Morphological and Molecular Evolution Are Not Linked in *Lamellodiscus* (Plathyhelminthes, Monogenea)

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**Abstract**

*Lamellodiscus* Johnston & Tiegs 1922 (Monogenea, Diplectanidae) is a genus of common parasites on the gills of sparid fishes. Here we show that this genus is probably undergoing a fast molecular diversification, as reflected by the important genetic variability observed within three molecular markers (partial nuclear 18S rDNA, Internal Transcribed Spacer 1, and mitochondrial Cytochrome Oxidase I). Using an updated phylogeny of this genus, we show that molecular and morphological evolution are weakly correlated, and that most of the morphologically defined taxonomical units are not consistent with the molecular data. We suggest that *Lamellodiscus* morphology is probably constrained by strong environmental (host-induced) pressure, and discuss why this result can apply to other taxa. Genetic variability within nuclear 18S and mitochondrial COI genes are compared for several monogenean genera, as this measure may reflect the level of diversification within a genus. Overall our results suggest that cryptic speciation events may occur within *Lamellodiscus*, and discuss the links between morphological and molecular evolution.

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**Introduction**

Describing new species solely on the basis of their morphology is often not straightforward, and especially so for small-bodied organisms that display few morphological features on which to rely. A good illustration is highlighted in monogenean parasitic flatworms, where the main morphological structures used for species identification, namely the hard parts of the host attachment apparatus (haptor) and male copulatory organ, often require expert advice to discriminate closely related species, and although displaying phylogenetic conservatism in some genera [1], may display variations with environmental conditions [2–4] and host species [5,6], eventually leading to speciation [7]. It is not clear whether morphological variation within a species should be linked to an ongoing speciation process, if it emerges as a combination of inter-individual variation and (potentially host-induced) polymorphism in the population, or involves any combination of these factors. In the specific case of monogeneans, the haptoral parts, because they are used by the parasite to attach to its host, are likely to be more strongly affected by phenotypic plasticity in generalist species (i.e. using several host species with varying gill characteristics [8]), even if this process appears to be limited [9]. It is now well described that species can engage in phenotype switching to cope with complex (in the case of parasites, multi-hosts) environments [10], which result in the coexistence of potentially different forms of the same species [11]. In this case, the existence of different morphotypes would not correspond to different species, rendering molecular evaluation of the taxonomic situation necessary. This problem is obviously more difficult to tackle when there are few characters on which identification can be conducted, and when these characters are directly under environmental control (as is the case for the hard haptoral parts of the monogeneans).

*Lamellodiscus* (Monogenea, Diplectanidae) are gill parasites of sparid fish throughout the world [12]. In the past ten years, over twelve new species have been described within this genus in Mediterranean and African fishes [13–19]. These species were described solely on the basis of very few morphological variations in comparison to previously known *Lamellodiscus* species. The morphological differences between recently described species and their already described counterparts are often tedious to observe in light microscopy, making them highly questionable. The difficulties in species assignment in *Lamellodiscus* were highlighted in previous molecular analyses, which showed that some species like *Lamellodiscus vagula* and *L. obtusum*, because of their high similarities in sequences coding for 18S and ITS1 (% differences are respectively 0 and 0.27), could be synonymous species [20]. A more striking example is *Pionastria echeneis*, that belongs to the genus *Lamellodiscus* [21], despite its blaring morphological divergence from previously known *Lamellodiscus* species. Most notably, *F. echeneis* attachment apparatus only harbors one lamellodisc, instead of two for all other *Lamellodiscus* species. The above examples stress that morphology should not be viewed as a consistently reliable tool in systematic investigation, and recent studies showed how this conclusion applies for other monogeneans [22] and free-living animals [23].

Beyond the use of molecular data for species-assignment purposes, a recent study by Hansen and colleagues [24], looking
for differences between the two monogeneans Gyrodactylus thynanni and Gyrodactylus salmonis, revealed the existence of several lineages, unveiling a higher than expected diversity. Bakke and colleagues [25] reported a similar result, proposing that there could be as many as 20000 Gyrodactylus species, due to their fast ability to diverge both on molecular and morphological characters. Due to the fact that gyrodactylids pose severe economic problems in aquaculture, they have been more extensively studied than any other monogenean genera, which explain that few data are available for other monogenean genera.

In this study, we used three genetic markers, the 3’ extremity of the 18S rRNA gene, the Internal Transcribed Spacer 1, and approximately 300 base pairs within the first subunit of the mitochondrial Cytochrome C Oxidase I, COI, to estimate the level of divergence at the intra- and interspecific levels in Lamellodiscus. We focused on recently described species from the morphological group ignoratus [14] that are characterized by simple lateral dorsal bars in the haptor and an en lyre (made of two loosely bound hard parts resembling the shape of a lyre) male copulatory organ. Several features of L. ignoratus s.l. (sensu lato, i.e. the group comprised of L. ignoratus s.s., L. falcus, L. nefarii, L. confusus and L. diplodi) make it a suitable group for such a study: its four taxa are discriminated by discrete morphological differences. Finally, these species occur on a limited number of spardin hosts: Diplodus sargus, D. vulgaris, D. annularis, D. puntazzo, Lithoglyphus mormyrus and Salpa salpa.

Our goals were (i) to assess the taxonomic status of these recently described Lamellodiscus species: L. nefarii, L. falcus, L. confusus, L. diplodi; (ii) to check whether or not these species are closely related to L. ignoratus (sensu stricto, henceforth referred to as L. ignoratus s.s.), thus comparing the relative merits of morphological and molecular investigation of species status in this genus; and (iii) to evaluate the level of molecular diversity in Lamellodiscus, within and between species and discuss how it can assist in species assignment problems.

Materials and Methods

1 Fish and parasite sampling

Fishes were sampled near Banyuls-sur-Mer (42°28′47″N, 3°08′10″E), by free diving. Two hosts species were collected, Diplodus sargus and D. vulgaris, as they are known to harbor several Lamellodiscus species belonging to the L. ignoratus s.l. subgroup [12]. Immediately after capture, fish were killed by a sharp blow on the top of the head, and dissected. Gills were removed, and examined at most 30 minutes after removal, under a light stereomicroscope (Olympus SZ61), to check for the presence of Lamellodiscus.

Parasites were isolated from the gills, and placed on a slide to be examined under light microscope (Olympus CX41, 400 times magnification). Species identification was carried out based on the shape of the opisthaptor and male copulatory organ [15]. Parasites were the preserved and stored individually in 96% ethanol before DNA extraction.

2 DNA extraction and amplification

DNAs were extracted from dried samples in a mixture of 70 μl of Chelex [100 mg/ml] and 15 μl of Proteinase K (10 mg/ml) at 55 °C for one hour. Reactions were then stopped at 100 °C for 15 minutes and kept at 4 °C until used.

Three markers were used in our analysis: the 3’ terminal fragment of the 18S rDNA (18S), the Internal Transcribed Spacer 1 (ITS1) and partial mitochondrial gene Cytochrome Oxidase I (COI). Until now, only the 18S had been used for phylogenetic analysis in Lamellodiscus [20,26,21].

The 18S-ITS1 fragment was amplified in one round with primers L7 (forward, 5’-TGATTTTTGTICGTGTTATTTCGAT-3’) and IR8 (reverse, 5’-GCTAAGCTGCGTTCTTATCGA-3’) as designed by Verneau and colleagues [27] and Šimková and colleagues [28] while COI was amplified with primers LCO1P (forward, 5’-TTTTTTGGGCATCCTGAGGTTTAT-3’) and HCox1P (reverse, 5’-TAAAGAAGAACAATATGAAAATG-3’), after Littlewood and colleagues [29]. PCR were performed using the following cycles: 6 minutes at 95 °C, then 35 cycles as follows: 1 minute at 95 °C, 1 minute at 48°C, and 2 minutes at 72°C. A final elongation was conducted for 10 minutes at 72°C. PCR fragments were run in 1% agarose gels and purified using Nucleospin Extract II Gel extraction kit (Macherey-Nagel). They were sent to Macrogen Inc. (Korea) for sequencing. Sequences for this study were deposited in GenBank with numbers EU259028 to EU259032 and JF427625 to JF427661.

3 Distance computation and phylogenetic analysis

Due to the difficulty to align ITS1 even within a single genus, the following analyses were done on COI and 18S only. GenBank [30] was first queried to retrieve 18S and COI sequences from monogeneans (species for which at least 3 sequences were available were included). ClustaW2 [31] was used to align all sequences for each marker with default settings, using the alignment of Lamellodiscus species as a reference (for both 18S and COI). The ambiguously aligned parts were removed using Ghblocks [32,33], which retained 473 unambiguous positions out of 537 in the original 18S sequences. Uncorrected pairwise distances (excluding indels) were computed using the dist.dna function of the APE package [33] for R 2.9.0 [35]. Numbers of sequences by genus and species are listed in Tables 1 and 2.

Due to the difficulty of obtaining enough sequences of COI in Lamellodiscus, the phylogenetic reconstruction was computed on the 18S fragment only. Evolutionary models were tested using ModelTest [36] and selected with regard to their AIC score, using PAUP* 4.0b10 [37]. Trees were inferred using two probabilistic approaches: maximum likelihood with a non-parametric bootstrap validation using PhyML [38] (using a GTR model with 49% of invariant sites and a Gamma shape parameter of 0.46), and Bayesian inference (using MrBayes 3.1.2 [39,40], using the same portion of the 18S and COI sequences from monogeneans as designed by Verneau and colleagues [27] and Šimková and colleagues [28]). Each time the phylogenetic pattern obtained casted doubt upon the taxonomic status of a group of individuals, ITS1 sequences were manually aligned to help in determining whether they belong to the same species, as it has been previously suggested that ITS1 could be aligned within but not between Lamellodiscus species [20,4]. However, given that this criterion deserves a more formal investigation, ITS1 is used along with other criteria such as genetic distance and phylogenetic pattern to assess species status.

Results

1 Phylogeny of Lamellodiscus

Our updated Lamellodiscus phylogeny (Figure 1– the ML version is presented, as both reconstruction methods gave congruent topologies), using the 3’ end of 18S ribosomal DNA carries new information regarding the previous phylogeny obtained by Desdevies and colleagues [42], using the same portion of the 18S ribosomal DNA (but based on fewer species and using only one
individual per species). The \( L. \text{ignoratus} \) s.l. group is not supported, with a bootstrap value of 17\% for its most basal node (PP=0.5). Within this group, individuals from several putative species (both previously known and recently described from morphology) cluster together. Moreover, individuals from the same species are not clustered in this tree (for example, the sequence obtained for \( L. \text{elegans} \)). The clusters of individuals for which this alignment was attributed to the various species of the \( L. \text{furcatus} \) are poorly supported by the molecular phylogeny, while \( L. \text{neifari} \) is supported.

### 2 Intraspecific and interspecific pairwise distances

From the partial 18S, the mean of uncorrected pairwise distances between all sequences available for \( L. \text{furcatus} \) is 5.7\%. The distance between \( L. \text{elegans} \) (AY038195) and \( L. \text{pansis} \) (AY038198), the most divergent sequences, is 9.2\%. For the least divergent sequences, \( L. \text{fratenum} \) (AY038191) and \( L. \text{ergensi} \) (AY038190), the distance is 0.6\%. Within \( L. \text{ignoratus} \) s.l. individuals (\( n = 17 \)), we were able to align all ITS1 sequences in two groups (containing 11 and 6 individuals), suggesting that all specimens within these groups belong to the same species (named \( L. \text{ignoratus} \) and \( L. \text{neifari} \)). This highlights incongruences between morphological and molecular identifications. The mean distance for 18S of \( L. \text{ignoratus} \) s.l. considered as a single taxonomic entity is 2.46\%.

Pairwise distances for the markers \( \text{COI} \) and 18S are listed in Tables 1 and 2, respectively. Variability within the genus \( L. \text{furcatus} \) is the highest observed in our sample for the \( \text{COI} \) gene. Concerning the 18S, the genus \( L. \text{furcatus} \) is more variable than any other monogenean genera, except \( \text{Gyrodactylus} \), as indicated by the higher genetic distances. The amount of variability correlates with the taxonomic level for \( \text{COI} \) (i.e., isolates are less variable than species, and species less than genera), but not for 18S. It should be noted that this result is likely to be influenced by the fact that sampling effort was stronger on some monogenean genera, and that should be kept in mind when interpreting these observations.

### Discussion

1 Cryptic speciation in \( L. \text{furcatus} \)?

Because of their strong potential for diversification, monogeneans are a promising model to study biodiversity issues [43]. This assumption is supported by the estimation of 25000 monogenean species by Rohde [44]. Note that Bakke and colleagues [25] estimated 20000 species in the genus \( \text{Gyrodactylus} \), only making it one of the most speciose animal genera known. \( \text{Gyrodactylus} \) is one of the most studied monogenean genera, because of the impact of some species in aquaculture [43,46], but data for other monogeneans are lacking. Here we suggest that \( L. \text{furcatus} \), compared to other monogeneans, is characterized by a high molecular diversity at both infraspecific and intrageneric level. Among the two main morphologically defined groups, \( \text{ergensi} \) was poorly supported by the molecular phylogeny, while \( \text{ignoratus} \) forms an unsupported cluster of individuals. In addition, the situation within each putative group is complex: phylogenetic support is weak within each group, where distinct morphs are found, among which some are close to each other from molecular data (grey boxes in Fig. 1). Within the \( \text{ergensi} \) group, the small sample size precludes any meaningful observation. Within the \( \text{ignoratus} \) group, while supported nodes exist, they are not compatible with groups that could be delineated using morphological characters. For example, individuals from \( L. \text{ignoratus} \) s.s. are interspersed in the 18S.
Figure 1. Phylogeny of several Lamellodiscus species obtained by maximum likelihood and Bayesian inference. As topologies obtained with both reconstruction methods gave congruent topologies and similar branch lengths, the most resolved tree, obtained by maximum likelihood, was retained and is presented here. Bootstrap values (1000 replicates) and posterior probabilities (>0.5) are indicated at each node. The clusters of individuals for which the alignment of ITS1 was possible are outlined in grey boxes. Thick black lines indicate ergensi and ignoratus groups.
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ignoratus s.l. group, and one of the L. abbreviatus individuals is outside a strongly supported clade containing the other L. abbreviatus isolates. Based on partial 18S rRNA gene sequences, several species appear to be either not monophyletic (e.g. L. ignoratus, L. ergensi) or invalid (such as L. coronatus which clusters within L. furcosus individuals), which suggests that Lamellodiscus could be either more diversified than expected, or that there is a gap between the morphological characterization of species and their evolutionary relatedness (these two propositions not being mutually exclusive). This claim is supported by the magnitude of pairwise distances observed, particularly at the lower taxonomic levels, between Lamellodiscus individuals.

Three main factors can be invoked to explain the putative high ability for diversification in monogeneans, reflected in the high molecular and morphological diversity observed in Lamellodiscus. First, habitat heterogeneity is likely to be greater for small-bodied organisms (such as Lamellodiscus), thus favoring their diversification [47–49]. Poulin [50] observed this pattern in monogenean ectoparasites: there are far more small-bodied fish monogeneans than large-bodied ones. Second, monogeneans have a direct life cycle. Life-cycle complexity has been suggested to affect speciation rates in parasites [43]. Within Platycladoumbes, which share a common origin of the parasitic lifestyle, the Monogenea is the only group in which an adaptive evolutionary radiation has occurred [51]. Because of their small body size and direct life cycle, monogeneans have an important potential for diversification [28]. Finally, the genus Lamellodiscus appears to be composed of more species with a wide host range than any other monogenean genera, which has been suggested to add some molecular variability [8] and potential for speciation [6,52–54].

Relying on morphology alone led previous researchers to consider as belonging to the same species some morphs that were found in different clades in our molecular phylogeny. This is emphasized by the situation of L. ignoratus s.l., where none of the described species receives support based on the molecular data. This situation could be interpreted in two ways. First, most species in the ignoratus group might be paraphyletic. By paraphyletic, we mean that within a cluster of related individuals belonging to the same species, one or several individuals from another species branch out. The existence of paraphyletic species has already been observed both for free-living [55] and symbiotic [56–58] taxa. In our sample, some pairwise genetic distances between individuals from different species (e.g. 0.6% between L. fraternus and L. ergensi based on partial 18S rDNA) were found to be lower than some intra-specific distances. This situation strongly suggests that two or more species are not a monophylum [59]. Second, Lamellodiscus may contain more species that our current estimation. Several studies aimed to characterize new species in this genus in the last few years [13,15,17], based on very small morphological variations. However, these recently described species are not easily differentiated from others (neither from morphological nor molecular analyses), and according to the molecular evidence presented here, it might be more conservative to consider that they are species inquirenda (i.e. species of doubtful identity).

The fact that the recently described species are not necessarily valid must not lead us to lump all L. ignoratus s.l. individuals into a single species. Indeed, pairwise distances within L. ignoratus s.l. are higher than for any other species pairs (Table 1), and comparable to those observed in other genera (the partial 18S rDNA diversity of L. ignoratus s.l. is comparable to what was observed between Dactylogyrus spp. and between Polystronoides spp., see Table 2), and while they do not correspond to what could be delineated based on morphological character, there are some supported clades in the ignoratus group. This result suggests that several species could exist in the L. ignoratus s.l. group, but due to a high diversity and putative cryptic speciation, an intensive sampling is still needed to gather enough data to detect them.

The precise knowledge of which taxa are species is crucial, because species, contrary to higher level taxa, are not only an outcome of evolution, but are also directly involved in evolutionary processes [60,61]. We face the same problem in Lamellodiscus: our current view of the evolution of this genus [42] was inferred according to what we thought to be species; if what we called species was rather an assemblage of dissociated taxonomical units, some of the mechanisms thought to act in Lamellodiscus evolution (such as radiation by host switch followed by speciation) need to be re-evaluated in the light of revised species delineation and an updated phylogeny. Before assessing intraspecific and intragenic genetic diversity, it is important to be sure which taxa are given the species status. In such a situation, it could be useful to use the Least-inclusive taxonomic unit (LITU) concept [62], that is considering several individuals as forming a clade, without making further assumptions about the taxonomic position of this clade. Our results suggest that the ignoratus group is highly diversified, and is likely to be formed by several OTUs; we suggest to give this group the status of LITU, and to wait for further investigation to determine its exact taxonomic status. According to these results, our view of the taxonomy and, consequently, of the evolution of Lamellodiscus needs to be reassessed.

2 Phylogeny and morphology seem to be unlinked in Lamellodiscus

The molecular variability (as approximated by the pairwise distance at several taxonomical depths) in Lamellodiscus was compared to what was found in other monogenean genera. For CO1, Lamellodiscus shows the most important interspecific distances; for 18S, Lamellodiscus is nearly twice as variable as Dactylogyrus, but less than half as variable as Gyrodactylus, thought to be the most variable monogenean genus [63]. Despite this important molecular variability, however, there are very little clearly distinguishable morphologies within the genus Lamellodiscus. Amine and Euzet [14] defined two morphological groups in this genus, named ignoratus (formed by L. ignoratus s.l., L. fraternus, L. knoeppfleri and L. erythini) and ergensi (formed by L. ergensi, L. sanfilippoi, L. kechemirae and L. baeri). Our results (Figure 1) are congruent with this classification, with two notable exceptions: L. knoeppfleri, L. fraternus and L. erythini were found to belong to the ergensi group. According to our phylogeny, the ignoratus group is only formed of the taxa belonging to L. ignoratus s.l. Within each group, however, there is no link between morphological features and phylogenetic position, mostly because none of the individuals of a single putative species cluster together. A similar situation was reported by Hay and colleagues [64]. They observed that the tuatara, Sphenodon punctatus, while being a living fossil (its morphology is strikingly similar to the fossil specimens), and having a slow metabolism, a long generation time, and a slow rate of reproduction, is the species having the highest rate of molecular evolution observed amongst vertebrates. Hence, a high molecular divergence is not necessarily linked to important morphological changes, and the assumption that rates of molecular and morphological evolution are inherently correlated [65] is likely to be untrue in certain genera, which could be the case in Lamellodiscus.

Given that we were able to align the ITS1 of several individuals, we are able to make suggestions as to the species status of some morphotypes. We found molecular evidences that, despite some morphological divergences on the shape of the hard parts and copulatory organs, L. furcosus and L. coronatus form a single species (that we call L. furcosus). The ITS1 of L. ergensi, L. oliveri, L. fraternus and L. gussevi can be aligned, suggesting that all of these morphotypes should be considered as a single species, that we call...
sequences are available to cover a whole family [69], thus of 17%. However, the latter result may be due to the fact that few pattern is the Polystomatidae family, with an intra-family distance spp.) to 19% (for Lamellodiscus

3 Genetic diversity in Lamellodiscus and other monogeneans

During this study, we assessed mean uncorrected genetic pairwise distances based on two molecular markers (the 5′ end of the 18S gene, and about 300 base pairs within COI) on several monogenean genera. For the COI gene (Table 1), it seems that the mean pairwise distance is an appropriate reflection of the taxonomic position: intra-specific uncorrected pairwise genetic distances range from 0% (in Euzylhalistomataoides grandi individuals from the same isolate) to 8% (between individuals of Protopolyxystoma simplus); intra-generic distances range from 13% (for Protopolyxystoma spp.) to 19% (for Lamellodiscus). The notable exception to this pattern is the Polystomatidae family, with an intra-family distance of 17%. However, the latter result may be due to the fact that few sequences are available to cover a whole family [69], thus potentially decreasing the mean distance, and emphasizing the need to gather more genetic data at broad taxonomical scales. Another explanation is that larger bodied monogeneans might be less speciose than smaller bodied organisms. Another explanation is that chelonian polystomes arose very early, in the Lower Triassic, namely 200 Million years ago [70]. This may explain large divergences observed between species of different genera. For the 18S gene (Table 1), however, the pattern of correspondence between taxonomic position and mean pairwise distance is lost. While some species display very few variations (the sequences we retrieved for Dactylogyrus crucifer and D. visutula showed no differences), others (such as G. salaris and G. thymallii thought to be a single species [71]) harbor a level of intra-specific divergence comparable to superior to the one found in the Lamellodiscus and Gyrodactylus genera. Altogether, these results indicate that analyses of pairwise genetic distances to assess taxonomical status, although used in diverse biological systems [72–74], should be conducted cautiously as not all markers display the same behavior of congruence between genetic distance and taxonomical rank.

It seems that the evolutionary rate of some markers, such as 18S rDNA, is lineage specific (e.g. Gyrodactylus seems to evolve faster than Lamellodiscus, itself evolving faster than Dactylogyrus), whereas in other markers, such as COI, mean distance correlates with taxonomical position. These results can be due to different evolutionary rates in these markers (COI is mitochondrial and coding, 18S is nuclear and structural), but may also be linked to sampling effort: where some genera have undergone an important sampling effort (e.g. Gyrodactylus), few molecular data are yet available for others or, when they are, they often come from a single study, often limited to a single geographic area despite the broad geographical distribution of Lamellodiscus [75]. The question of whether the current amount of available data allows us to capture the majority of the genetic diversity in monogeneans remains pending. Moreover, it is likely that the lifestyle of the various taxa will matter in determining the genetic diversity; for example, how viviparous and egg laying monogeneans differ in this extent is yet to be investigated.

4 Concluding remarks

This study suggests that the degree of variability displayed by the different markers used here is impacted by the taxonomical position of the group investigated. Here, this variability is linked to the taxonomical position for COI, but not for 18S. In comparison to the important genetic variability displayed by Lamellodiscus, there is a relative morphological conservatism, suggesting the action of environmental (host-induced) selection pressures on the shape of several haptoral parts.

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Author Contributions

Conceived and designed the experiments: TP OV YD. Performed the experiments: TP OV. Analyzed the data: TP OV YD. Wrote the paper: TP OV YD.

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