy (introd)
K. panizzae sp.	Venice lagoon	I	1		
K. punctatissima Canestrini, 1864			AF067273 AF067260	D/F	Northeastern Italy, west Slovenia, north Dalmatia
Padogobius nigricans Canestrini, 1867			AF067270 AF067257	D/F	Only in rivers of west central Italy
P. martensii Günther, 1861			AF067274 AF067261	D/F	Italian rivers of the northern Adriatic, Dalmatian rivers Zrmanje and Krka
Gobius paganellus Linnaeus, 1758			AF067271 AF067258	D/E rocky	Eastern Atlantic, Mediterranean and Black Sea

B = Belgium; Fr = France; N = Norway; NL = The Netherlands; I = Italy; G = Greece; D = demersal; M = Midwater; E = euryhaline; S = stenohaline/marine; F = freshwater; B = brakish

An approximately 400 bp fragment of 12S and a 600 bp fragment of 16S mtDNA was amplified using the following PCR primers: 16SH 5'-CCGGTCTGAACTCAGATCACGT-3', 16SL 5'-CGCCTGTTTATCAAAAACAT-3' (Palumbi et al., 1991), 12SH 5'-TGACTGCAGAGGGTGACGGGGCGGTGTGT-3', 12SL 5'-AAAAAGCTTCAAACTGGG ATTAGATACCCCACTAT-3' (Kocher et al., 1989). Amplification reactions consisted of 1x PCR buffer, 1.25 mM $MgCl_2$, 1 μM of each primer, 1

unit *Taq* polymerase, 200 µM of each dNTP, 1 µl lysate and mQ H_2O. The mixtures of 25 µl were layered with mineral oil, heated for 4 min at 97°C and subjected to 35 cycles as follows: 95°C for 1 min, 64/54°C (12/16S) for 45 s, 72°C for 45s, followed by a final extension step at 72°C for 7 min. The PCR products were purified and directly sequenced in both directions as described above.

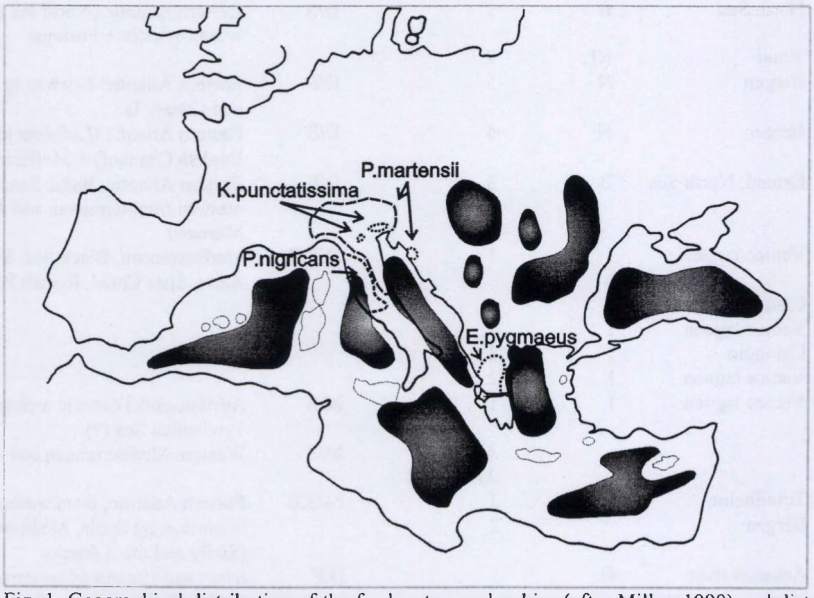

Fig. 1. Geographical distribution of the freshwater sand gobies (after Miller, 1990) and distribution of the late Miocene evaporates, indicated in grey (after Banarescu, 1992/Hsü, 1974).

2.3. Alignment of sequences

The first dataset consisted exclusively of sand goby species (see Table 1), and *Gobius paganellus* (AF067271, AF067258), *Padogobius nigricans* (AF067270, AF067257) and *Padogobius martensii* (AF067274, AF067261) were taken as outgroup. These sequences were aligned using the Clustal X multiple sequence alignment program (version 1.81, Thompson et al., 1997). In a second dataset the following GenBank sequences were included: *Gobius niger*, *Gobius buchichi*, *Gobius auratus*, *Zebrus zebrus*, *Zosterisessor ophiocephalus* and *Bovichtus variegatus* (AF067254 - AF067266; AF067257 – AF067279; Z32721, Z32702). In this case the sequences were aligned using the program SOAP (Löytynoja and Milinkovitch, 2001). It generates alternative CLUSTAL W alignments by using all possible combinations of gap opening penalty values ranging from e.g. 7-15 and gap extension penalty values ranging from e.g. 3-7. The program then identifies the "unstable-hence-unreliable"

characters by comparing the different alignments. These particular regions are then excluded and the file is exported into NEXUS or PHYLIP file formats. Afterwards, PAUP can re-include those characters and the impact of unstable sites on phylogeny reconstruction can be evaluated. Exclusion of the unstable characters in the 12S-16S alignment resulted in a 750 bp fragment.

2.4. Phylogenetic analyses

The 12S and 16S fragment were treated as one dataset since the incongruence-length difference test (Farris et al., 1995) implemented in PAUP* provided no evidence for significant difference in the phylogenetic signal of both regions. First, a consensus tree was made from the topologies obtained by TREE-PUZZLE 5.0 (Schmidt et al., 2002), maximum parsimony (MP), maximum likelihood (ML) and Neighbor-Joining (NJ) using PAUP* v. 4.01b (Swofford., 2001). This tree was used as input tree in the PAUP* command block from ModelTest 3.06 (Posada and Crandall, 1998). The parameters and likelihood scores were estimated upon that tree, and then the program chooses the model of DNA evolution that best fits the data based on the likelihood scores (LK). The parameters estimated under this best-fit model were entered in the ML search and nearest-neighbour-interchange branch swapping was performed. The respective parameters where then optimised upon this tree through successive iteration. Trees were statistically tested by calculating *P* values for the ML tree. With MP the exhaustive search method was performed using the branch and bound algorithm (100 replicates). In these analyses gaps were treated as fifth character or as missing data; all sites were equally weighted. The minimum-evolution search was conducted (1000 replicates of tree-bissection reconnection branch swapping) from a matrix of ML genetic distances calculated under the optimised model. The base composition for all sequences was compared using a 5% χ^2 test on the average composition (TREE-PUZZLE 5.0). The molecular-clock hypothesis was tested assuming the HKY model (Hasegawa, Kishino and Yano, 1985) and γ-distributed rates across sites, with the likelihood ratio test for the clock hypothesis implemented in TREE-PUZZLE.

The split decomposition method in the program SplitsTree 3-1 (Huson, 1998) does not attempt to force data onto a tree, providing an indication of how tree-like the data is. The evolutionary data is transformed into a sum of "weakly compatible splits" and then represented by a so-called splits graph. For ideal data, this graph is a tree, whereas less ideal data gives rise to a tree-like network that can be interpreted as possible evidence for different

and conflicting phylogenies. The phylogenetic content of a sequence alignment can also be visualized by the likelihood mapping analysis implemented in TREE-PUZZLE (Strimmer and von Haezeler, 1997). This method distinguishes between phylogenetic signal producing treelike topologies and phylogenetic noise, producing star- and/or netlike topologies. Plotting transitions and transversions against divergence of the complete dataset did not show any sign of saturation (DAMBE v4.0.75, Xia and Xie, 2001). In order to test for rate constancy among the different goby lineages, the two-cluster and branch length tests were performed using Lintree (Takezaki, Rzhetsky and Nei, 1995). Using constraint analyses in PAUP*, different topological constraints were constructed and compared using the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999). PAML v.3.1 (Yang, 2001) was further used for its implementation of a local clock model (Yoder and Yang, 2000).

3. Results

3.1. Characteristics of the ITS1, 12S and 16S sequences

The amplified ITS1 region varied considerably in length between the *Pomatoschistus* spp. The smallest fragment was found in *P. pictus* (694 bp), followed by *P. microps* (729/752 bp), *P. knerii* (734 bp), *P. marmoratus* sp. 1 and sp. 2 (752/748 bp respectively), *G. flavescens* (781 bp), *P. lozanoi* (820 bp), *P. norvegicus* (817 bp) and *P. minutus* (813 bp). They are deposited in GenBank under the following accession numbers: x. These interspecific length differences are due to two large insertions of about 29 and 39 bp, besides smaller insertions of eight and nine bp in the latter three sequences. *Gobiusculus flavescens* showed a similar pattern of (smaller) insertions, while *P. microps* and *P. marmoratus* showed only insertions of about two to five basepairs. In the sequence of *P. marmoratus* a $(GA)_{4,5,7}$ repeat was found. Also intraspecific length differences were found: two clones sequenced from one *P. lozanoi* specimen differed in 371 bp, due to a complete deletion of the central part of the sequence. The beginning and end of the fragment was identical in the two clones. Also *P. microps* showed intra-individual variation, the p-distance between the two clones was about 2%, resulting from point mutations and an insertion/deletion of a $(GAGAGGGAGA)_2$ repeat. Excluding all ambiguous regions resulted in a 614 bp fragment. The base composition of that fragment was biased towards [GC], with a percentage of 69.1%. (base composition p-value of 63-99%; with gamma shape parameter = 0.3; transition:transversion ratio = 1.6; clock not rejected). The pairwise distances between sister taxa were comparable to those generated with the 12S and 16S sequences, but the ITS1 distances between the *P. minutus* and *P.*

microps complex were considerably higher (about 12%). The topology (Fig. 2) is well resolved and independent of the treebuilding method used. Although the phylogenetic analyses do not seem to be influenced by the intraspecific and intra-individual variations, only 12S and 16S were used for further analyses.

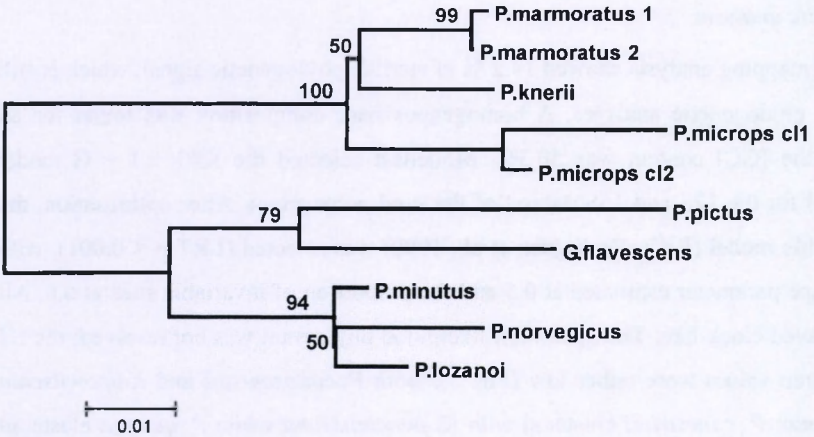

Fig. 2. NJ phylogram constructed with the ITS1 sequences of *Pomatoschistus* spp.

The amplified 12S and 16S fragments were about 400 and 580 bp respectively (GenBank, Accession Nos x). The specimens from *P. minutus elongatus* collected in the Adriatic Sea (Chioggio) differed in two transitions from the sequence of Penzo et al. (1998). In comparison with the specimens sequenced from the North Sea, 12 substitutions, two transversions and one insertion/deletion event (12S and 16S) were found. The 12S fragments of *P. minutus* collected from the North Sea (Belgium and Norway) were identical but differed in one substitution with the specimen from the Mediterranean Sea (Etang de l'Arnel). The 16S fragment was only sequenced from specimens from the Belgian North Sea and Mediterranean Sea (Etang de l'Arnel and Sète): they differed in one transition, while the specimen from Etang de l'Arnel showed 2 unique substitutions. *Gobiusculus flavescens* collected from Trondheim and Bergen (Norway) differed in one transition and one transversion in the 16S fragment (12S was not compared).

The sample of Venice lagoon appeared to be a mixture of species. One of the sequences was, besides one transition, identical to the *P. marmoratus* sequence of Penzo et al (1998) and will be referred to as *P. marmoratus*. Although morphologically not distinguishable from *P. marmoratus*, two other genotypes were found, here referred to as *P. marmoratus* 1 and *P.*

marmoratus 2, differing from 0.4 - 1.0%. Another specimen was determined as *K. panizzae* and showed 0.4% difference in the 16S fragment compared to the *K. panizzae* sequence of Penzo et al (1998); it will be referred to as *K. panizzae* sp. A last specimen was identified as *P. knerii.*

3.2. Phylogenetic analyses

Likelihood mapping analysis showed 14.2 % of starlike phylogenetic signal, which is still reasonable for phylogenetic analyses. A homogenous base composition was found for all members and the [GC] content was 50.3%. Modeltest selected the K80 + I + G model (Kimura, 1980) for the 12S and 16S dataset of the sand goby group. After optimisation, the general reversible model (REV, Rodriguez et al., 1990) was selected (LRT $p < 0.001$), with the gamma shape parameter estimated at 0.5 and the proportion of invariable sites at 0.6. All sequences behaved clock-like. The maximum-likelihood phylogram was not resolved; the NJ and MP bootstrap values were rather low (Fig. 3). Both *Pomatoschistus* and *Knipowitschia* were paraphyletic: *P. canestrinii* clustered with *K. punctatissima* while *P. quagga* clustered with *K. pannizae.* The bootstrap support was in both cases very low. The branch leading to the latter two species branched off earlier than the remaining goby species, but the branch length did not differ significantly from zero ($p = 0.403$). *Gobiusculus flavescens* clustered within the *Pomatoschistus* clade. Enforcing a monophyly of all *Pomatoschistus* species decreased the LK significantly at a level of 5%, not at a level of 1% (SH test: $p = 0.02$). The most strongly supported cluster is the *P. minutus* complex comprising *P. minutus, P. lozanoi, P. norvegicus* and *P. minutus elongatus* from the Adriatic Sea. Based on the pairwise distance matrix (Table 2, see appendix), the genetic differentiation within this cluster was rather low (about 1.3%). *Pomatoschistus microps* clustered with *P. marmoratus* and *P. knerii* although its position was not fully resolved. A topological constraint based on the morphological diagnostic characters as defined by Miller (1986) decreased the likelihood significantly ($p < 0.01$). Forcing the three freshwater species in a monophyletic group required one extra step in parsimony analysis and a SH test showed that this actually increased the likelihood score ($p < 0.01$). The three species with a midwater lifestyle (*G. flavescens, P. quagga* and *P. knerii*) clustered in three distinct clades. According to the two-cluster test *K. pannizae* evolved slower than *P. quagga*, and *P. minutus elongatus* slower than the remaining *P. minutus* complex, but both values were not significant. The root-to-tip distance for *K. panizzae, K. punctatissima, E. pygmaeus* and *P. canestrinii* differed from the average, although not

significantly. When allowing a different rate for the freshwater species in the local clock model of PAML, a faster evolutionary rate than the rest of the sand goby clade was calculated, although this did not improve the LK. The linearized tree based on the 12S and 16S sequences is shown in Fig. 4.

When including all other goby sequences, SOAP excluded the unstable regions resulting in a 750 bp fragment. TREE-PUZZLE showed a deviating base composition for *G. niger* and *Z. zebrus*; the clock hypothesis was rejected. The split graph (Fig. 5) represents the 'sand goby' group as a closely related monophyletic group, quite distant from all other gobiids, and the phylogeny resembled a star phylogeny. According to the two-cluster test, *K. panizzae* evolved significantly slower while *G. niger, G. buchichi* and *Z. ophiocephalus* evolved significantly faster.

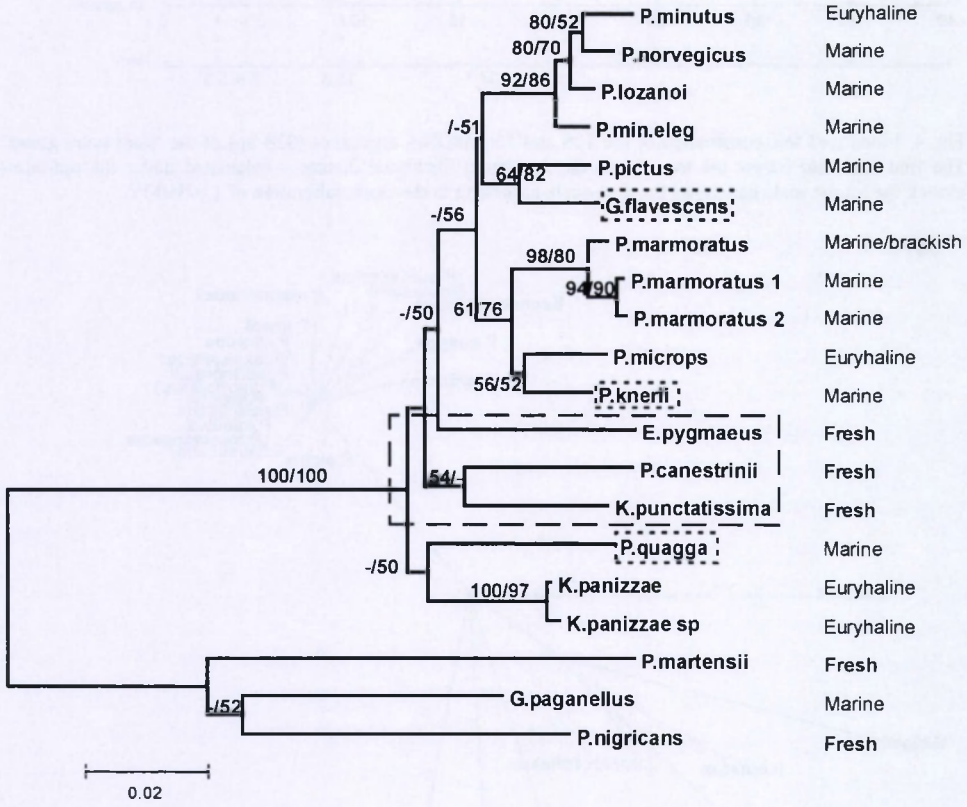

Fig. 3. Maximum-likelihood phylogram of 12S and 16S sequences (800 bp) of the 'sand goby group' constructed with PAML (Yang, 2001). (lnL = -3023.04; rmatrix = (2.1 7.6 4.3 1.4 7.7); $\alpha = 0.2$; $\rho = 0.84$; rates: 1, 0.4). Bootstrap values are shown for the MP/NJ analyses. (MP: 142 parsimony informative sites; tree length = 376; C.I. = 0.56; R.I. = 0.60). Sand gobies with a freshwater and a midwater habitat are framed; salinity tolerances are presented.

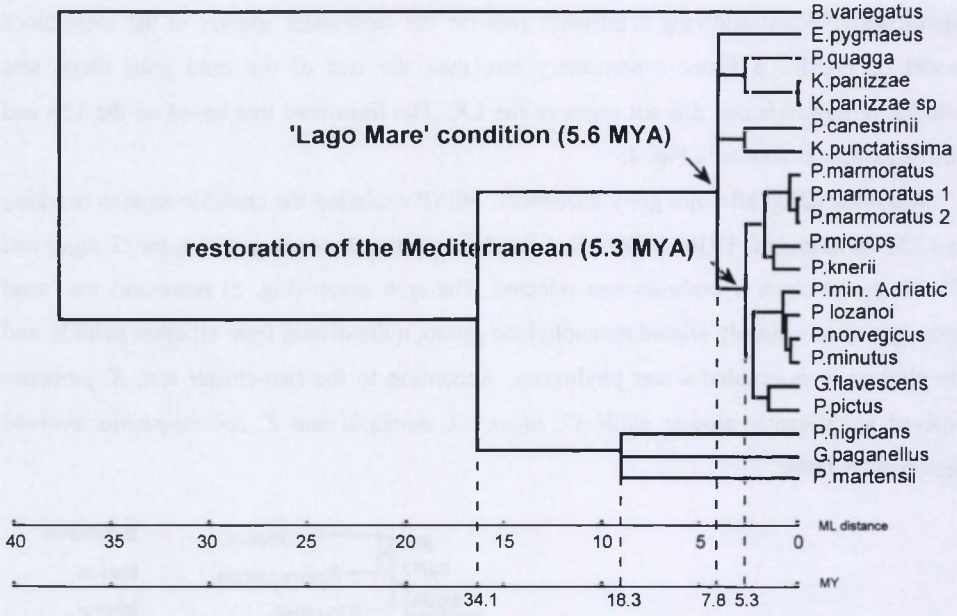

Fig. 4. Linearized tree constructed of the 12S and 16S mtDNA sequences (820 bp) of the 'sand goby group'. The first scale bar below the tree shows the maximum likelihood distances calculated under the optimized model; the lowest scale bar shows the time-scale according to the clock calibration of 1.07%/MY.

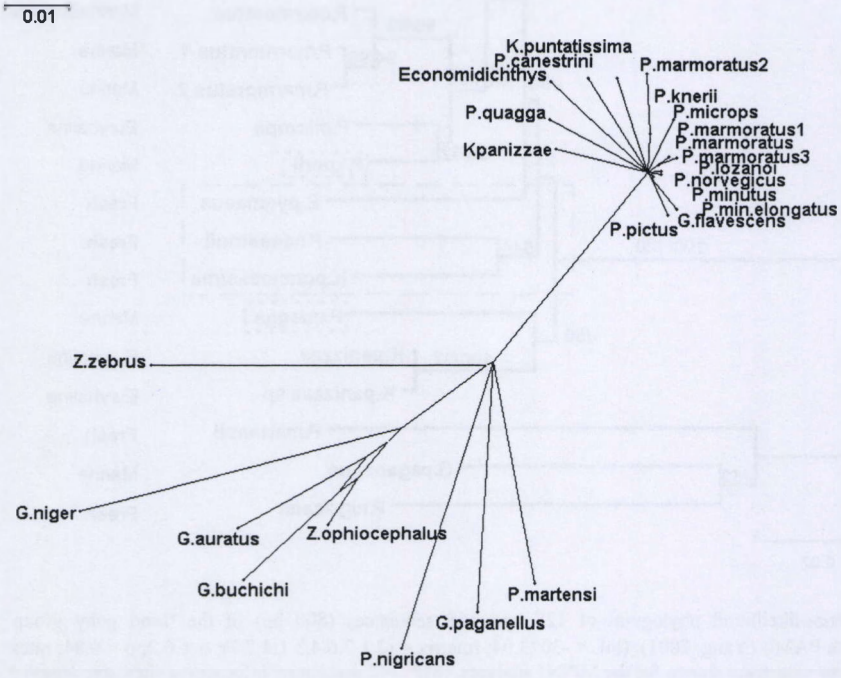

Fig. 5. Splits graph obtained from the 12S and 16S sequences (820bp) of the 'sand goby group' and related gobiids, Fit = 60.3; 1000 bootstrap samples.

4. Discussion

4.1. Phylogenetic relationships: morphology versus DNA

The molecular phylogeny agrees with the morphological criteria in the sense that the 'sand goby' group forms a monophyletic group of genetically closely related species, i.e. morphologically characterized by a distinctive sensory papillae pattern. This overall grouping was also confirmed by allozyme studies (McKay and Miller, 1997). With respect to the interrelationships within this group, some conflicts arose. Miller (pers. comm.) placed *P. marmoratus* together with the *P. minutus* complex, based on a shared character: the villi on the pterygoid membrane. An allozyme study of McKay and Miller (1997) supported this view. The present phylogenetic analyses however, showed *P. microps* and *P. marmoratus* as most closely related. This relationship was also found in an allozyme study of Wallis and Beardmore (1984) where they grouped *P. microps* and *P. marmoratus* within the *P. microps* complex. The position of *P. knerii* also conflicts with the view of Miller (pers. comm.). Based on morphology it was supposed to cluster with *Knipowitschia* while it grouped together with *P. microps* and *P. marmoratus* (Fig. 3). *Pomatoschistus microps* would belong to a separate group together with *P. tortonesii* and *P. bathi* (Miller, pers. comm.), but unfortunately, no material of these latter species was available. The clustering of *G. flavescens* within the *Pomatoschistus* clade suggests that it actually belongs to this genus. It clustered with *P. pictus* and although the bootstrap values were only moderate, this relationship was also found in the ITS1 phylogeny. The SH test showed that enforcing monophyly of *Pomatoschistus* significantly decreased the likelihood of the tree at a level of 5%. Both studies by McKay and Miller (1997) and Penzo et al. (1998), on allozymes and mtDNA sequencing respectively, reached the same conclusion. The position of *Economidichthys pygmaeus* could not be resolved, as such, its sister relationship with *Knipowitschia* (Miller, 1990) could not be confirmed. However, due to the low bootstrap values, no final conclusions can be made regarding the precise interrelationships. The SplitsTree analysis (Fig. 5) suggests that the whole group might have evolved simultaneously, which might explain the low bootstrap levels and the fairly low consistency index.

The backbone constraint analysis showed that the morphological phylogeny was significantly worse than the phylogeny obtained in this study ($p < 0.01$). This might suggest that convergent evolution might have played an important role in this goby group. For example the adaptation to a midwater lifestyle, implying a slightly emarginated caudal fin with black caudal spot, big lateral situated eyes and reduced transverse *c* rows occurred three times

independently in *G. flavescens, P. quagga* and *P. knerii* (Fig. 3). Stenohaline species are also found scattered throughout the tree: *P. norvegicus, P. pictus* and *P. quagga.* The same is true for the euryhaline species, being *K. panizzae, P. microps* and *P. minutus*, the latter two being at the same time most widely distributed species.

4.2. Phylogenetic relationships: nuclear DNA versus mtDNA

Since the ITS1 rDNA region showed intra-individual differences for some species, we did not continue sequencing the remaining goby species. However, the differentiation so far detected within *P. microps* seems to have accumulated after the speciation between *P. microps* and *P. marmoratus.* As such, ITS1 might be still useful for phylogeny reconstruction. However, more clones have to be sequenced before conclusions can be made. The obtained phylogeny based on nine species was in agreement with the 12S-16S phylogeny. The fact that phylogenies constructed with independent markers are in agreement with each other reinforces the reliability of the inferred phylogeny. Intraspecific and intra-individual length differences in ITS1 were mainly due to the presence of microsatellites. It thus seems that the homogenizing processes involved in concerted evolution, are not operating uniformly within *Pomatoschistus.* Sequence variation might exceed homogenisation due to factors such as dispersal of rDNA on various chromosomes (Van Herwerden et al., 1999; Vogler and De Salle, 1994). Although the genetic distance between *P. microps* and the *P. minutus* complex is relatively low (about 4% based on the 12S and 16S fragment), the karyological differentiation is remarkable (Webb, 1980). Regarding the cause and consequences of these karyological transformations, several hypotheses have been put forward. It might be that these transformations played a primary role in the origin of these species, or alternatively, they might be an incidental accompaniment of successful isolation. However, although the diploid complement of the goby *Aphia minuta* ranged from 44 to 41, it was in this case suggested that structural heterozygotes are fertile and that these chromosomal changes are not involved in speciation processes (Caputo et al., 1999). The third hypothesis proposes that the different karyotypes are adaptive to environmental conditions and thus being controlled by selection. Wallis and Beardmore (1984) found a correlation between the environmental heterogeneity experienced by the goby species and genetic variation at the level of enzyme loci and Webb (1980) found an increase in chromosome number with increasing environmental heterogeneity. *Pomatoschistus microps* occurs in estuaries, experiencing much more environmental changes than the stenohaline *P.*

norvegicus. However, before going deeper into the role of chromosomal rearrangement and concerted evolution in the sand goby group, more species and more clones have to be sequenced.

Many studies describing intra-individual ITS1 variation, dealt with complexes of sibling species (Vogler and DeSalle, 1994: Tang et al., 1996). As such, interbreeding might allow new alleles of the ITS1 to be introduced into a species at a level high enough to partially counteract the effects of concerted evolution (Tang et al., 1996). Hybridisation has been reported for *Tridentiger* and *Pomatoschistus* gobies (Mukai et al., 1997; Wallis and Beardmore, 1984).

4.3. Miocene origin of the goby ancestral fauna

The earliest fossil remains of gobies in the area covered by the Tethys were reported from the Eocene (54 to 38 MYA) sediments of Monte Bolca, Italy (Simonovic, 1999 and references therein). During the Oligocene and early Miocene, the Tethys covered the modern Indo-West Pacific Region and the modern Mediterranean Sea (see Fig. 6), enabling a reasonable exchange of fauna between both regions (Harzhauser et al., 2002). Until the early Middle Miocene, a tropical equatorial current flowed from the Pacific-Indian Ocean to the western Pacific areas promoting dispersal of fauna in an easterly direction. Around 16 million years ago (MYA), the point-of-no-return was reached by the closure of the Eastern Mediterranean seaway when Arabia was connected with Eurasia. From this point, the fauna of the modern Mediterranean Sea and the modern Indo-West Pacific Region evolved independently from each other.

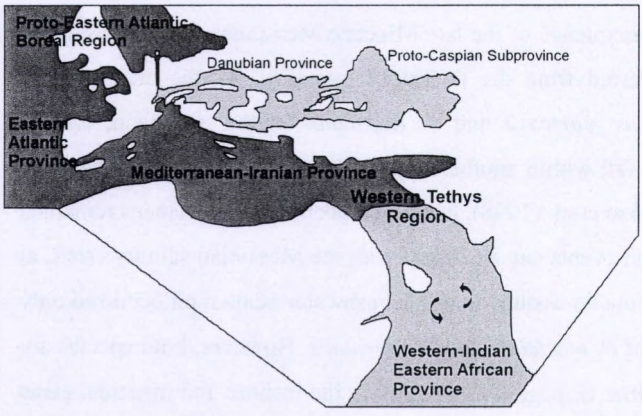

Fig. 6. Marine biogeography of the circum-Mediterranean area in the Oligocene - early Miocene. The Western Tethys Region is divided into the Mediterranean-Iranian Province and the Western Indian-Eastern African Province (after Harzhauser, 2002).

The present study confirmed the sand gobies as a distinct assemblage without an obvious sister group. The evolutionary gap between this line and the other Atlantic-Mediterranean gobiines is fairly high (see the splitsgraph in Fig. 5). According to McKay and Miller (1997) the most likely sister group to the sand gobies have to be looked for in the Indo-Pacific region, namely the genus *Nesogobius*. Unfortunately, no GenBank sequences were available for these species. However, the 12S region has been sequenced of members of the closely related genus *Tridentiger*, also considered as a putative sister group. Indeed, based on the gamma corrected pairwise genetic distances (not shown), *T. brevispinis* and *T. kuroiwa*, euryhaline and freshwater gobies from Japan and Korea, appeared most closely related to the sand gobies. Phylogeny reconstruction (on 300 bp of the 12S region) clustered *Tridentiger* together with the sand gobies, however more genes have to be sequenced to confirm this outcome. The eastern Pacific *Gillichthys mirabilis* might be a derivate from these western Pacific species that spread around the North Pacific when it was warmer (Miller, pers. comm.). Based on a 510 bp 16S fragment, it appeared more closely related to the sand gobies than the Atlantic-Mediterranean gobiines included in this study. These findings support the view of McKay and Miller (1997), and closure of the Atlantic-Mediterranean part of the earlier Tethys might be the major vicariant event that irrevocably separated the sand-goby and *Nesogobius-Tridentiger* stocks.

For now, there is no obvious geological or hydrographic event that could account for the separation of the sand gobies from the other Atlantic-Mediterranean gobiid lines, which apparently occurred before the separation of the Atlantic-Mediterranean from the Indo Pacific region. According to Miller (1990), the divergence of the freshwater Mediterranean (*Padogobius*) and West Balkanian (*Economidichthys*) lines from the Ponto-Caspian sister groups (*Knipowitschia*) was a consequence of the late Miocene Messinian salinity crisis (see below). However, as can be inferred from the linearized tree (Fig. 4), the origin of the freshwater lifestyle of *Padogobius martensii* and *P. nigricans* on the one hand, and *K. punctatissima* on the other hand, fall within another time frame. This was already stated by McKay and Miller (1997) and Penzo et al. (1998), but the respective circumstances remained unclear. Only one of the speciation events can be linked with the Messinian salinity crisis, at most. It would be most parsimonious to assume that the freshwater adaptation occurred only once in the ancestral population of *P. martensii* and *P. nigricans*. However, both species are more closely related with the marine *G. paganellus*, found in the inshore and intertidal areas of the Eastern Atlantic, Mediterranean and Black Sea (Miller, 1986) than they are to each other. Moreover, the morphological and genetic differentiation is fairly high, which is why

they previously were assigned a different generic rank (but united in the genus *Padogobius* by Bianco and Miller, 1990). Furthermore, although they occupy similar ecological niches in stream habitats, both species are geographically isolated from each other (Miller, 1990; see Fig. 1). Taking all facts together, it is most likely that speciation occurred in fully marine condition, before the Messinian salinity crisis. Only secondary, *P. nigricans* and *P. martensii* might have, triggered by the Messinian salinity crisis, invaded the Italian freshwater systems in the East and West respectively, while *G. paganellus* probably survived in the Atlantic Ocean.

4.4. The Messinian salinity crisis: triggering (basal) speciation in the 'sand goby' group?

About 5.96 MYA, the Mediterranean Sea passed through a salinity crisis during which most of its basins desiccated and turned into a desert (Hsu et al., 1977; Krijgsman et al., 1999). This period lasted almost seven hundred thousand years, and most of its ancient Indo-Pacific ancestral fauna got extinct. Closure of connection between the Mediterranean and the Atlantic Ocean about 5.59 MYA was followed by a large fall in water level and Sarmatic drainage into the desiccated basin lead to the origin of hyper- and hyposaline lakes. Canyon incision in the Aegean region for example, most likely caused the transition to 'Lago Mare' (sea-sized lake) conditions by capturing freshwater of the Black Sea drainage (Krijgsman et al., 1999). According to Miller (1990), this might have created an intense selection pressure leading to freshwater adaptation and colonization by stocks of euryhaline lacustrine gobies.

Taking the isolation of the Mediterranean Sea and subsequent origin of the Lago Mare system at 5.59 MYA, as a calibration point of the origin of the freshwater lifestyle, the patristic distances of the linearized tree (Fig. 4) would be translated into a rate of 1.53%/MY. The discontinuous distribution of the feshwater species may reflect their origin from congeneric stock isolated in different Lago Mare systems and subsequent isolation by Atlantic marine transgression (Miller, 1990). As such, *E. pygmaeus* might have originated in the big fresh water lake in the Aegean region and afterwards migrated into the freshwater system of Greece while the ancestor of *K. punctatissima* and *P. canestrinii* could have originated in a Lago Mare capturing fresh water from the Po-Delta.

With the opening of the Straits of Gibraltar (5.33 MYA) and subsequent re-flooding of the Mediterranean basins, the ancestral population of *K. punctatissima* and *P. canestrinii* was split up; one population leading to *K. punctatissima* retained the freshwater lifestyle while the other population, leading to the euryhaline *P. canestrinii*, had to adapt to the marine

environment. Furthermore, gobiine ancestors from the eastern Atlantic re-colonized the newly formed Mediterranean Sea. Adaptation to new ecotopes and free ecological niches might have lead to a radiation resulting in the present day fauna of which many are endemic to the Mediterranean Sea (Ahnelt, 1995). Since the low support of the relationships among taxa can't be attributed to the saturation of the 12S and 16S sequence data, the resulting "star" phylogeny might suggest that these goby species have speciated simultaneously, triggered by the same event. If the re-flooding of the Mediterranean at 5.33 MYA would be equated with the diversification of the remaining goby clade, this would invoke a slightly slower rate of 1.07 %/MY. This apparent difference in evolutionary rate can be tested by using the local clock model in PAML. Allowing a different rate for the freshwater species resulted in a faster rate than the remaining clade, however, this did not affect the likelihood score. This might be explained by a statistical flaw in the program (although allowing other taxa to vary in rate did not gave the same result), or this might suggest that the signal produced by the 12S and 16S fragment is not strong enough implying that more basepairs are required.

The estimates of the molecular clock agree with a general clock for vertebrate mtDNA.(1-2%/MYA, ref). Of course, many controversies exist regarding the molecular clock. The mtDNA clock is assumed to tick slower in poikilotherm fishes (Martin et al., 1992; Cantatore et al., 1994), however, it might be postulated that the fast generation time (about 1-2 years) and small body size (*Economidichthys* comprises the smallest European freshwater fish) would compensate for the slower clock generally assumed for fish (Martin and Palumbi, 1993).

It would be interesting to include the other freshwater species *Knipowitschia thessala* found in the Thessaly river system in Greece and the widespread euryhaline Ponto-Caspian *Knipowitschia caucasica*. According to Miller (1990) the latter species is closely related to *K. panizzae*. However, based on allozyme studies (Wallis and Beardmore, 1984) it shares common ancestry with *P. microps* and *P. marmoratus* since the divergence of the remaining *Pomatoschistus* spp. Molecular sequencing might clarify this issue and provide more 'test material' for the above scenario. If this scenario approximates the actual history of the gobies, then it must be concluded that the present taxonomy does not reflect the evolutionary history of the gobies (e.g. the clustering of *P. quagga* and *K. panizzae*).

4.5. The Pleistocene epoch as a trigger for speciation in the P. minutus complex

The shallowest relationships were found within the *P. minutus* complex and between the *P. marmoratus* genotypes sampled in the Adriatic. Applying a clock of 1 or 1.53 %/MY point into the direction of Pleistocene speciation, about 1.35 - 0.88 MYA. The Pleistocene glaciations were the most significant historical events during the evolutionary lifespan of most species. This period is named the "Great ice age", comprising up to 20 glaciation events, spaced out with warmer interglacial periods. These Pleistocene climatic cycles are believed to have speed up the speciation process in present day sister taxa (Avise and Walker, 1998). During glaciation, populations were forced into separated refugia, initiating allopatric speciation. This scenario could account for the speciation within the *P. minutus* complex. Examples of refugia could be found in the Bay of Biscay (Nesbo et al., 2000) and more down towards North Africa. However, another explanation might involve sympatric speciation by ecological specialization to different niches that came available during the interglacial phases. According to Wallis and Beardmore (1984b) it seems that speciation and habitat diversification may have been connected. *Pomatoschistus minutus* is euryhaline and can be found both in estuaries and open sea. *Pomatoschistus lozanoi* is more stenohaline and has a more epibenthic lifestyle compared to *P. minutus* (Hamerlynck and Catrijsse, 1994; Geets). Finally, *P. norvegicus* distinguishes itself by occupying the deeper parts, up to 200 m depth (Miller, 1986).

The Pleistocene cycling had also dramatic biogeographic consequences in the Mediterranean. The contacts of the Mediterranean Seas with the open ocean caused a fluctuation in the temperature and salinity of the Mediterranean (Por, 1975). About 25.000 to 18.000 years ago the fall in sea level narrowed the Siculo-Tunisian Strait, isolating the Adriatic from the rest of the Mediterranean. The shallower differentiation between *P. marmoratus* genotypes sampled in the Adriatic (0.4 - 1.0%, p-distances 16S) might be induced by the isolation of the Adriatic during the last Ice age. Today this area is still isolated by a topologically controlled cyclonic gyre in the South Adriatic pit (Magoulas et al., 1998), possibly limiting dispersal of pelagic larvae. This isolation is also reflected by the Anchovy distribution in the Adriatic (Magoulas et al., 1998).

However, the differences between the specimens sampled from the Adriatic and the rest of the Mediterranean is of another magnitude. They were similar to the interspecific differences between *P. minutus*, *P. lozanoi* and *P. norvegicus* and far exceeded the geographic variation found for *P. minutus*. Only two transitions in the 12S and 16S fragment

were found between *P. minutus elongatus* from the Mediterranean (France) and *P. minutus minutus* from the North Sea (Belgium and Norway), while 12 substitutions, two transversions and one insertion/deletion event (12S and 16S) were found when compared with the Adriatic specimens. Also at the allozyme level distinct differences between them were found (Wallis and Beardmore, 1984). These findings indicate that *P. minutus* from the Adriatic should be regarded as a distinct species, by analogy with *P. minutus, P. lozanoi* and *P. norvegicus*. During the early Pleistocene, a subpopulation of the ancestor of the *P. minutus* complex might have been isolated in the Adriatic, as a consequence of sudden drop in sea level, followed by an independent evolution. At the same time, speciation of the *P. minutus* complex in the Atlantic gave rise to *P. minutus*, *P. norvegicus* and *P. lozanoi* as described above. Another such species pair described in the literature is *P. pictus pictus* and *P. pictus adriaticus* (Miller, 1986). Unfortunately, no specimens were available for sequencing.

This hydrographic isolation of the Adriatic might also explain why *P. microps* does not occur there, despite its very wide distribution, from Norway to Morocco, including the Baltic and western Mediterranean Sea (and Mauritania and Canary Islands). The role of *P. microps* might be replaced by *P. marmoratus* in the Adriatic and partially in the remaining Mediterranean as well.

The low geographic variation between *P. minutus* specimens of the Western Mediterranean and the Atlantic Ocean can be attributed to the pelagic larval stage of the gobies, which enables them to migrate over large distances. Apparently, the Almeria-Oran front does not act as a zoogeographic barrier to drifting *P. minutus* larvae. Moreover, large effective population sizes are supposed to counteract differentiation caused by genetic drift or differential selection (Ward et al., 1994).

4.6. Reconstruction

The sand gobies have a tropical origin tracing back to the Oligocene – early Miocene, when an open seaway between the modern Indo-Pacific and Mediterranean region allowed exchange between both region's. West-east oriented surface currents along the northern shores of the Tethys facilitated the distribution of European gastropods as far southeast as Pakistan (Harzhauser et al., 2002). A similar scenario could explain the presence of *Tridentiger*, the proposed sister group of the sand gobies as far north in Japan. The separation of the Atlantic-Mediterranean part from the earlier Tethys (about 16 MYA) can be used as a calibration point for the speciation between the sand gobies and members of the genus

Tridentiger. Based on the Kimura-2p gamma corrected distances of the 12S fragment, the estimated rate approached about 0.8%/MY. When using the 16S fragment and *Gillichthys mirabilis* as a representative of the eastern Pacific stock, a clock of about 0.9% was obtained. Our estimated rates of 1 – 1.5%/MY (see above), suggests that exchange might have been possible somewhat after 16MYA. The freshwater species included in this study all have a restricted distribution, reflecting their dichotomous origin in different hyposaline lakes. While the ancestors of the freshwater diverged in the hyposaline lakes during the crisis, the species pairs *K. punctatissima-K. panizzae* and *P. canestrinii-P. quagga* might have diverged through allopatric separation after the restoration of the Mediterranean. During that time, the Atlantic population could re-colonize the Mediterranean Sea basins, giving rise to the many endemic taxa known today. The origin of the shallowest clades goes back to the Pleistocene epoch, probably initiated by allopatric speciation in separate refugia.

In the study by Penzo et al. (1998), a rate of 4.7 times faster than in other vertebrates was invoked to reconcile the history of the gobiids with the paleo-geological history of the Mediterranean. However, applying this rate would imply that the separation of sand-goby and *Tridentiger* stocks occurred only 3-4 MYA, when the Mediterranean was already isolated from the Indo-Pacific region. Sampling more loci might provide an independent assessment of the above-presented reconstruction.

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Appendix

Table 2. Kimura-2 parameter distances with gamma correction ($\alpha = 0.6$; pinvar = 0.5), based on the 12S and 16S mtDNA fragments of the sand gobies.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 *P.microps*	0	18	21	34	33	33	32	30	31	42	40	41	42	43	43	102	106	111
2 *P.knerii*	0.025	0	24	34	30	28	29	26	29	40	42	40	38	39	41	100	105	111
3 *P.marmoratus*	0.030	0.035	0	33	31	27	24	26	31	42	47	43	37	38	46	103	109	112
4 *P.minutus*	0.053	0.053	0.051	0	10	11	14	27	30	40	47	46	42	42	51	107	110	115
5 *P.norvegicus*	0.051	0.046	0.047	0.014	0	8	12	29	30	40	44	43	41	41	48	104	104	112
6 *P.lozanoi*	0.051	0.042	0.041	0.015	0.011	0	8	23	28	42	43	41	36	36	45	104	108	111
7 *P.minut.elong.*	0.049	0.044	0.036	0.019	0.016	0.011	0	23	30	41	44	41	36	38	45	99	104	107
8 *P.pictus*	0.045	0.039	0.039	0.041	0.044	0.034	0.034	0	18	40	43	39	38	38	46	105	107	114
9 *G.flavescens*	0.046	0.043	0.046	0.045	0.045	0.042	0.045	0.025	0	39	40	41	34	34	42	106	109	118
10 *E.pygmaeus*	0.071	0.066	0.070	0.066	0.066	0.070	0.067	0.066	0.063	0	46	43	44	44	48	107	110	116
11 *P.canestrinii*	0.068	0.071	0.082	0.082	0.075	0.073	0.075	0.072	0.065	0.077	0	36	43	43	49	105	111	117
12 *K.puntatissima*	0.068	0.066	0.072	0.077	0.070	0.067	0.067	0.063	0.066	0.072	0.057	0	42	44	44	101	103	111
13 *K.panizzae*	0.070	0.061	0.060	0.070	0.067	0.057	0.057	0.062	0.053	0.073	0.072	0.069	0	2	35	99	105	104
14 *K.panizzae* sp.	0.072	0.063	0.062	0.070	0.067	0.057	0.061	0.062	0.053	0.073	0.072	0.073	0.003	0	37	101	107	106
15 *P.quagga*	0.072	0.067	0.078	0.089	0.081	0.075	0.075	0.078	0.068	0.081	0.084	0.074	0.055	0.058	0	106	113	114
16 *G.paganellus*	0.266	0.257	0.267	0.284	0.264	0.269	0.251	0.275	0.273	0.283	0.280	0.250	0.249	0.256	0.29	0	63	74
17 *P.martensii*	0.292	0.291	0.300	0.313	0.279	0.300	0.284	0.298	0.300	0.305	0.318	0.261	0.275	0.282	0.326	0.122	0	81
18 *P.nigricans*	0.295	0.304	0.297	0.310	0.294	0.296	0.281	0.309	0.325	0.330	0.344	0.289	0.273	0.281	0.319	0.154	0.175	0

CHAPTER 8

TANGLED TREES: COMPARING THE PHYLOGENIES OF *GYRODACTYLUS* AND ITS GOBY HOST

Tine Huyse and Filip A.M. Volckaert

Abstract: The co-evolutionary history of *Gyrodactylus* spp. and their goby hosts was investigated using both topology-based and distance-based approaches. Independent phylogenies were constructed on the ssrRNA V4 region and the complete ITS rDNA region for the parasites, and on fragments of the 12S and 16S mtDNA for the hosts. The overall fit between both trees was found to be significant according to the topology-based programs (TreeMap and Treefitter); the distance based method (Parafit) found only a significant fit when the gill and fin parasites were analysed separately (the separate datasets included more basepairs). TreeMap and Treefitter postulated seven to eight co-speciation events, in combination with several duplications and a few host-switchings. The monophyletic group of host-specific gill parasites appeared to have evolved from a host transfer from *G. arcuatus*, which parasitizes three-spined stickleback, onto the gobies, followed by several host-switching events between the respective goby hosts. The timing of these events dates back to the late Pleistocene. ParaFit suggested that the host-associated species complexes found in the fin parasites had evolved through co-speciation, whereas the number of co-speciation events was not significant according to TreeMap. These conflicts reflect the differences between the underlying methods used. The occurrence of multiple parasite lineages on a single host and the lower host-specificity displayed by the fin parasites, is known to complicate the comparison in topology-based programs, whereas distance-based programs are not affected. Furthermore, these findings confirm that host-specificity and co-speciation are not always correlated with each other.

1. Introduction

The distribution of parasites is shaped by the immediate environment but is at the same time a product of a long ancestry reflecting associations of millions of years (Manter, 1966). Both factors influence parasite evolution, although their relative contribution may vary according to the particular host-parasite system. The reproduction and transmission strategy of the parasite play a major role. Vertically transmitted parasites tend to evolve together with their hosts (Clark et al., 2000), whereas host-switching is more common in horizontally transmitted parasites (Lopez-Vaamonde et al., 2001; Roy et al., 2001; Desdevises et al., in press). In the strict definition, co-evolution happens when two or more species influence each other's evolution (Ridley, 1996). In the definition of Brooks and McLennan (1991), co-evolution includes co-adaptation (mutual adaptation of host and parasite to each other) and co-speciation (joint speciation of host and parasite). In this paper we refer to co-evolution at the macro-evolutionary scale, where a complete agreement of host and parasite phylogenies is an indication of strict co-speciation. Molecular phylogenies are used as a tool to infer the relative contribution of the past (phylogenetic) and present day (ecological) events.

The best-studied example of strict host-parasite co-speciation is the pocket gopher-lice system (Hafner and Nadler, 1988; Hafner et al., 1993; Page, 1994; Hafner and Page, 1995). These wingless lice are restricted to pocket gophers, where they spend their entire life-cycle (Page and Hafner, 1996). Asocial hosts and a low mobility of the parasite seem to be prerequisites for co-evolution to occur. A related group of lice that can move independently of the host do not show such mirror-image phylogenies (Ridley, 1996). A high phylogenetic host-specificity and close relationships between parasite and host should promote co-evolution (Connell, 1980). According to Humpery-Smith (1989) phylogenetic specificity is exhibited by parasites manifesting 1) low pathogenicity, 2) high host-specificity maintained through time, 3) extensive colonization of a host group and 4) having a life-cycle closely linked with the host. All these characteristics are displayed by the *Gyrodactylus* spp. parasitizing the *Pomatoschistus* gobies (Huyse et al., submitted). These parasitic flatworms belong to the order of the Monogenea, which means that they only require one host to fulfil their life-cycle. However, in contrast to these ectoparasitic lice, gyrodactylids are quite mobile since they mainly depend on host-host contact for dispersal. They may survive for some time independently of their host and some kind of 'swimming behaviour' has been observed (Cable et al., 2002). As such; they can also be found on non-optimal or temporary host species (Malmberg, 1970). Moreover, their capacity of producing a viable deme from a

single colonization event is thought to promote speciation by host-switching (Brooks and McLennan, 1993). Several instances of ecological radiations are found in nature; e.g. the *G. wageneri* group primarily infects cyprinids but they are also found on sticklebacks, percids and cottids (Harris, 1993).

In a recent review, Bakke et al. (2002) pointed to the lack of host-parasite studies within the genus *Gyrodactylus*. According to Zietara and Lumme (in press) this is related to the limited set of morphological systematic characteristics among *Gyrodactylus*. This excludes phylogenetic comparisons below the level of *Gyrodactylus* subgenera and fish orders or families. However, according to Page et al. (1996) it is better to sample a closely related group than members from a larger group, to avoid misinterpretation or non-detection of events such as sorting and duplications. The prerequisites for co-evolutionary studies are (1) the availability of a sound alpha taxonomy of both host and parasite, (2) robust phylogenies of hosts and parasites, (3) although not essential, molecular phylogenies are preferably based on homologous characters, (4) wide taxon sampling, and (5) quantitative comparison of host and parasite trees by means of explicit statistical tests.

We use as a model the *Gyrodactylus* parasites living on gobies of the genus *Pomatoschistus*. Huyse et al. (submitted) studiend the phylogenetic relationships of *Gyrodactylus* using the complete ITS rDNA region and the V4 region of the ssrRNA. In order to minimize sampling bias, sampling has been extended over time and space, throughout the natural geographical distribution of the fish hosts. The phylogeny of the host has been reconstructed from 12S and 16S mtDNA fragments, and in order to compare homologous characters, the ITS1 locus has been sequenced as well. It has been shown that all gyrodactylids displayed phylogenetic host-specificity towards gobies of the genus *Pomatoschistus*. The fauna could be split in two groups, differing in certain genetic and ecological parameters. Group A represented a monophyletic group of closely related and host-specific species, mainly infecting gills, while host-switching was more frequent in Group B, dominantly found on fin and skin. It remains to be tested, whether this difference in niche is reflected in their evolution and distribution on the host species. Did this monophyletic group of gill parasites (indeed) evolve through co-speciation with the host, as generally expected for host-specific parasites (Poulin, 1992; Kearn, 1994)? And contrarily, did the group of the more generalist fin parasites evolve through repeated host-switching events instead of co-speciation?

2. Material and Methods

2.1. Host and parasite data

A wide range of *Gyrodactylus* species have been collected on *Gobiusculus flavescens* and several *Pomatoschistus* species (Gobiidae, Teleostei) along the North-Eastern Atlantic continental shelf and in the Mediterranean Sea (see Huyse et al., submitted). The *Gyrodactylus* parasites of the three-spined stickleback, *Gasterosteus aculeatus* were collected as outgroup species. Phylogenetic relationships of the *Gyrodactylus* parasites were inferred from the V4 region of the ssrRNA and the complete ITS rDNA region obtained in a previous study (Huyse et al., 2002). Analyses have been conducted on three datasets consisting of all species pooled, and Group A and B separately. Within the respective groups the 5.8S sequence was identical, so only the ITS1, ITS2 and V4 sequences were aligned using Clustal X v. 1.81 (Thompson et al., 1997). When pooling groups, the highly variable ITS1 region was skipped and only the V4, 5.8S and ITS2 sequences were aligned using the program SOAP (Löytynoja and Milinkovitch, 2002). This program identifies unstable sites, which can be easily excluded and re-included during subsequent analyses. The phylogenetic relationships of the goby hosts were derived from ITS1 rDNA and 12S and 16S mtDNA (Huyse et al., in prep).

2.2. Phylogeny reconstruction

First, a consensus tree was made from the topologies obtained by TREE-PUZZLE 5.0 (Schmidt et al., 2002), maximum parsimony (MP), maximum likelihood (ML) and Neighbor-Joining (NJ) using PAUP* v. 4.01b (Swofford., 2001). This consensus tree was used as input tree in the PAUP* command block from ModelTest 3.06 (Posada and Crandall, 1998). The parameters and likelihood scores were estimated upon that tree, and then the program uses the likelihood scores (LK) to select the model of DNA evolution that best fits the data. The parameters estimated under this best-fit model were entered in the ML search and optimised trough successive iteration. Trees were statistically tested by calculating *p* values for the ML tree. MP trees were inferred with the branch and bound algorithm (100 replicates). In these analyses gaps were treated both as fifth base and as missing data, all sites were equally weighted and different transition:transversion (ti/tv) ratios were applied; 10:5 for 5.8S and V4 region and 1:5 for ITS2. A minimum-evolution search was conducted (1000 replicates of tree-bisection reconnection branch swapping) from a matrix of ML genetic distances calculated under the optimised model. The molecular-clock hypothesis was tested assuming

the HKY model (Hasegawa, Kishino and Yano, 1985) and γ-distributed rates across sites, with the likelihood ratio test (LRT) for the clock hypothesis implemented in TREE-PUZZLE. Shimodaira-Hasegawa (SH) tests were used to compare alternative MP and ML topologies obtained during the analyses (Shimodaira and Hasegawa, 1999), as implemented in PAUP*. If no significant differences were found, only the ML topology was used; otherwise all topologies were investigated.

2.3. Testing for co-evolution

Nowadays, several methods for testing co-speciation are available, most of which have been reviewed by Paterson and Banks (2001). Four of these methods were used to analyze the host-parasite interactions in the present system. The first method TreeMap 1.1 (Page, 1994), reconciles the host and the parasite tree by introducing four types of events: co-speciation (C), host-switching (H), duplication or intra-host speciation of the parasite (D) and sorting, extinction of the parasite lineage (S). Using a parsimony argument, the program tries to explain the differences between both phylogenies by postulating the fewest possible number of these events, and maximizing the number of co-speciation events. A randomisation test was performed to assess if both phylogenies are more similar to each other than expected by chance alone. The probability of obtaining the observed number of co-speciation events was then calculated by randomising both host and parasite trees 1000 times to generate a null frequency distribution; the proportional-to-distinguishable model was chosen to generate random trees. Complete resolved trees are necessary, but alternative tree-topologies can be imported and evaluated. Recently, the beta version of TreeMap 2.0 is released (Page and Charleston, 2002 available at http://evolve.zoo.ox.ac.uk/software/TreeMap/main.html), which uses the algorithm Jungles (Charleston, 1998) to find all optimal solutions by exhaustive search. It also allows an assignment of different costs to each of the four cophylogenetic events, a feature also available in the program Treefitter 1.1 (Ronquist, 2001, available at http://www.ebc.uu.se/systzoo/research/treefitter/treefitter.html). The optimal reconstruction is the one that minimizes the global cost. Treefitter uses a permutational procedure to statistically test the overall cost and contribution of each type of event. Several costs have been applied to assess their effect on the reconstruction. For example, the default settings are C=0, D=0, S=1 and H=1, while the TreeMap setting correspond with C=-1, D=0, S=0 and H=0, maximizing co-speciation events.

Whereas methods are topology-based (except for TreeMap 2.0β), the program ParaFit (Legendre et al., 2002) makes use of genetic or patristic distances, thus overcoming the need of well-resolved topologies. It tests the null hypothesis (H_0) that the evolution of the two groups, revealed by the two phylogenetic trees and the set of host-parasite links, has been independent. It combines the information from three data matrices: (1) the observed host-parasite association links, (2) principal coordinates representing the phylogenetic distances among the parasites and (3) the transpose of the matrix of principal coordinates representing the host phylogenetic distances. These distance matrices can be computed from sequence data, DNA/DNA hybridization data or morphological characters. In the present study both the ML genetic distances and the patristic distances inferred from the host and parasite phylogenies were used.

Host-switching and co-speciation can also be detected by analyzing genetic distances themselves (Paterson and Banks, 2001), on the condition that hosts and parasites evolve at a constant rate (which may differ in both groups). Therefore, the genetic distances of a homologous rDNA fragments, i.e. the ITS1 region, from both the host and the parasite were compared in Table 1.

3. Results

3.1. Phylogenetic analyses: are the phylogenies resolved?

A total of about 800 bp of 12S and 16S mtDNA were used for the construction of the host phylogeny. Since the ML, MP and NJ topologies were not significantly different ($p > 0.05$), the ML tree was used as input-file. The parasite tree, including Group A and B, was constructed from 675 bp of 5.8S and the V4 and ITS region, and was not completely resolved. Separate analyses included a total of 1125 bp, resulting in fully resolved phylogenies. These phylogenies were used as backbone constraints in the analysis on the complete dataset.

3.2. Comparison of ITS1 variation between host and parasite

The ITS1 sequences of the gobies and the *Gyrodactylus* spp. of Group A were behaving clock-like according to the LRT in TREE-PUZZLE, in contrast to Group B. The genetic distances constructed from the ITS1 region of the hosts *Pomatoschistus minutus* and *P. microps*, and their respective parasites are presented in Table 1. Each time, the parasite sister species were compared (i.e. comparison within Group A and B, respectively). The ITS1

variation was too high to allow unambiguous alignment between species belonging to different groups (e.g. *G. gondae* and *G. rugiensis*). With exception of *G. rugiensoides* and *G. micropsi*, the variation was much greater for the host-pair than for the respective parasite species-pairs.

Table 1. Uncorrected p-distances (excluding gaps) constructed from the ITS1 sequences of *P. minutus* and *P. microps* and their respective *Gyrodactylus* parasites.

Host/parasite species	p-distance
P. minutus - *P. microps*	11.6%
G. gondae - *G. branchialis*	1.3%
G. micropsi - *G.* cf *micropsi* 2	3.6%
G. rugiensoides - *G. rugiensis*	2.1%
G. rugiensoides - *G. micropsi*	13.0%

3.3. Is there evidence for co-evolution?

TreeFitter

Using the default settings, the fit between the host and parasite phylogenies showed that the overall cost is significantly lower than expected by chance alone ($p = 0.01$; 1000 permutations). As such, there is a phylogenetic structure in the association. However, it could not be determined which co-phylogenetic event contributed to this since none of the p-values were significant. Lowering the cost of host-switching from 2 to 1.5, resulted in significant values for the number of host-switching events (6-8 events; $p = 0.008$) and the number of co-divergence events (6-8 events, $p = 0.034$). Applying TreeMap settings, the global fit between the two trees was not significant anymore ($p = 0.157$). By assigning a cost of 1 to sorting and host-switching events, the fit was significant ($p = 0.017$) with host-switching as the main factor contributing to this (4-6 events; $p = 0.023$). If co-speciation and sorting were assigned a very high cost (Fitch optimisation), the significant values disappeared, confirming the signal of co-speciation in the present host-parasite system.

TreeMap 1.0.

Without invoking any host-switching event, TreeMap had to introduce seven co-speciation events, nine duplications and 27 sorting events to reconcile both trees. By adding host-switching events (using a heuristic search) seven co-speciation events, eight duplications, one host-switching and 23 sorting events were postulated. By randomising host and parasite trees with the proportional-to-distinguishable option, a null frequency distribution was generated (Fig. 1). The observed number of co-speciations appeared significantly higher ($p = 0.01$) than expected by chance. The percentage of co-speciating nodes (i.e. the number of co-speciating nodes divided by the total number of nodes in the

parasite phylogeny, multiplied by 100) amounted to 44 %. The confrontation of the ultrametric host and parasite tree, with the respective host-parasite associations is shown in Fig. 2.

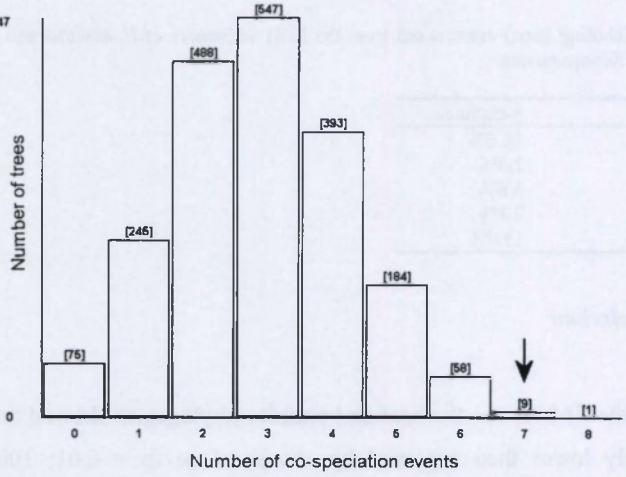

Fig. 1. Distribution of the number of co-speciation events generated by 1000 randomizations of *Gyrodactylus* and *Pomatoschistus* trees (TreeMap 1.1, Page, 1994). The arrow indicates the number of observed co-speciation events inferred for the complete dataset (p = 0.01).

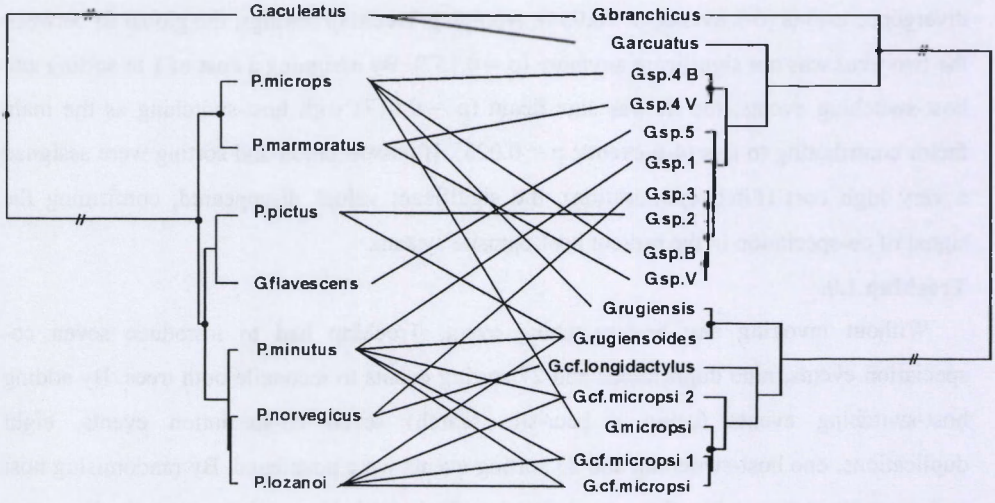

Fig. 2. Evolutionary patterns of host association in *Gyrodactylus* spp. Comparison of the goby host (left) and *Gyrodactylus* (right) ultrametric trees, constructed from 12S and 16S mtDNA and the V4 and ITS region, respectively. TreeMap 1.1 (Page, 1994) called upon 7 co-speciation events (denoted as a black circle; p = 0.01) to reconcile both trees. Branch lengths are proportional to the amount of evolutionary change, expect for the branches connecting the ingroup with the outgroup sequences. Branch lengths of hosts and parasites was significantly correlated (r=0.99; p = 0.034).

TreeMap 2.0β

Both host and parasite trees behaved clock-like as shown by the LRT performed in TREE-PUZZLE. As such, clock-like trees were calculated and imported in the timed analyses where branch lengths can be taken into account during reconstruction. Both host and parasite trees were 'scaled' by dividing the branch lengths by the respective evolutionary rate. Unfortunately, each analysis was aborted before ending, possibly due to a software bug. In the non-timed analysis, 22 optimal reconstructions were found. If the costs of duplications, losses and host switches are equated, six out of 22 came out as minimum-cost reconstruction. The optimal solutions postulated 16 co-speciation, 5 losses, 6 switches and 16 duplication events (Fig. 3). The randomisation test on the complete dataset suggested that the global fit between the host and parasite tree was statistically significant ($p = 0.02 \pm 0.01$). When the dataset was split up, only the association between Group A and the host tree appeared to be significant ($p = 0.01 \pm 0.01$), while the level of congruence between Group B and the host tree was not higher than expected by chance ($p = 0.11 \pm 0.03$).

Parafit

For each of the three datasets, both phylogenetic and patristic distances were used as input matrix. The global test of co-speciation on the complete dataset of phylogenetic distances showed that there was no global association between hosts and parasites (Fit = 0.134, p = 0.095). Using patristic distances did not influence the results (Fit = 0.066; p = 0.094). Considering the individual host-parasite links, only the links between the outgoup host and parasites were significant. However, when a separate matrix was constructed from the complete dataset of Group B (about 1300bp), the result of the global test was highly significant (Fit = 0.218, p = 0.001). Significant host-parasite links are amongst others: *G.* cf. *micropsi* and *P. minutus/P.lozanoi*; *G.* cf. *micropsi 2* and *P. minutus/P. lozanoi*; *G. rugiensoides* and *P. minutus/P. lozanoi/P. pictus.*

In contrast, when taking the gill parasites separately, only the link between *G. gondae and P. minutus/P.lozanoi* appeared significant (p = 0.023), besides the *G. arcuatus* and *G aculeatus* (p = 0.029). However, the overall signal was also significant, but the fit between both matrices was very low (Fit = 0.002, p = 0.029).

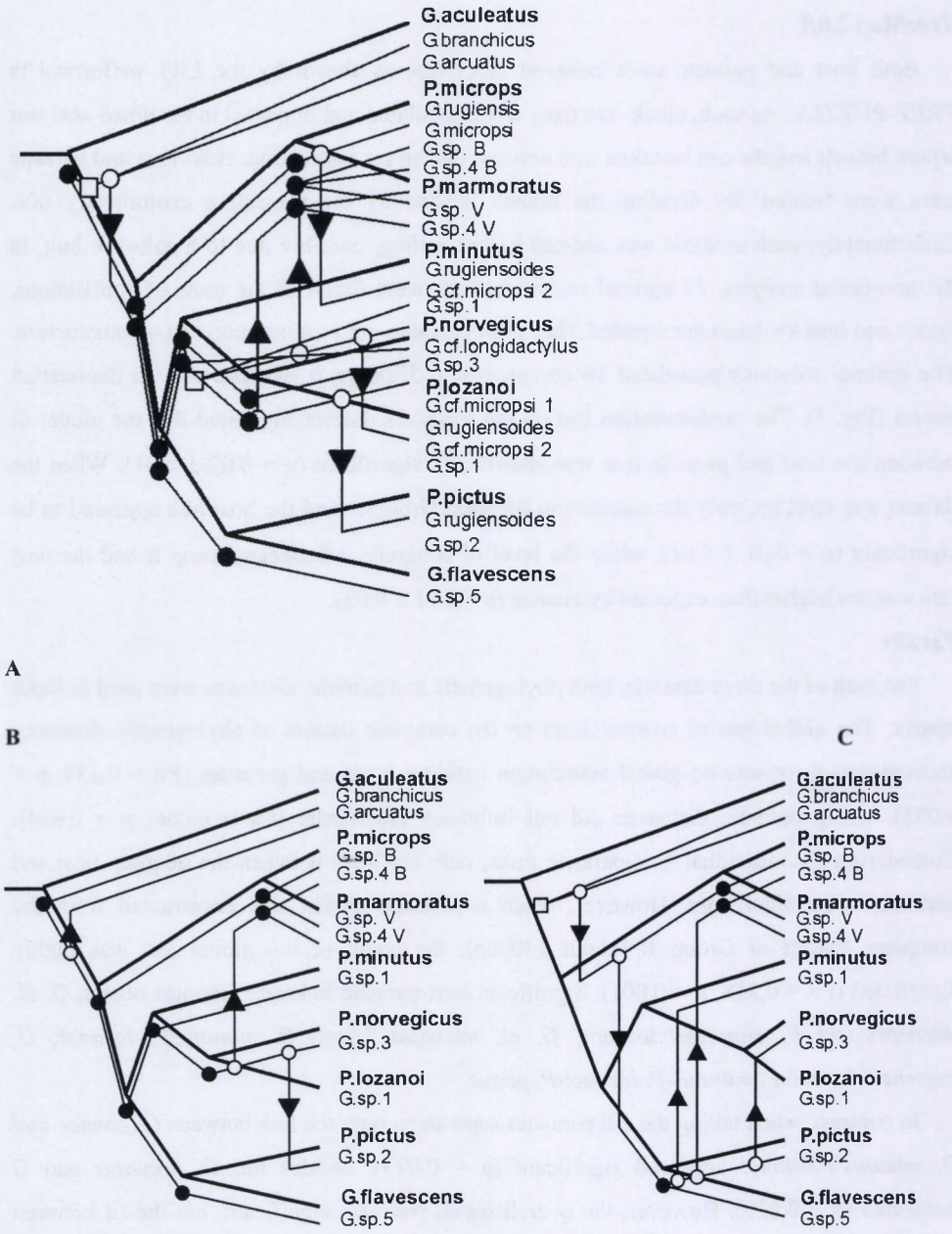

Fig. 3 Reconciliation by TreeMap 2.0β (Page and Charleston, 2002) of goby host and *Gyrodactylus* phylogenies. **A** shows the optimalisation for the complete dataset (p = 0.02 +/- 0.01); **B** and **C** represent two alternative reconstructions for Group A (p = 0.01 +/- 0.01). Arrows indicate a host-switching event; black circles indicate co-speciation; grey circles sorting; and white squares and circles indicate duplication events.

Table 2. Probabilities computed by ParaFit (999 permutations); the H_0 hypothesis of the global test is that the evolution of hosts and parasites has been independent (see bottom of the Table); the H_0 hypotheses in the tests of the individual host-parasite association links is that the link under test is random (Legendre et al., 2002). Probabilities in bold are significant at a level of 5%.

Gyrodactylus spp.	Host	Probability
G.branchicus	*G. aculeatus*	0.001
G. cf. *micropsi* 1	*P. lozanoi*	0.037
G. cf. *micropsi*	*P. minutus*	0.015
G. cf. *micropsi*	*P .lozanoi*	0.034
G. micropsi	*P .microps*	0.062
G. cf. *micropsi* 2	*P .minutus*	0.015
G. cf. *micropsi* 2	*P .lozanoi*	0.036
G. cf. *longidactylus*	*P .norvegicus*	0.062
G.rugiensis	*P .microps*	0.069
G.rugiensoides	*P .minutus*	0.013
G.rugiensoides	*P .lozanoi*	0.023
G.rugiensoides	*G .pictus*	0.030
Global test		0.001

4. Discussion

The evolutionary associations between *Gyrodactylus* spp. and their goby hosts appeared significant according to the topology-based programs TreeMap and Treefitter. The distance-based method TreeMap pointed to many instances of co-speciation, in combination with several duplications and a few host-switching events. According to Treefitter, both co-speciation and host-switching had a significant contribution, when the default cost of host-switching was slightly lowered. Biological background information on host and parasite is needed to be able to assign a cost to each specific event (Paterson and Banks, 2001). One can imagine that features such as dispersion mode of the parasite, its reproduction mode and host abundance are highly correlated with the likelihood of the various co-phylogenetic events. Taking the dispersing capabilities of *Gyrodactylus* into account, lowering the host-switching cost should be justified. In contrast to the aforementioned topology-based programs, the program ParaFit suggested that there was no significant fit between both datasets. These contrasting results most likely reflect the differences between the underlying methods (topology *versus* distance). However, when more basepairs were included in the analyses of the gill and fin parasites (Group A and B) separately, the respective fits were significant.

Although host-switching is expected to be an important speciation mode in *Gyrodactylus* (Brooks and McLennan, 1993; Harris, 1993; Kearn 1994; Bakke et al., 2002; Zietara and Lumme, in press), quantitative comparisons of host-parasite phylogenies are lacking. Zietara

and Lumme describe host-switching followed by adaptive radiation as the process behind the *G. wageneri*-species group diversification. The impressive colonizing capability of *Gyrodactylus* is documented by several switches between families of cyprinids, percids, esocids, and gasterosteids (Harris, 1993), while host transfer between different fish orders (Perciformes and Anguilliformes) has been described as well (Huyse et al., submitted). However, phylogenetic radiations have also been described, e.g. the *G. flesi*-species group infecting only pleuronectiform fishes (Bakke et al., 2002), but no molecular studies are available yet. The only quantitative host-parasite study of the monogenean-fish association has been conducted by Desdevises et al. (in press). No strongly significant signal of co-speciation could be detected, either by ParaFit, TreeMap or Treefitter. They conclude that specialization is mainly influenced by ecological factors. They found that all solitary fish species harbored only one parasite species, while the highest species richness was found on all gregarious species living closely together, with enhanced transmission between sympatric fish hosts.

So far, congruence is imperfect or absent for most kind of interactions (Poulin, 1998). Most associations represent a combination of co-speciation and host-switching (Page and Hafner, 1996; Lopez-Vaamonde et al., 2001; Roy et al., 2001; Ricklefs and Fallon, 2002; Weiblen and Bush, 2002). Examples of strict co-speciation can be found in systems where host-switching is prevented by the asocial life-style of the host and the low mobility of the parasite. Examples are found in rodent-lice and seabird-lice systems (Page and Hafner, 1996; Paterson et al., 2000; Paterson and Banks, 2001), and in insect-symbiont associations where the bacteria, needed for host reproduction, are transmitted maternally (Clark et al., 2000).

4.1. Is there evidence for co-evolution in the gill group of Gyrodactylus*?*

TreeMap suggested a significant fit between the host and parasite tree. According to the program the number of co-speciation events was higher than expected by chance, although host-switching events were suggested as well. Since this analysis does not take branch lengths into account, the closely related (sub-)species pairs found on *P. microps* and *P. marmoratus* are designated as co-speciation events. However, when ultrametric host and parasite trees are compared (Fig. 2); the branch lengths within both *Gyrodactylus* pairs are clearly shorter than those of the respective host pairs. This may explain why the significant fit disappears when only genetic distances were taken into account by the program Parafit. The low level of differentiation in the parasites, compared to the hosts, suggests an association by

recent host-switching rather than co-speciation. However, if the hosts would evolve much faster than their respective parasites, the association might still have evolved through co-speciation, as suggested by TreeMap and Treefitter. It is important to note that different gene fragments have been compared (12S and 16S mtDNA for the host *versus* ITS rDNA for the parasite), most likely evolving at a different rate. In general, the 12S and 16 mtDNA genes are suitable for phylogeny reconstruction above the genus level, while rapidly evolving ITS regions are useful for fine-scale comparisons (Hillis et al., 1996). However, this is not a general rule applying for all organisms. It is therefore difficult to make such comparisons and it highlights the need of homologous gene fragments in both host and parasite species (see below).

Despite the monophyly of Group A and its inclusion of highly host-specific gill parasites (Huyse et al., subm), host-switching appears to be the important mode of speciation in this system (Fig. 3). A fairly early host-switch is suggested, from *G. aculeatus* onto the ancestors of *P. microps* and *P. marmoratus*. From there it spread onto the other *Pomatoschistus* species by means of several host transfers. *Gyrodactylus* sp. 1 parasitizing both *P. minutus* and *P. lozanoi* might be the consequence of current host-switching between these sympatric hosts or it might be an instance of 'inertia' (Paterson and Banks, 2001), where a parasite species remains the same despite speciation of its host.

4.2. Is there evidence for co-evolution in the fin group of Gyrodactylus*?*

In contrast to the previous case, TreeMap found no congruence between host and parasite trees while the fit was highly significant according to Parafit. Page (1993) pointed out that the presence of one or more parasite lineages on the same host results in incongruent host and parasite phylogenies. As can be seen from Fig. 2, up to four *Gyrodactylus* spp. were present on a single host species. Also, the lower host-specificity complicates the comparison of the host and parasite phylogenies. *Gyrodactylus rugiensoides* for example, is found on *Pomatoschistus minutus, P. lozanoi* and *P. pictus*. However, a lack of pronounced host specificity does not exclude the possibility that a parasite has evolved with one of its hosts and has colonized the other hosts without speciating (Brooks and McLennan, 1993). This shows that host-specificity is not a prerequisite for co-speciation. According to Brooks and McLennan (1993), the traditional assumption about host-specificity and congruence between host and parasite phylogenies is a widely held belief. They pointed to the host-specificity paradox: "specialist parasite species are the least likely to colonize new hosts, but they are the

ones most likely to speciate as a result of any such switch since they should be more sensitive to changes, while generalists are most likely to colonize new hosts, but they are the least likely to speciate as a result of the interaction".

The *P. minutus* complex consists of the closely related gobies *P. minutus, P. lozanoi* and *P. norvegicus*. *Pomatoschistus minutus* and *P. lozanoi* occur in sympatry which might explain why they share so many *Gyrodactylus* spp. (e.g. *G.* sp. 1, *G. rugiensoides, G.* cf. *micropsi*). However, the fact that *P. lozanoi* also harbours a unique parasite *G. longidactylus* (Geets et al. 1998), proves that host-switching does not always occur whenever possible. *Pomatoschistus norvegicus*, which is more isolated by occupying the deeper sections of the continental shelf up to 200 m depth (Miller, 1986), is found to be infected with a similar species here referred to as *G.* cf. *longidactylus*. Morphological analysis showed distinct differences between both species (pers. data); a molecular comparison is in progress. This indicates the possibility of another host-associated species complex as previously described for the species pairs *G. rugiensis* and *G. rugiensoides*, and *G. micropsi* and *G.* cf. *micropsi* (Huyse and Volckaert, 2002).

The dominant mode of speciation in the present system appeared to be allopatric. However, one instance of recent intra-host speciation might have led to *G.* cf. *micropsi* and *G.* cf. *micropsi* 1, who are each other's closest relative found on the same host. The programs TreeMap and Treefitter also indicated the importance of historical duplication or sympatric speciation events. This can be expected from the biology and population structure of *Gyrodactylus* characterized by high host specificity and auto-infection of their hosts. It has been stated that if sympatric speciation occurs, it is most likely in parasite groups like monogeneans (Brooks and McLennan, 1993; Gusev, 1995; Poulin, 1998; Poulin 2002).

4.3. Tentative timing of events

It is always very difficult to estimate the evolutionary rate for a certain gene fragment, especially when no fossil data are at hand. In case of the tiny, soft bodied *Gyrodactylus* flatworms, only well documented (recent) vicariance events can provide criticial information. Recently such an attempt has been made by Zietara and Lumme (in press), their estimate was based on the divergence of *G. aphyae* living on the minnow *Phoxinus phoxinus* on the opposite sides of the Baltic - White Sea watershed. Connecting this divergence with the divergence time of the host, a rate of 5.5%/million years (MY) was obtained. If this clock is applied, most of the (sister) speciation events in Group A and B fall within the Late

Pleistocene period. In case of the hosts, speciation is thought to have occurred in the early Pliocene, with exception of the *P. minutus* complex that originated in the Pleistocene (Huyse et al., in prep). In this case, all recent speciation events in *Gyrodactylus* should be the result of duplication or host-switching. The genetic distance between *G. arcuatus* and the *Gyrodactylus* spp. found on the gobies, would be translated into a speciation event of about 1.82 - 2.05 MY. Such timing would support the scenario of host transfer between three-spined stickleback and *Pomatoschistus* gobies (see Fig. 3B) when sharing the same refugium, e.g. the Bay of Biscay (Nesbo et al., 2000) during the Pleistocene ice ages. This event might have been followed by a combination of host-switching and co-speciation with the respective gobies. According to Parafit *G.* sp. 1 has evolved through co-speciation with its hosts *P. minutus* and *P. lozanoi.* Indeed, the *P. minutus* complex is thought to have speciated in the Pleistocene period as well (Huyse et al., in prep.), which corresponds with Fig. 3 B. Applying a clock would estimate the speciation between e.g. *G.* sp. 1 and *G.* sp. 5 (found on *G. flavescensis*) at 0.18 MY. However, since the speciation between *G. flavescens* and *P. minutus* is much older (Huyse et al., in prep), co-specation with the respective hosts as suggested by Fig. 3 B, is rejected. Rather, the scenario described in Fig. 3C, characterized by successive host-switching events between the goby species, is favoured. Only the deeper nodes in Group B, e.g. the speciation between *G. micropsi* and *G. rugiensis* (estimated at 3.51MY), would correspond with the estimated speciation between their hosts *P. microps* and *P. minutus* (Huyse et al., in prep).

Another estimate can be achieved by extrapolating clock estimates from other animal groups. Schlötterer et al. (1994), suggested a rate of 1.2%/MY for the ITS1 region of *Drosophila.* Applying this clock would result in speciation events shifted to the early Pleistocene – Pliocene, favouring the scenario of Fig. 3B. However, it was found that the percent variation in the ITS1 locus from *P. minutus* and *P. microps* was much greater than the variation between the respective parasites (Table 1). This suggests a more ancient speciation of the hosts then of its parasites, unless the ITS1 region of the parasite evolves slower. In a study on salmonids (Pleyte et al., 1992) a rate of 5%/100.000 years was suggested. If this clock also holds for gobies, this would mean that the parasite evolves slower than its host. There are however, several reasons why we should not use ITS1 as marker for such purposes. First of all, more information has to be gathered concerning the prevalence, magnitude and origin of the intra-individual variation in the fish ITS1 region (Huyse et al., in prep). Moreover, the ITS1 region did not evolve clock-like for all *Gyrodactylus* species, precluding comparison of relative rates in different species groups.

4.4. Considerations and future perspectives

There are maximum-likelihood methods available (Huelsenbeck et al., 1997; 2000) that can test the robustness of the molecular data used for phylogeny reconstruction. This is very useful as a complementary test to the topology-based programs, which do not take the support of co-speciating nodes into account. One major drawback is that duplications and sorting events are not considered and the methods are still in their infancy (Paterson and Banks, 2001). Here, we used the program ParaFit as an alternative and complementary approach to the complex study of co-evolutionary associations.

Once substantial co-speciation has been found, a comparison of the evolutionary rate in the same gene, over the same period of time, in distantly related organisms as host and parasites, may reveal underlying evolutionary processes with a high degree of universality (Page and Hafner, 1996). Due to the limitations of the ITS1 locus (see above), attempts have been made to find another homologous marker. Currently, the 16S mtDNA is being sequenced for *Gyrodactylus* spp. Distances between a 350 bp fragment sequenced in *G.* sp. and *G.* sp. 2 reached 3.7%, while their respective hosts, *P. microps* and *P. pictus*, differed 3.6% in a 550 bp fragment of 16 mtDNA (Kimura 2-paramter model). Although preliminary, these promising results should shed more light on the present *Gyrodactylus-Pomatoschistus* system.

Page et al. (1996) suggested host transfer experiments to identify constraints on natural host-switching. In this particular system, it might be very interesting to conduct experiments in order to explain the absence of some parasite taxa on certain host species. For example; *G. longidactylus* is host-specific to *P. lozanoi* (Geets et al., 1998), transferring this species onto *P. minutus* might provide information on the degree of host specificity. Both host species are very closely related and occur in sympatry. If *G. longidactylus* is unable to survive on *P. minutus*, this suggests a specificity on a very high level, i.e. although both hosts are still able to hybridise in nature, they emit different chemical cues discernable to the parasite. This experiment can be repeated with *G.* cf. *longidactylus* found on *P. norvegicus.* In this way, it can be tested whether chemical cues rather than niche separation of the host is responsible for the absence on *P. lozanoi* and *P. minutus.*

Expanding the analysis to other closely related sand gobies, might shed light on the importance of accidental host-switching and primary host species. Preliminary analysis showed specimens of *Pomatoschistus knerii and Knipowitschia panizzae* collected in the Venice lagoon (Italy), to be infected with *G.* sp. 5, which is normally found on the fins of *P.*

marmoratus. *Economidichthys pygmeaus* collected in the Acheron river in Greece, was also found to be infected with two *Gyrodactylus* species, found on the gills and fins respectively. Until now, only morphological data are available but genome sequencing is in progress.

In conclusion, the evolutionary history of *Gyrodactylus* spp. and their goby hosts has gone through periods of co-speciation, followed by (and intermingled with) periods of successive host-switching events. These switches have most likely been triggered by alternating Pleistocene ice ages. Eventually a new period of co-evolution may appear, but considering the colonizing capacities of *Gyrodactylus*, future host-switches are bound to happen.

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In this study we focussed on the history and evolution of host-parasite associations between the monogenean ectoparasite *Gyrodactylus* and its gobiid hosts. Host-parasite systems are intrinsically interesting because they reflect intimate long-term associations between organisms that are biologically and genetically very distinct from each other (Page and Hafner, 1996). We reconstructed their shared history by comparing the molecular phylogenies of the host and parasite lineage. In order to achieve this, several conditions had to be fulfilled (Page et al., 1996): (1) a sound alpha taxonomy of both host and parasite taxa, (2) wide taxon sampling, (3) robust phylogenies of hosts and parasites, preferentially reconstructed from molecular data, (4) quantitative comparison of host and parasite trees by means of explicit statistical tests and (5) host transfer experiments. These prerequisites are addressed in the following paragraphs, with special attention to the results obtained in this study. Future perspectives will be discussed at the end.

(1) A sound alpha taxonomy of the host and the parasite

A first prerequisite for studies on phylogeny and co-evolution is a sound knowledge of the *Gyrodactylus* fauna present on the specific host species. Therefore we first made an inventory of the *Gyrodactylus* species living on *Pomatoschistus* species, which led to the description of several new species (Chapter three and four). Believing in an added value of a complementary approach, we combined morphological, morphometric, statistical and molecular analyses. In a previous study by Harris et al. (1999) morphological and molecular analyses were combined in describing new species. Here we presented the first study assessing the validity of molecular markers, comparative morphometric analyses and statistical classifiers in discriminating and describing closely related *Gyrodactylus* species.

The implementation of molecular tools has fuelled taxonomical and systematic debates and often led to substantial taxonomic revisions (e.g. Burridge and White, 2000; Jousson et al., 2000; Lazoski et al., 2001; Desdevises, 2001). In classical morphological analyses, cryptic speciation may lead to an underestimation of the number of species while phenotypic plasticity may induce the reverse effect. Therefore some authors feel that DNA sequences

should be used as the universal reference standard (Tautz et al., 2002), while others argue that genetic divergence in itself is not useful for identifying species (Ferguson, 2002). Irrespective of this controversy, taxonomic names and phylogenetic hypotheses are necessary tools to monitor parasite biodiversity (Brooks and Hoberg, 2001). But what is the required minimum amount of morphological and or molecular differentiation to be recognized as a new species? According to Milinkovitch et al. (2000), co-variation between *a priori* morphological designations and a minimum of one molecular character should be a valid basis for species recognition. In case of the closely related species described in chapter four, significant morphological differences were first described by Geets et al. (1999), and thereafter assessed by molecular tools. In Chapter three, the species description stemmed from an opposite situation: molecular differentiation led us to re-examine the morphology of two apparently cryptic species.

Poulin (2002) found that the body size of monogenean species correlates negatively with their year of description; hence it are essentially the smallest taxa that await discovery. The size of the marginal hook sickles of the species described in Chapter four is indeed among the smallest (less than 2.5 µm) described in *Gyrodactylus* (eg. Malmberg, 1970). In the case of *Gyrodactylus* spp. this might be linked to the fact that they are easily overlooked due to their small body size, and it is rather recently that *Gyrodactylus* research has been given more attention, especially since *G. salaris* caused major losses in the salmon industry. We might state that 'detection lies in the eye of the beholder', but besides sampling effort it also depends on the equipment and methodology used.

Despite its high species richness, *Gyrodactylus* has a low morphological diversity, showing more anatomical conservatism in its attachment and copulatory apparatus than any other group of monogenean parasites (Kearn, 1994). The number of useful taxonomic characteristics is limited due to adaptations for viviparity and progenesis (Cable, 1999). Species discrimination is mainly based on the shape and size of the opisthaptor, which consists of a single pair of hamuli and 16 marginal hooks (Malmberg, 1970; 1998). The interspecific morphometric differences are often consistent and useful for species discrimination, even in the case of closely related species. However, caution has to be taken regarding the intraspecific variation induced by climate and habitat (Malmberg, 1970; Geets et al., 1999). The difference in size is also an indicator of the conditional status of the species. In non-optimal conditions (in terms of salinity, temperature or the 'wrong' host species) the size of the ophisthaptoral parts are smaller as a result of a reduced time of embryogenesis (Dmitrieva and Gerasev, 2002). Therefore it might be interesting to test whether this

'environmental stress' is also reflected in differences between the left and the right anchor, also known as functional asymmetry (Lens and Van Dongen, 2002; Raeymaekers, pers. comm).

The question arises what might be the most time-and cost-effective way to discriminate among closely related *Gyrodactylus* species. Kay et al. (1999) constructed a classification system with the use of statistical classifiers. This allows discrimination of the pathogen *G. salaris*, based on measurements of the marginal hook alone when using scanning electron microscopy. Applying this method in Chapter three on light microscopy-based images, resulted in a perfect discrimination of *G. rugiensoides* from its close relative *G. rugiensis*. For species descriptions light microscopy and scanning microscopy-based analyses are indispensable, but in our opinion too labour intensive and costly for routine species identification. Therefore we would choose for a standardized molecular strategy based on enzyme restriction with species-specific markers. Of course, caution has to be taken regarding the specificity of the markers. Recently it turned out that DNA probe hybridisation to the amplified V4 region misidentified *Gyrodactylus teuchis* samples as *G. salaris* (Cunningham et al., 2001).

(2) Wide taxon sampling

This is the first study that compared the prevalence and the molecular and morphological variation of *Gyrodactylus* species over such a wide geographic range: we found a broad-scale *Gyrodactylus-Pomatoschistus* association, ranging from the Mediterranean Sea (Chapter five), along the European coasts up to and in the Baltic Sea (Chapter six). In Chapter five both morphometric and molecular (ITS rDNA) analyses have been carried out on the Mediterranean populations and a comparison has been made with the same species collected from the North Sea (Belgium). Surprisingly, the *Gyrodactylus* populations from the Mediterranean and the North Sea were strikingly similar in their morphological characteristics and almost no geographical differentiation was found throughout the whole ITS region. Collecting in different seasons (with a temperature range of 10.7 – 18°C) and localities (with salinity ranging from 10 to 32 ppm) hardly affected the size and shape of the morphological characters. The lack of morphological and molecular variation is all the more surprising considering the fact that the host *P. microps* shows considerable population differentiation in the cytochrome *b* mtDNA between Eastern Atlantic, Mediterranean and Adriatic populations (Gysels et al., in prep.). This might suggest that the ITS rDNA region of

Gyrodactylus is not sensitive below species level. In the literature, low intraspecific and geographic variation was found for *G. arcuatus, G. branchicus, G. gondae* and *G. pungitii* (Zietara et al., 2000; 2002), but no intraspecific variation could be found in the ITS2 sequences of *G. kobayashii* from the U.K. and Australia (Cable et al. 1999), nor in the ITS sequences of *G. anguillae* collected on *A. anguilla* from Spain and Australia, and on *A. australis*, *A. reinhardtii* and *A. rostrata* (Hayward et al. 2001). Mitochondrial DNA markers are therefore expected to provide more information on the population-level differences within these species. The next step will involve a comparison of the populations using the COI mtDNA marker that recently became available (Meinila et al., 2002).

Using COI mtDNA might reveal whether *G. arcuatus* is comprised of several strains, as suggested for *G. salaris* (Meinila et al., 2002). Asexual reproduction involved in the reproduction of to the first-born daughter would lead to populations made up of numerous asexual clones, strictly preserving the heterozygosity of the mother while parthenogenesis in the second-born daughter would gradually eliminate the variation and increase the level of homozygosity. Infrequent sex would restore heterozygosity and variation and populations would consist of a greater number of clones, with a smaller average size (Harris, 1993). This author found a positive correlation between the degree of sexual reproduction and the amount of morphological variation, and a negative correlation with the degree of host specificity. The pathogenic *G. salaris*, for instance, shows frequent sex, relatively low host specificity and a high variance in anchor dimensions (5-15% of the mean). So far, no geographic variation was found at the ITS level (see Chapter two). *Gyrodactylus arcuatus* is considered as a cyclic parthenogen (Harris, 1993), and it shows, hitherto, the highest intra-specific genetic variation observed in *Gyrodactylus* (Zietara et al., 2000). Inter-populational differentiation for morphological characters is also very high (see Chapter four). According to Bakke (2002) this species can be regarded as a generalist, found on many 'accidental' host species. The newly described species of Chapter four belong to the *G. arcuatus*-species group and the morphological variation was also very high. Furthermore, the presence of a vesicula seminalis containing sperm cells indicates that sexual reproduction might occur in natural populations of these species as well. This could be tested using microsatellite markers. These markers would create many interesting possibilities and help to understand the complex population biology of these remarkable *Gyrodactylus* species.

In this study, sampling has been mainly carried out in spring and autumn, although it has been shown that the prevalence of *Gyrodactylus* species studied by Geets (1998) varies seasonally. Therefore we might expect more species to be discovered when sampling is

extended over time. The same is true if more localities would be included, both on a regional and on a global scale. For example, it would be very interesting to include molecular data on the North American *Gyrodactylus* fauna found on e.g. three-spined sticklebacks (Cone and Wiles, 1985), which are morphologically highly similar to the species described in our study. This might allow us to reveal the origin and history of the *G. arcuatus*-species group. It would also be interesting to expand the analysis to more species of the 'sand goby' group. Molecular analysis showed specimens of *Pomatoschistus knerii* and *Knipowitschia panizzae* collected in the Venice lagoon (Italy) to be infected with identical *Gyrodactylus* spp. as found on *P. marmoratus*. *Economidichthys pygmeaus* collected in the Acheron river in Greece was found to be infected with two *Gyrodactylus* species, on the gills and fins respectively. Preliminary morphological analysis points into the direction of two new species, but more analyses are needed, complemented with sequencing of the ITS region.

(3) Robust phylogenies of hosts and parasites

Evidence about the nature of the speciation events requires a robust phylogeny of both host and parasite. The ssrRNA V4 region and the complete ITS rDNA region of all *Gyrodactylus* spp. found on the *Pomatoschistus* gobies were sequenced for phylogenetic analyses (Chapter six). Also samples of different geographic localities were included in the analysis. As observed in Chapter two, the overall variation within *Gyrodactylus* was almost impossible to evaluate due to the highly unreliable sequence alignment when all species were pooled. The ITS and 5.8S rDNA variation of the subgenera *G.* (*Limnonephrotus*), *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*) reached the upper limits reported for the most related genera such as *Echinistoma* or *Schistosoma*. This was solved by taking a gradual approach (Chapter two) or by depicting the most conservative fragments using appropriate alignment software (SOAP, Löytynoja and Milinkovitch, 2002; Chapter six). These findings support the idea that *Gyrodactylus* species are much older and genetically more differentiated than might be deduced from their morphological similarity. Nevertheless, we obtained a robust phylogeny of the *Gyrodactylus* spp. parasitizing the gobies, which was used in Chapter eight.

On the basis of six main types of protonephridial systems, Malmberg (1970; 1998) subdivided *Gyrodactylus* into six subgenera: *G.* (*Gyrodactylus*), *G.* (*Mesonephrotus*), *G.* (*Metanephrotus*), *G.* (*Paranephrotus*), *G.* (*Neonephrotus*) and *G.* (*Limnonephrotus*). A complex excretory system, as found in the subgenus *G.* (*Gyrodactylus*), is considered

primitive, while the simplest systems, found in e.g. *G.* (*Limnonephrotus*), are regarded more advanced. The excretory system of *G.* (*Mesonephrotus*) may have given rise to the system of *G.* (*Metanephrotus*) by a reduction of the lateral flame cells. This excretory system may have developed into that of *G.* (*Neonephrotus*) through the excretory bladders specializing for a constantly pumping function. However, in the molecular analysis this subgenus clustered in the middle of *G.* (*Paranephrotus*), suggesting that the transformation of big bladders into continuously pumping bladders occurred very recently. By mapping morphological characters on the molecular phylogenetic tree, we evaluated the evolution of these traits. A small excretory bladder appeared to be the ancestral character state in *Gyrodactylus* as suggested by Malmberg (1970). The evolution of big bladders apparently happened more than once, so that *G.* (*Paranephrotus*) is likely to be paraphyletic, and excretory bladders disappeared at least twice: in *G.* (*Gyrodactylus*) and in *G.* (*Limnonephrotus*). A complex excretory system characterized by many flame bulbs and lateral flame cells was confirmed to be primitive; the number of flame bulbs decreases along the lineage leading from *G.* (*Gyrodactylus*) to the other subgenera. A further simplification of the excretory system, by the loss of all lateral flames, evolved twice: in the lineage leading to *G.* (*Metanephrotus*) and the lineage leading to *G.* (*Limnonephrotus*) and *G.* (*Paranephrotus*).

For the construction of a molecular phylogeny of the host species, namely the sand gobies, we used both nuclear DNA (ITS1 locus) and mtDNA (12S and 16S fragments) as independent estimates. Considerable ITS1 length differences, primarily due to the presence of several tandem repeats, were found between species and even within individuals. Therefore, phylogenetic analyses focused on fragments of the 12S and 16S mtDNA region; 14 goby species have been sequenced. The four genera clustered as a monophyletic group, in congruence with morphological analyses. With respect to the interrelationships some conflicts arose: *Gobiusculus flavescens* and *Knipowitschia punctatissima* clustered within the *Pomatoschistus* species, pointing to a paraphyletic origin of both genera or a flaw in the phenetic methodology used in goby classification. However, it was difficult to draw conclusions on the phylogenetic position of each species as the bootstrap values were rather low. This might reflect the actual speciation mode or the choice of the marker. In a study on Japanese gobiids (closely related to the Atlantic-Mediterranean gobies) the cytochrome *b* gene was used for phylogeny reconstruction, and the low bootstrap values were explained by a rapid or simultaneous speciation mode (Akihito et al., 2000). Sequencing of another gene fragment will help to reveal whether these goby species have evolved simultaneously, as a

result of the drastic alterations that affected the Mediterranean during and after the Messinian salinity crisis (about 5.9 my ago).

Pomatoschistus minutus elongatus has been described morphologically as a sub-species of *P. minutus* (Miller, 1986). However, the genetic differentiation in the 12S and 16S mtDNA region of *P. minutus elongatus* from the Adriatic was as high as the differentiation between the remaining species of the *P. minutus* complex. Therefore it should be considered as a distinct species.

Another aspect that deserves more attention is the intra-individual variation observed in the ITS1 sequences of *P. microps* and *P. lozanoi.* By sequencing more than two clones per specimen we will gain more insight in the extent of intra-individual variation in other species as well. If it remains restricted to *P. microps* and *P. lozanoi* and if the amount of intra-individual variation is less than the amount of interspecific variation, we may conclude that a recent chromosomal rearrangement was involved. In that case we can use the ITS region as a phylogenetic marker. By sequencing more sand goby species it can serve as an independent test of the obtained molecular phylogeny.

Finally, goby phylogeny would profit from additional sequences of the Indo-Pacific gobiids, which have been proposed as a sister taxon to the sand gobies (McKay and Miller, 1997). This would clarify the origin of the sand gobies and their position within the family of the Gobiidae.

(4) Quantitative comparison of host and parasite trees by means of explicit statistical tests

Parasites track their hosts with a degree of fidelity that depends on the relative frequency of four events: co-speciation, host-switching, duplication and sorting (Page and Charleston, 1998). By statistically comparing host and parasite phylogenies, the contribution of each event can be calculated. In the present system, co-speciation and host-switching had a significant contribution to the fit between the *Gyrodactylus* and *Pomatoschistus* phylogenies. As such, this association has evolved through co-evolution, although host-switching, especially between the various goby species, had an important influence as well. When a clock estimate on the ITS phylogeny was applied (Zietara and Lumme, in press), the host-switching events coincided with the Pleistocene period resulting in fairly recent *Gyrodactylus-Pomatoschistus* associations. The genetic distance between the two *Gyrodactylus* lineages parasitising the gobies, belonging to the subgenera *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*), were much higher. Therefore we suggest that they

represent two independent colonization events. For the most recent association, corresponding with the gill parasites of the subgenus *G.* (*Mesonephrotus*), a host switch was suggested from *G. arcuatus*, which parasitizes the three-spined stickleback, onto the *Pomatoschistus* gobies. The origin of the second association was less clear; molecular inventarisation of other sympatric fish species should provide clarification.

By assessing the amount of host-switching events in the history of host-parasite relationships, a better understanding of the nature of emerging diseases might be obtained (Ricklefs and Fallon, 2002). In this study we identified a host-switching event of a *G.* (*Paranephrotus*) species from the gobies to *Anguilla anguilla*. This remarkable host jump crossing the boundary of fish orders, illustrates the colonizing capacity of *Gyrodactylus* spp. Following speciation on the European eel, it probably gave rise to the wide-spread *G. anguillae*, which has been reported as a pest in the culture of anguillid eels (Hayward et al., 2001). It is noteworthy that *Gyrodactylus nipponensis,* found on the Japanese eel, showed a close relationship with *G. arcuatus* found on three-spined stickleback and the gill parasites of the *Pomatoschistus* gobies (see Chapter six).

Another example is provided by the epidemic spread of *G. salaris* from fish farms to wild fish, causing major salmon losses (for a summary see Malmberg, 1993; Bakke et al., 2002). Upon the introduction of rainbow trout *Oncorhynchus mykiss* from North America to Europe, it has been colonized by *G. salaris* and *G. derjavini*, the latter causing very high infection intensities in aquaculture (Malmberg, 1993). The spreading capacity is also linked with the tolerance to wide salinity and temperature ranges. We found *Gyrodactylus rugiensis* for example, in the oligohaline zone of the Western Baltic (Stockholm), in the intertidal zone in Ambleteuse (experiencing salinity values of 0 to 33 ppm), in fully marine areas such as the Belgian continental shelf of the North Sea, and in the Mediterranean Sea in the Vaccarès lagoon complex (salinity of 10 to 30 ppm). This allows *Gyrodactylus* spp. to readily invade new areas.

Co-speciation events represent temporal links between the host and parasite phylogenies, providing an internal time calibration for comparative studies of evolutionary rate in both groups (Page and Hafner, 1996). Since we found a significant signal of co-speciation, the next step would be to include a comparison of homologous genes in both organisms to test for rate correlates. This will make it possible to infer whether *Gyrodactylus* spp. indeed evolve faster than their fish host, as might be expected from their reproductive biology. The evolutionary rate of parasites is generally higher than that of the host (Hafner et al., 1994; Moran et al., 1995; Page et al., 1998; Paterson et al., 2000). In a co-evolutionary study on

seabirds and their lice for example, synchronous co-speciation was found, with the lice evolving 5.5 times the rate of their hosts (12S mtDNA; Paterson et al., 2000). According to Page et al. (1998) small effective population sizes of lice, in combination with founder events when transmitted to new host individuals might be responsible for this observation. In contrast, the cytochrome *b* gene of avian malaria parasites appeared to have evolved three times slower than their hosts (Ricklefs and Fallon, 2002).

The knowledge of past events of host-switching, together with molecular data from the hosts and parasites, might reveal sudden changes in evolutionary rates, following colonization events (Poulin, 1998). In accordance with Lumme and Zietara (in press), the two evolutionary *Gyrodactylus* lineages found on the gobies might also represent an adaptive radiation following host-switching. However, several conditions have to be fulfilled before one can speak of an 'adaptive radiation' (Schluter, 2001).

(5) Host transfer experiments.

The *Gyrodactylus-Pomatoschistus* association is an interesting system for host transfer experiments in order to explain the absence of some parasite taxa on certain host species. For example, it can be tested whether chemical cues rather than niche separation of the host is responsible for the absence on certain *Pomatoschistus* species. Some examples for experiments are given in Chapter eight. Several authors (Malmberg, 1970; Harris, 1985; Bakke 2002 and references therein) have shown that infection experiments with *Gyrodactylus* are feasible, and *Pomatoschistus* spp. can also be maintained under lab conditions. Host-specificity among monogeneans is governed by a number of dynamic interactions, which are outlined in an excellent review by Buchmann and Lindenstrøm (2002): (1) the parasite is able to recognize host molecules emitted over short distances, (2) when contacting the host, substances present in parasite and host must be compatible, (3) the anatomical state of the host substrate must fit the attachment organs of the parasite, (4) the successful propagation of the parasite after attachment depends on appropriate host stimuli perceived by the parasite, and (5) nutritive host material must be recovered and utilized by the monogenean and translocated for productive purposes. In order to meet the above conditions, the monogeneans need to avoid or exploit the various immune mechanisms used by teleosts (complement, lectins, specific antibodies, etc.).

Future perspectives

In the above paragraphs we briefly discussed further initiatives that might be taken to address particular questions. In general, we can move one step further by investigating whether the observed trends account for the remaining *Gyrodactylus* subgenera as well. We might suppose for example, that factors structuring host-parasite systems in freshwater habitats differ from those operating in marine systems. Going still one step further to the level of the Monogenea, a comparison can be made between different genera displaying alternative life-history traits (e.g. viviparity *versus* oviparity) and the possible correlation with co-evolutionary interaction with the host. Alternatively, going one step down by comparing the population structure of hosts and their parasites may reveal whether and how the observed co-evolutionary interactions are reflected at the micro-evolutionary scale. Also, in hybridisation zones between host taxa the rate and pattern of introgression can be compared (if hybridisation and introgression is present in parasites as well) to reveal common demographic patterns (Page and Hafner, 1996).

We conclude that the ecology and biology of both *Gyrodactylus* and the gobies provide numerous opportunities for host-specificity and host-specialization to develop, providing a high potential for co-evolutionary studies at the macro- and micro-evolutionary level.

SUMMARY

We used gill parasites belonging to the genus *Gyrodactylus* (Monogenea, Platyhelminthes) and living on gobies from the genus *Pomatoschistus* (Gobioidea, Teleostei) as a study system to obtain a better understanding of evolutionary host-parasite relationships. Monogenea show a direct life-cycle (they use a single host species to complete their life-cycle) and are generally accepted as having the highest degree of host specificity, hence forming an ideal model for testing co-evolution. Geets (1998) made an inventory of the *Gyrodactylus* platyhelminths parasitising the genus *Pomatoschistus* on the Dutch continental shelf. Identification solely based on morphometry turned out to be difficult because of intraspecific variation induced by climate and habitat (Malmberg, 1970; Geets et al., 1999). Furthermore, little is known of the developmental genetics of gyrodactylids and it is not clear to what extent characters such as hook size and shape are truly independent of each other (Cable et al., 1999). By now, molecular techniques have been widely accepted as an important tool in tackling taxonomic and systematic related questions. The ribosomal internal transcribed spacers (ITS1 and ITS2) have proven to be suitable markers to discriminate among closely related *Gyrodactylus* species (Cunningham et al., 1995, 1997). Therefore, we decided to combine morphological and molecular data throughout this study.

Although the ITS region can be used as a diagnostic marker, it was found to be too variable for confident alignment when members of the various *Gyrodactylus* subgenera were included. To minimize the problems of the alignment of the complete region encompassing the 5.8S gene and both spacers ITS1 and ITS2, a gradual approach is proposed in **Chapter two**. Since the 5.8S gene is the most conserved, it can be used to distinguish among subgenera, while the more variable ITS1 and ITS2 regions are suitable for phylogenetic reconstruction at the species level.

Chapter three drew attention to the presence of host associated species complexes in the *Gyrodactylus-Pomatoschistus* system. We assessed the validity of molecular markers, comparative morphometric analyses and statistical classifiers in discriminating apparently cryptic *Gyrodactylus* species. A new species *G. rugiensoides* has been described. Poulin (2002) found that the body size of new monogenean species correlates negatively with their

year of description; hence, it are essentially the smallest taxa are waiting for discovery. Accordingly, the size of the marginal hook sickles of the species described in **Chapter four** is among the smallest (less than 2.5 µm) described in *Gyrodactylus* (eg. Malmberg, 1970). Nevertheless, a combined morphological, morphometric and molecular approach clearly separated all four species.

So far, almost all investigations and species descriptions of *Gyrodactylus* were made in the temperate northern hemisphere. However, being such a highly speciose group distributed over 19 orders of bony fish (Bakke et al., 2002), their distribution is expected to be much wider than presently recorded. In **Chapter five** we confirmed the occurrence of *Gyrodactylus* in the Meditteranean Sea. A morphometric comparison between this fauna with populations collected in the North Sea showed that they were strikingly similar. Moreover, almost no geographical differentiation was found throughout the complete ITS region: one species showed three substitutions whereas the other three appeared identical.

In **Chapter six** the fine-scale *Gyrodactylus* associations within the genus *Pomatoschistus* were examined, in order to delineate the different factors shaping parasite evolution. In a previous chapter (Chapter three) it was shown that ecological factors played an important role in this particular host-parasite system, but phylogenetic aspects appeared to be important as well. More species were included and ecological parameters such as abundance, site- and host specificity were recorded. It was established that the gobies have been colonized by at least two independent evolutionary lineages of *Gyrodactylus*, belonging to the subgenera *G.* (*Mesonephrotus*) and *G.* (*Paranephrotus*). Allopatric speciation appeared to be the dominant mode of speciation in this host-parasite system, with an example of a host-switch to another fish order (Anguilliformes). Confirmed by the molecular analysis, a small excretory bladder appeared to be the ancestral character state in *Gyrodactylus*. The evolution of large bladders apparently happened more than once since *G.* (*Paranephrotus*) was likely to be paraphyletic, and excretory bladders disappeared at least twice: in *G.* (*Gyrodactylus*) and in *G.* (*Limnoneprotus*). A complex excretory system characterized by many flame bulbs and lateral flame cells was confirmed to be primitive, with a decrease in number along the lineage leading from *G.* (*Gyrodactylus*) to the other subgenera. A further simplification of the excretory system by the loss of lateral flames evolved twice: in the lineage leading to *G.* (*Metanephrotus*) and the lineage leading to *G.* (*Limnonephrotus*) and *G.* (*Paranephrotus*).

A molecular phylogeny of the sand gobies was proposed in **Chapter seven**, based on sequences of the 12S and 16S mtDNA region. The sand gobies clustered in a single monophyletic group, confirming morphological data. With respect to the interspecific

relationships however, some conflicts arose: *Gobiusculus flavescens* and *Knipowitschia punctatissima* clustered within the *Pomatoschistus* species, pointing to a paraphyletic origin of both genera or a discrepancy in the phenetic methodology used in goby classification. Furthermore, the differentiation between *P. minutus minutus* and *P. minutus elongatus* from the Adriatic is as high as the differences within the *P. minutus* complex. As such, it should be considered as a distinct species, by analogy with *P. norvegicus* and *P. lozanoi.* The "star" phylogeny suggests that these goby species have evolved simultaneously, most likely linked to the drastic alterations that affected the Mediterranean during and after the Messinian salinity crisis (about 5.9 MY ago).

Host choices by parasites and subsequent specialization are driven by historical factors or by ecological factors. In the first scenario a parasite evolves strictly in parallel with its hosts, while resource tracking is not always connected with host phylogeny. By confronting the phylogenies of Chapter six and seven, we were able to discriminate among both scenario's. **Chapter eight** presents the reconstruction of the co-evolutionary history of *Gyrodactylus* spp. and their goby hosts. We found a significant fit between host and parasite phylogeny, with a significant contribution of both co-speciation and host-switching. We could establish that the *Gyrodactylus* spp. and their goby hosts have evolved through periods of co-speciation, followed by and combined with periods of successive host-switching events, probably triggered by the Pleistocene ice ages. Eventually a new period of co-evolution may commence, but considering the colonizing capacities of *Gyrodactylus*, future host-switches are bound to happen, once the optimal circumstances are presented.

We may conclude that the ecology and biology of both *Gyrodactylus* and the gobies provide the opportunity for host-specificity and host-specialization to develop, offering high potential for co-evolutionary studies. Since we found a significant signal of co-speciation, the next step would include a comparison of homologous genes in both organisms to test for rate correlates. This will allow us to infer whether *Gyrodactylus* spp. indeed evolve faster than their fish host, as generally expected from their reproductive biology. From this point on, we can go one step further and investigate whether the observed trends account for the remaining *Gyrodactylus* subgenera as well. We might imagine for example, that factors structuring the present marine host-parasite system are different in freshwater systems. In contrast, we may also go one step down to the level of populations to assess whether and how the observed co-evolutionary interactions are reflected at the micro-evolutionary scale.

SAMENVATTING

Zo goed als alle biologische vraagstellingen moeten in een evolutionaire context geplaatst worden: ze kunnen niet beantwoord worden buiten het kader waarin we nagaan hoe organismen evolueerden tot het stadium dat we nu waarnemen. Fylogenetische relaties spelen daarom een cruciale rol in alle aspecten van biologische studies. Fylogenetische co-evolutie is het mechanisme waardoor fylogeniën van groepen van nauw interagerende taxa meer gelijken op elkaar dan te verwachten is door toeval. Zulke paren groepen omvatten gastheren en hun parasieten, organismen en hun genen, en geografische gebieden met de inwonende soorten (Page and Charleston, 1994, 1998).

In deze studie wordt er gebruik gemaakt van een gastheer-parasiet systeem, meer specifiek de interactie tussen ectoparasieten behorende tot het platwormgenus *Gyrodactylus* (Monogenea, Platyhelminthes) en de grondels behorende tot het genus *Pomatoschistus* (Gobioidea, Teleostei). Monogenea kenmerken zich door het gebruik van één enkele gastheer om hun levenscyclus te voleindigen (zeer hechte relatie gastheer-parasiet) (Harris, 1985; Kearn, 1994) en worden algemeen de hoogste graad van gastheerspecificiteit toegeschreven (Rohde, 1978). Hierdoor vormt het *Pomatoschistus-Gyrodactylus* systeem een ideaal model om co-evolutie en speciatieprocessen te toetsen.

Geets et al. (1998) stelden een eerste inventaris op van *Gyrodactylus* parasieten van *Pomatoschistus* spp. op het Nederlands continentaal plat. Determinatie gebeurde op basis van de vorm en afmetingen van de haken van het vasthechtingsorgaan. Omdat afmetingen van gelijkaardige *Gyrodactylus*-soorten vaak overlappen, is identificatie op basis van morfometrische studies moeilijk. Bovendien kunnen er intraspecifieke variaties optreden onder invloed van het klimaat en habitat (Harris, 1993; Appleby, 1996; Geets et al., 1999). Deze moeilijkheden beperken de studies naar speciatieprocessen, biogeografie, populatiestruktuur en gastheer-parasiet co-evolutie. Meer dan 400 *Gyrodactylus* soorten zijn reeds beschreven en niets is geweten van de omstandigheden waarbij deze radiaties zijn ontstaan (Harris, 1993; Rohde, 1996; Bakke et al., 2002). De reproductiebiologie van deze parasiet is zeer complex en sommige soorten zijn nagenoeg uitsluitend asexueel (Harris, 1993, 1998; Cable et al., 2002). De mogelijke toepassingen van gyrodactyliden in

evolutionair biologisch onderzoek zijn bijgevolg enorm. Voorwaarde is echter dat de status van de taxa kan bevestigd worden en dat de hypothetische evolutiepatronen in het genus getest kunnen worden m.b.v. onafhankelijke kenmerken. Een alternatieve benadering wordt nu mogelijk door de moleculaire biologie. De rDNA sequenties blijken zeer bruikbare merkers te zijn voor het bepalen van de fylogenetische status van de Platyhelminthes (Carranza et al., 1997; Littlewood et al., 1999a,b). Teneinde de co-evolutionaire geschiedenis van *Gyrodactylus* en zijn gastheer te achterhalen, maakten we zowel gebruik van de klassieke morfologie als van de evolutieve genetica. Morfologische kenmerken laten toe aan te sluiten bij de systematiek van de Monogenea zoals tot nu toe gepubliceerd, de moleculaire biologie verschaft *a priori* onafhankelijke criteria om hypotheses omtrent evolutie te testen, en om de divergentie tussen taxa te bepalen.

Een eerste bijdrage van dit onderzoek ligt bijgevolg in het verder uitdiepen van de fylogenie van het genus *Gyrodactylus* en in het uittesten van de evolutiegeschiedenis vooropgesteld door Malmberg op basis van morfologische kenmerken (1970, 1998). **Hoofdstuk twee** geeft hiertoe een eerste aanzet door de bruikbaarheid van ITS rDNA merkers voor de fylogenetische reconstructie na te gaan met betrekking tot de groep *Gyrodactylus*. Het was reeds aangetoond dat de ribosomale spacersequenties ITS1 en 2 als diagnostische merkers konden gebruikt worden (Cunningham et al. 1995, 1997; Zietara et al., 2000), maar hun rol voor het opstellen van een fylogenie bleef onduidelijk (Cable et al., 1999). Volledige ITS sequenties van soorten uit vier verschillende *Gyrdactylus* subgenera werden aan een grondige moleculaire analyse onderworpen. Het conservatieve gen 5.8S werd fylogenetisch informatief bevonden en kan als hulpmiddel gebruikt worden voor het bepalen van het subgenerisch niveau. Op het soortniveau echter, contrasteerde de morfologische en moleculaire variatie enorm. De morfologische variatie, uitgedrukt in de vorm en afmeting van het vasthechtingsorgaan, is zeer laag, dit in tegenstelling tot the moleculaire variatie, op het niveau van ITS1 en ITS2. De genetische verschillen tussen de subgenera bleek minstens even hoog te liggen als de generische verschillen in andere platwormgenera zoals *Echinostoma*, *Fasciola* en *Schistosoma* en zelfs hoger dan de variatie tussen bepaalde families van nematoden (Chen, Willis and Miller, 1996; Chilton, Gasser and Beveridge, 1997; Zhu, Gasser and Chilton, 1998). Dit suggereert dat *Gyrodactylus* soorten veel ouder en genetisch meer gedifferentieerd zijn dan hun morfologische verschillen doen vermoeden. Dit kan te wijten zijn aan het snel evoluerend karakter van het ITS gebied, of dit impliceert dat het genus *Gyrodactylus* samengesteld is uit groepen met een hoger taxonomisch niveau dan eerder werd aangenomen. Er werd reeds door Avise en Johns (1999) op gewezen dat

taxonomische groeperingen zoals 'genus' niet altijd equivalent zijn zodat een vergelijking met andere taxa bemoeilijkt wordt. Hoe dan ook, hier opteren we voor een graduele aanpak bij het opstellen van een fylogenie voor *Gyrodactylus* met behulp van ITS rDNA. Een eerste indeling in subgenera wordt gemaakt op basis van het 5.8S gen, waarna de variabelere ITS1 en ITS2 merkers informatie kunnen verschaffen omtrent de relaties op soortniveau.

Een tweede doel was het opstellen van een inventaris van de *Gyrodactylus* fauna voorkomend op de grondels van het genus *Pomatoschistus*. Vermits tot nu toe weinig onderzoek op deze groep was uitgevoerd, waren er een aantal nieuwe soorten die moesten beschreven worden. Bij deze soortbeschrijvingen trachtten we steeds complementair te werk te gaan, gebruik makend van zowel morfologische, morfometrische als moleculaire karakteristieken. **Hoofdstuk drie** wees op het bestaan van gastheer-geässocieerde soortcomplexen in het *Gyrodactylus-Pomatoschistus* systeem. Het diagnostisch vermogen van multivariaat analyse, statistische 'classifiers' en moleculaire merkers werd geëvalueerd met betrekking tot ogenschijnlijk cryptische soorten, en een nieuwe soort *Gyrodactylus rugiensoides* Huyse en Volckaert, 2002 werd beschreven. De naam verwijst naar zijn opvallende gelijkenis met *G. rugiensis* Gläser, 1974, een vinparasiet van *Pomatoschistus microps*, waardoor ze vroeger als één soort beschouwd werden. De gastheerspecificiteit lag lager dan algemeen verwacht voor *Gyrodactylus* soorten. De parasiet werd zowel op *P. pictus* als op de nauwverwante *P. minutus* en *P. lozanoi* gevonden. Vermits *P. pictus* en *P. minutus* niet mekaars meest verwante soort zijn, maar wel sympatrisch voorkomen, werd besloten dat ecologische factoren zoals gastheer habitat een belangrijke invloed hebben. De nieuwe soort kan echter wel een fylogenetische gastheerspecificiteit toegeschreven worden daar ze enkel grondels van het genus *Pomatoschistus* infecteert.

Uit een studie van Poulin (2002) bleek de grootte van Monogenea soorten negatief gecorrelleerd te zijn met het jaartal van hun beschrijving, of met andere woorden, het zijn voornamelijk de allerkleinste soorten die nog ontdekt moeten worden. Zodoende zijn de marginale haken van de soorten beschreven in **Hoofdstuk vier** de kleinste (minder dan 2,5 µm) ooit beschreven in *Gyrodactylus* (e.g. Malmberg, 1970). *Gyrodactylus branchialis* sp.n., *G. gondae* sp.n., *G. flavescensis* sp.n. and *G. arcuatoides* sp.n. zijn beschreven op de vijf nauwverwante grondelsoorten *P. microps*, *P. minutus*, *P. lozanoi*, *Gobiusculus flavescens* en *P. pictus* respectievelijk. Alle vier soorten waren uiterst gastheerspecifiek en vertoonden morfologisch gezien een enorme gelijkenis met *G. arcuatus* van de driedoornige stekelbaars, hoewel de genetische verschillen (op basis van het V4 en ITS gebied) vrij hoog lagen. De onderlinge genetische verschillen waren algemeen lager dan vermeld in de literatuur, maar dit

is hoogstwaarschijnlijk gekoppeld aan het feit dat dit de eerste studie is die *Gyrodactylus* soorten van nauwverwante gastheersoorten vergelijkt.

Tot nog toe beperkte de inventarisatie van *Gyrodactylus* soorten zich tot de gematigde zone van het noordelijk halfrond. De soortenrijkdom en het feit dat *Gyrodactylus* spp. voorkomen op 19 ordes van beenvissen (Bakke et al., 2002) doen nochtans vermoeden dat hun verspreidingsgebied veel verder strekt dan tot nog toe onderzocht. Zo was de Middellandse Zee was tot dusver een onontgonnen terrein maar in **Hoofdstuk vijf** konden we bevestigen dat ook daar *Gyrodactylus* mariene vissen infecteert. Een morfometrische vergelijking met deze fauna en populaties van de Noordzee wees op een onverwachte overeenkomst tussen beide populaties. Ook toonde het ITS gebied amper geografische differentiatie: slechts een enkele soort vertoonde drie substituties in tegenstelling tot de andere drie soorten die identiek bleken te zijn. Dit lag niet meteen in de lijn der verwachtingen daar de gastheer, *P. microps*, een duidelijke geografische structuur vertoont in de verspreiding van mtDNA haplotypes (Gysels, pers. comm.). Daarom zou het interessant zijn om de analyse te herhalen met gevoeligere merkers zoals COI mtDNA, die recent ontwikkeld zijn (Meinila et al., 2002).

Om na te gaan hoe parasieten ontstaan en geëvolueerd zijn, moet er van zowel de gastheer als van de parasiet een fylogenie opgesteld worden. In **Hoofdstuk zes** hebben we ons toegelegd op de parasiet, gebruik makend van de ribosomale spacersequenties ITS1 en ITS2, in combinatie met het variable V4 gebied van de grote rRNA subeenheid. Fylogenetische reconstructies stellen ons in staat om het relatieve aandeel te bepalen van recente (ecologische) en historische (fylogenetische) factoren. Uit de resultaten bleek dat de grondels door minstens twee onafhankelijke *Gyrodactylus* lijnen werden gekoloniseerd, behorende tot het subgenus *G.* (*Mesonephrotus*) en *G.* (*Paranephrotus*). Allopatrische speciatie bleek het grootste aandeel te hebben in de ontstaansgeschiedenis van *Gyrodactylus*, ofwel door co-speciatie met de grondels ofwel door een ecologisch fenomeen, namelijk gastheerwissel. Op basis van deze gegevens kon vastgesteld worden dat de kieuwparasieten hoogst waarschijnlijk ontstonden als gevolg van een gastheerwissel van *G. arcuatus* levend op de driedoornige stekelbaars, naar de grondels. Of deze gebeurtenis gevolgd werd door co-speciatie of verdere gastheerwissel kan enkel uitgemaakt worden als we beschikken over een moleculaire fylogenie van de gastheer (zie verder). De dispersiecapaciteit van *Gyrodactylus* werd tevens geïllustreerd door een opmerkelijke gastheerwissel tussen de grondels (Perciformes) en de Europese paling *Anguilla anguilla* (Anguilliformes). Dit onderstreept tevens het belang van gastheerwissel bij het ontstaan van nieuwe pathogene soorten.

Vervolgens konden we met behulp van deze moleculaire fylogenie de hypothetische evolutiepatronen testen die vooropgesteld werden door Malmberg (1970; 1998). Het genus *Gyrodactylus* is namelijk op basis van het type excretiestelsel opgedeeld in zes subgenera. De complexe systemen worden als primitief beschouwd (*G.* (*Gyrodactylus*)) terwijl de eenvoudigste systemen meer geëvolueerd zouden zijn (*G.* (*Paranephrotus*) en *G.* (*Limnoneprotus*)). Dit kon echter niet helemaal bevestigd worden door onze resultaten, bovendien bleek het subgenus *G.* (*Paranephrotus*) parafyletisch te zijn. Het subgenus met het meest complexe excretiesysteem groepeerde inderdaad het meest basaal, maar een vereenvoudiging door het verlies van laterale vlamcellen vond plaats in twee onafhankelijke evolutionaire lijnen. Een kleine excretieblaas bleek eveneens ancestraal te zijn, maar de evolutie naar grote blazen heeft minstens tweemaal plaatsgevonden en de excretieblaas verdween volledig in de zoetwater subgenera *G.* (*Gyrodactylus*) en *G.* (*Limnoneprotus*).

De fylogenie van de gastheer werd ten dele beschreven door Wallis en Beardmore (1984) op basis van allozymes. Echter, om tot een volwaardige vergelijking te komen moeten beide fylogeniën gebaseerd zijn op homologe merkers. Daarom werd er in **Hoofdstuk zeven** een fylogenie opgesteld gebruik makend van zowel nucleaire (ITS1) als mitochondriale (12S en 16S) merkers. Ondanks de intra-individuele verschillen tussen verschillende clones van *P. microps* en *P. lozanoi* respectievelijk, kwam de fylogenie gebaseerd op beide merkers helemaal overeen. De zandgrondels groepeerden als één monofyletische groep, overeenkomstig met de morfologische gegevens. De onderlinge relaties waren echter minder duidelijk en het genus *Pomatoschistus* bleek parafyletisch te zijn. In de veronderstelling dat het mtDNA aan een snelheid van 1,0 – 1,5% divergentie per miljoen jaar (MY) evolueert, kon er een verband gelegd worden tussen het ontstaan van de zoetwater adaptatie bij grondels en de Messiniaanse saliniteitscrisis die 5,9 MY geleden de Middellandse Zee reduceerde tot enkele hypo- en hypersaline 'meren'. Met de heropening van de straten van Gibraltar konden de Noord-Atlantische voorouders van de grondels de Middellandse Zee herkoloniseren.

Door vergelijking van de fylogeniën uit hoofdstuk zes en zeven was het mogelijk om de geschiedenis van de *Gyrodactylus-Pomatoschistus* associaties te reconstrueren. Statistische testen toonden in **Hoofstuk acht** aan dat de fylogeniën van *Gyrodactylus* en de grondels met elkaar overeenstemden. Zowel co-speciatie als gastheerwissel bleken hiertoe een significante bijdrage te leveren. We konden verder besluiten dat een gastheerwissel van *G. arcuatus* van de driedoornige stekelbaars aan de oorsprong lag van de huidige verdeling van *Gyrodactylus* kieuwparasieten over de grondels. Dat de gastheerwissel recent was viel af te leiden uit de lage genetische differentiatie (0,7 – 1,8% in het gehele ITS en V4 gebied) tussen de

parasieten onderling. Daaropvolgend is elke parasiet op de respectievelijke gastheer gespecieerd, en dit kan uiteindelijk resulteren in een stabiele co-evolutie. Zulk een opeenvolging van co-evolutie en gastheerwissel wordt vaker waargenomen in gastheer-parasiet relaties. Het ontstaan van de vinparasieten viel moeilijker af te leiden en een uitbreiding van de inventarisatie van sympatrische vissoorten zou uitsluitsel kunnen geven. Vaststaat dat gastheer-geassocieerde speciatie hier een heel belangrijke rol heeft gespeeld, wat niet belet dat ook een gastheerwissel van grondels naar de Europese paling *A. anguilla* plaatsvond. Hoogstwaarschijnlijk heeft dit geleid tot het ontstaan van *G. anguillae*, een welgekende plaag in de aquacultuur van paling, wijdverspreid over alle continenten, behalve Afrika (Hayward et al., 2001). Dit is de tweede *Gyrodactylus* soort, naast *G. salaris*, die na introductie in natuurlijke populaties nieuwe epidemies uitlokt. Dit illustreert de kolonisatie capacititeit van *Gyrodactylus* en onderstreept het belang van gastheerwissel bij het ontstaan van nieuwe pathogene soorten.

Vermits deze studie heeft aangetoond dat de evolutie van *Gyrodactylus* en zijn gastheer wel degelijk bepaald wordt door co-evolutionaire interacties, kunnen we in een volgende stap nagaan of de evolutiesnelheid in homologe genen (bvb 16S mtDNA) gecorrelleerd zijn. Dit zal ons toelaten om na te gaan of *Gyrodactylus* sneller evolueert dan zijn grondels, zoals de reproductie strategie doet vermoeden. Van hieruit kunnen we één stap verder gaan en onderzoeken of de geobserveerde tendensen doorgetrokken kunnen worden naar andere *Gyrodactylus* subgenera. Factoren die mariene gastheer-parasiet systemen structureren zouden bijvoorbeeld kunnen verschillen in zoetwater ecosystemen. We kunnen ook een stap lager gaan tot het niveau van populaties om na te gaan of en hoe de geobserveerde co-evolutionaire interacties weerspiegeld zijn op micro-evolutieve schaal.

GLOSSARY AND ABBREVIATIONS

Abundance = mean number of individuals of a particular parasite species per host examined.

Allopatric speciation = speciation via geographically separated populations.

Apomictic parthenogenesis = parthenogenesis lacking meiosis, and therefore lacking any opportunity for recombination.

Asexual reproduction = any reproductive process which does not involve the union of gametes.

Biological species concept = concept of species, according to which species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups (Mayr, 1942).

Cladistic species concept = concept of species, according to which a species is a lineage of populations between two phylogenetic branch points (or speciation events).

Co-evolution = evolution in two or more species in which the evolutionary changes of each species influence the evolution of the other species.

Concerted evolution = the process which results in internal homogeneity for sequence variants of many multigene families within an organism or a species, resulting in the tendency of a family of repeated DNA sequences to evolve in unison. Rectification mechanisms include gene conversion and unequal crossing over. All the members of a repeat family undergoing convergent evolution can be considered to be descended from a single member at some point in the past.

Co-speciation = joint speciation of closely associated organisms.

Cyclical parthenogenesis = life-cycle in which a phase of mictic (bisexual) reproduction alternates with a phase of parthenogenetic reproduction.

Direct life-cycle = the parasite uses only one host to fulfil its life-cycle, opposed to an indirect life-cycle involving a *secondary* host (see *host*).

DNA = deoxyribonucleic acid, the nucleic acid forming the genetic material of all cells, some organelles and many viruses.

Duplication = the occurrence of a second copy of a particular sequence of DNA. **Duplication (parasite)** = intra-host speciation. The parasite lineage speciates independently of the host and both the new parasite species remain on the host.

Generalist (parasite) = parasite species infecting many, unrelated host species.

Host = organism supporting a parasite in or on its body and to its own detriment. A **primary** or **definitive** host is that in which a parasite reproduces sexually or becomes sexually mature; a **secondary** or **intermediate** host is that in which a parasite neither reproduces nor attains sexual maturity, but which generally houses one or more larval stages of the parasite.

Host range = the number of host species infected by a certain parasite species irrespective of how heavily and frequently the various host species are infected, whereas *host-specificity* takes intensity and/or *prevalence* of infection into account.

Host-specificity = the restriction of a parasite species to a certain host species.

Host-switching (speciation by) = the parasite lineage speciates but one of the new parasite species switches to another host species.

ITS = internal transcribed spacers, separating the *rRNA* genes. See rDNA and rRNA.

Mean intensity = mean number of individuals of a particular parasite species per host.

Morphological species concept = concept of species, according to which species are the smallest groups that are consistently and persistently distinct, and distinguishable by ordinary means (Conquist, 1988).

Opisthaptor = the posterior attachment of a monogenean.

Parapatric speciation = speciation in which the new species forms from a population contiguous with the ancestral species' geographic range.

Parasite = organism living in (**endoparasite**) or on (**ectoparasite**) another organism, its *host*, obtaining nourishment at the latter's expense.

Peripheral isolate speciation = a form of *allopatric* speciation in which the new species is formed from a small population isolated at the edge of the ancestral population's geographic range. Also called peripatric speciation.

Parthenogenesis = the development of an organism from an unfertilised egg.

Phenetic species concept = concept of species, according to which a species is a set of organisms that are phenetically similar to each other.

Phylogenetic species concept = concept of species, according to which a species is the smallest biological entity that is diagnosable and/or monophyletic (Cracraft 1989).

Phylogeny = "Tree of life"; branching diagram showing the ancestral relations among species or other taxa. It shows, for each species, with which other species it shares its most recent common ancestor.

Polyembryonie = cloning at the egg or embryonic stage by cleavage or budding; primary embryos may give rise to secondary embryos, and secondaries to tertiaries, depending on the organism.

Prevalence = number of individuals of a host species infected with a particular parasite species divided by the number of hosts examined (usually expressed as a percentage).

Progenesis = the process by which development is cut short by precocious sexual maturity.

Protogyny = a condition in which the female parts develop first.

rDNA = family of chromosomal DNA sequences encoding *ribosomal RNA*. The genes coding for 17-18S, 5.8S, and 25-28S rRNA are arranged as tandemly repeated units which are co-transcribed and contain, in addition to the rRNA genes, the so-called nontranscribed spacer (NTS). Each unit starts

with an external transcribed spacer (ETS) and the rRNA genes are separated from each other by two internal transcribed spacers (ITS1 and ITS2).

Ribosomal RNA (rRNA) = kind of RNA that constitutes the ribosomes and provides the site for translation.

RNA = ribonucleic acid. Messenger RNA, *ribosomal RNA*, and transfer RNA are its three main forms. They act as the intermediaries by which the hereditary code of the DNA is converted into proteins. In some viruses, RNA is itself the hereditary molecule.

Sorting events = instances were parasites are not found to be associated with a particular host species due to extinction, sampling error or uneven parasite distribution.

Specialist (parasite) = parasite species infecting only one (or a few closely related) host species.

Speciation = the separation of populations of plants and animals, originally able to interbreed, into independent evolutionary units which can interbreed no longer, owing to accumulated genetic differences.

Species = an important classificatory category, which can be variously defined by the *biological species concept*, *cladistic species concept*, *phenetic species concept*, *morphological species concept*, *phylogenetic species concept*, etc.

Sympatric speciation = speciation via populations with overlapping geographic ranges.

Viviparity = reproduction in animals whose embryos develop within the female parent and derive nourishment by close contact with her tissues.

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Abbreviations of *Gyrodactylus* terminology

Features of the attachment organ (opisthaptor):

LAP = Length of anchor point

LA = Total length of anchor

LAS = Length of anchor shaft

LAR = Length of anchor root

LVB = Length of ventral bar

BWVB = Basal width of ventral bar

MWVB = Median width of ventral bar

VBM = Length of ventral bar membrane

TLVBM = Total length of ventral bar membrane

(median width of ventral bar+ length of ventral bar membrane)

LMH = Total length of marginal hook

LH = Length of marginal hook handle

LSI = Length of marginal hook sickle

DWSI = Distal width of marginal hook sickle

PWSI = Proximal width of marginal hook sickle

LOOP = Length of marginal hook filament loop

APERTURE = Marginal hook sickle aperture distance

TOE = Marginal hook toe length

G. sp. = *G. branchialis* described in Chapter 4.

G. sp. 1 = *G. arcuatus*-like = *G. gondae* described in Chapter 4.

G. sp. 2 = *G. arcuatoides* described in Chapter 4.

G. sp. 3 = undescribed *Gyrodactylus* spp. found on *Pomatoschistus norvegicus*

G. sp. 4 = *G.* cf. *harengi*, found on the fins of *Pomatoschistus microps*

G. sp. 5 = *G. flavescensis* described in Chapter 4.

G. sp. 6 = undescribed *Gyrodactylus* spp. found on *Gobiusculus flavescens*

G. cf. *micropsi* = undescribed *Gyrodactylus* spp. found on *Pomatoschistus minutus* and *P. lozanoi*

G. cf. *micropsi* 1 = undescribed *Gyrodactylus* spp. found on *Pomatoschistus lozanoi*

G. cf. *micropsi* 2 = undescribed *Gyrodactylus* spp. found on *Pomatoschistus lozanoi*

G. cf. *longidactylus* = undescribed *Gyrodactylus* spp. found on *Pomatoschistus norvegicus*

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"So, naturalists observe, a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em;
And so proceed *ad infinitum*".

Gulliver's Travels, Jonathan Swift (1667-1745)