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1 Host specificity of monogenean ectoparasites on fish skin and gills assessed by a
2 metabarcoding approach

3

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13

14 Supplementary file associated with this article.

15

16 Abstract

17 Monogeneans are highly diverse fish ectoparasites with a direct life cycle, widely distributed and
18 are known to generally display strict host specificity. Factors related to the hosts and the parasite
19 have been suggested to explain this high specificity. Monogeneans have also been observed to
20 colonize fish species not in their natural host range under experimental conditions. We developed
21 a specific metabarcoding protocol and applied it on the Sparidae-*Lamellodiscus* host-parasite
22 system, to assess parasite diversity on skin and gills of several sparid host species. We first
23 demonstrated that the use of a metabarcoding approach provided a better understanding of the
24 diversity of monogeneans associated with teleost skin and gills than traditional approaches based
25 on morphological identification. We identified a high diversity of both expected and unexpected
26 (never observed on this host species) *Lamellodiscus* spp. on each host species and on skin and gills.
27 No significant difference in parasite diversity was found between skin and gills. These results
28 suggest that the establishment of the observed host specificity in monogeneans relies on multiple
29 levels of regulation, involving the survival capacity of the larvae and host recognition mechanisms.

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36 *Keywords:* Monogenean, Teleost, Specificity, Metabarcoding approach, Diversity, Host-parasite
37 interactions

38

39 1. Introduction

40 Understanding patterns and mechanisms of host recognition and host specificity among
41 parasites has been a dynamic research area in recent decades (e.g. Poulin, 1992; Whittington,
42 2000; Sasal et al., 2004). Host specificity is considered to be the result of various processes and
43 ecological (Poulin, 1992; Sasal et al., 1998a, 1999), evolutionary (Adamson and Caira, 1994; Sasal,
44 1998b), physiological (Desdevises et al., 2002) or immunological (Buchmann et al., 1999) factors
45 have been suggested to act on the observed host specificity of different parasite species
46 (Whittington et al., 2000). In this study, we defined host specificity (or host range) as the number
47 of known host species in/on which a parasite species may occur (Poulin, 1998). A parasite infecting
48 only one host species is a specialist, whereas a parasite infecting several host species is a
49 generalist (Humphery-Smith, 1989).

50 Among the many organisms that parasitize teleost fishes, the class Monogenea mainly
51 includes ectoparasites and is often abundant on fish skin and gills. Monogeneans are generally
52 highly host specific compared with other parasite groups such as copepods (Poulin, 1992). With
53 the notable exception of viviparous gyrodactylids, monogeneans are oviparous and have a direct
54 life cycle (i.e. they only parasitize one host during their life cycle). Adult monogeneans lay eggs in
55 the water column that generally attach to various supports before hatching into ciliated larvae,
56 called oncomiracidia. These free-swimming larvae actively search for a specific host(s) and
57 colonize its (their) skin (Kearn, 1967; Buchmann and Lindenstrøm, 2002). The larvae then lose their
58 ciliature, some of them remain on the skin but most mature ones migrate from the skin to the gills
59 of their fish host (Tinsley and Owen, 1975; Tinsley, 1983; Kearn, 1985). Several studies showed
60 that monogenean hatching is not continuous throughout the day but displays particular egg laying
61 and hatching rhythms that maximize opportunities to infect fish hosts (Kearn, 1973). Egg laying
62 and hatching rhythms have been shown to respond to many environmental factors such as light,
63 mechanical disturbances, water currents and chemical and osmotic stimuli generally generated by

64 the host (Kearn, 1974, 1980; Kearn and Macdonald, 1976; Macdonald and Jones, 1978; Tinsley,
65 1978; Whittington and Kearn, 1986, 1988, 1989, 2011).

66 Several studies demonstrated that the attraction and specificity of monogeneans are
67 governed by factors present within the host surface mucus (Kearn, 1974; Du Preez and Kok, 1997;
68 Buchmann, 1999; Yoshinaga et al., 2002). Since the 1960s, it has been shown that monogenean
69 larvae are able to distinguish their specific hosts from other organisms and inorganic substrates.
70 Kearn (1967, 1974) showed experimentally that the oncomiracidia of *Entobdella solea* were
71 attracted by agar blocks that had been in contact with the cutaneous mucus of *Solea solea*
72 (Soleidae, Teleostei), their specific host, more than by mucus from other fish species, suggesting
73 the presence of specific chemicals in the host mucus that induce attraction and attachment of
74 specific monogenean larvae. Other studies showed that some monogenean larvae, known as
75 strictly host specific, are able to parasite other fish species under experimental conditions but only
76 for a brief time (Whittington et al., 2000). For example, Le Brun et al. (1988) infected gills of seven
77 different fish species (six cyprinids and one centrarchid) with eggs of *Diplozoon paradoxum*, a
78 specific monogenean species of *Abramis brama* (Cyprinidae). They observed (by dissecting gills)
79 that oncomiracidia of *D. paradoxum* succeeded in attaching to the gill filaments of all fish species.
80 However, oncomiracidium development and maturation were only observed on *A. brama*,
81 whereas their number decreased rapidly in other fish species until their complete disappearance
82 within a few days (Le Brun et al., 1988). These observations suggest that different factors are
83 involved in monogenean specificity and its establishment has multiple levels of regulation,
84 including host recognition and the ability of larvae to survive and mature on their fish host (on
85 both skin and gills). According to these studies, it can be hypothesized that the monogenean host
86 range is large when the larvae colonize host skin and decreases with larval maturation. However,
87 the establishment and the determinants of the specificity and attraction of monogeneans toward
88 their fish hosts is still poorly understood (Whittington et al., 2000; Buchmann and Lindenstrøm,

89 2002; Simkova et al., 2006). Until now, these two mechanisms have only been investigated by
90 analyzing the diversity and abundance of monogeneans based on morphological identification.
91 The aim of this study is therefore to address the hypothesis that monogenean larvae have a wider
92 host range than adults by using a new molecular approach.

93 To investigate how monogenean host specificity establishes during the infection in nature,
94 it is necessary to characterize and compare monogenean species diversity on the fish skin, mostly
95 infected by oncomiracidium larvae, and gills which are infected by both larvae and adults.
96 Traditionally, recovering monogenean individuals (and more generally parasites) is performed by
97 dissecting the host, followed by parasite identification based on morphological criteria, generally
98 on adult forms (Chisholm and Whittington, 1998; Öztürk and Özer, 2014). Adult monogeneans are
99 identified by morphological criteria based on the shape of sclerified parts of the opisthohaptor and
100 the male copulatory organ (e.g. Amine et al., 2006, 2007). However, studying monogenean
101 diversity also implies the recovery and identification of the other life stages: oncomiracidium
102 larvae and eggs. For most monogenean species, larval and egg stages are often difficult or nearly
103 impossible to observe in situ due to their very small size, and morphological characters of these
104 developmental stages are generally insufficient to discriminate species.

105 The use of metabarcoding approaches using the 18S rRNA gene is booming in the field of
106 eukaryotic diversity, allowing a more in-depth investigation of eukaryotic communities including
107 all life stages (Leung et al., 2009; Bass et al., 2015; Huver et al., 2015; Lott et al., 2015; Aivelo and
108 Medlar, 2018; Scheifler et al., 2019). However, when applied to parasite communities, because the
109 18S rRNA gene is shared between the host and its eukaryote parasites, and host tissues are
110 present in large amounts in samples, it is critical to limit, if not avoid, the amplification of host
111 DNA. This generally could lead to an underestimation of parasitic diversity (Leray et al., 2013; Liu
112 et al., 2019). The use of such methods for study of host-parasite interactions remains a challenge.
113 In the present study, in order to compare monogenean diversity between fish skin and gills, we

114 designed a new metabarcoding protocol involving the use of specific primers for Platyhelminthes
115 (Littlewood and Olson, 2001) and restriction enzymes to cut host DNA sequences to hamper their
116 amplification (Flaherty et al., 2018).

117 We focused on a well-known fish-parasite interaction in the Mediterranean Sea, between
118 Sparidae (Perciformes, 19 species in Mediterranean Sea, (Chiba et al., 2009) and their specific
119 monogenean gill ectoparasites belonging to the *Lamellodiscus* genus (Diplectanidae; 28 species
120 were recorded in the Gulf of Lion). The specificity of each *Lamellodiscus* sp. (adult individuals)
121 within fish gills has been intensively studied in Mediterranean sparids and can thus be considered
122 well known (Euzet and Oliver, 1966, 1967; Oliver, 1968, 1973, 1974; Oliver 1987, Les Diplectanidae
123 Bychowsky, 1957 (Monogenea, Monopisthocotylea, Dactylogyridea), Systématique, Biologie,
124 Ontogénie, Écologie. Essai de phylogénèse. PhD Thesis, Université des Sciences et Techniques du
125 Languedoc, France; San Filippo, 1978, Microhabitat des Monogènes Dactylogyroidea parasites
126 branchiaux de Téléostéens Mugilidae et Sparidae. PhD Thesis, Université des Sciences et
127 Techniques du Languedoc, France; Euzet, 1984; Euzet L., Combes C., Caro C., 1993. A check list of
128 monogenea of Mediterranean fish. Second International Symposium on Monogenea,
129 Montpellier/Sète, France; Neifar, 1995, Contribution à l'étude de la biodiversité des monogènes
130 parasites de poissons du secteur nord-est de la Tunisie. Master Thesis, Université de Tunis II,
131 Tunisia; Desdevises et al., 2001; Amine et al., 2006, 2007; Boudaya et al., 2009; Diamanka et al.,
132 2011) (Supplementary Table S1). Two host species, *Boops boops* and *Dentex dentex*, were never
133 found to be parasitized by *Lamellodiscus* while the host specificity of *Lamellodiscus* spp. in the
134 other sparid species is highly variable (specific richness from one to 13 *Lamellodiscus* spp.,
135 Supplementary Table S1). Until now, there has been no report characterizing the diversity of
136 *Lamellodiscus* spp. on fish skin.

137 The aim of this study was to use our new metabarcoding protocol to assess the
138 *Lamellodiscus* spp. diversity on sparid skin and gills, to shed light on the establishment process of

139 the parasite on its specific host(s). Our objectives were to test whether this novel metabarcoding
140 protocol was able to detect more parasite species than a standard approach. We also wanted to
141 assess if this sequencing technique was able to identify expected *Lamellodiscus* spp. (i.e. the same
142 species observed by morphological identification or known to parasitize a particular host fish
143 species) associated with gills and skin mucus of teleosts and/or unexpected species, which would
144 colonize the external surfaces for a short period.

145

146 2. Materials and methods

147 2.1. Ethics statement

148 The Oceanological Observatory of Banyuls-sur-Mer, France, holds the authorization for
149 fishing and housing wild Mediterranean teleosts (Inter-regional direction of Mediterranean Sea).
150 Wild fish were caught (see below for details) in accordance with the European Union Regulations
151 concerning the protection and welfare of experimental animals (European directive 91/492/CCE).

152

153 2.2. *Fish sampling*

154 Fish sampling was conducted between April 2018 and July 2019 in the Bay of Banyuls-sur-
155 Mer (northwest Mediterranean, France) (Table 1). For all fish individuals, a gill fishing net was
156 placed overnight between 0 and 6 m deep. Approximately 6 h later, fish were collected from the
157 net, handled with gloves and put into individual plastic bags right after collection from the net.
158 They were immediately brought from the vessel to the laboratory for dissection. Skin mucus and
159 gill mucus (collected from one gill arch) were collected per fish individual with a sterile spatula and
160 scissors. We collected the same gill arch from all individuals, while the seven other arches were
161 used to determine *Lamellodiscus* spp. diversity and abundance. Samples were immediately put
162 into sterile tubes and frozen at -80°C until DNA extraction. A total of 43 fish individuals from 10
163 sparid species (Table 1) were sampled for their skin and gill mucus.

164

165 2.3. *Characterization of gill parasites*

166 *Lamellodiscus* individuals were sampled on each fish individual under a dissecting
167 microscope from the seven gill arches not used to perform sequencing (see below). To determine
168 *Lamellodiscus* spp. (including *Furnestinia echeneis*, as the phylogenetic analysis by Desdevises
169 (2001) supported that this monospecific genus is included in the *Lamellodiscus* clade), we
170 identified each individual based on the opisthohaptor and copulatory organ morphology under an
171 optical microscope (Euzet and Oliver, 1966; Oliver, 1987, PhD Thesis, cited earlier; Euzet et al.,
172 1993, Second International Symposium on Monogenea, cited).

173

174 2.4. *DNA extraction and 18S rRNA amplification*

175 DNA was extracted by using the Quick-DNA Fecal/Soil Microbe MiniPrep Kit (Zymo
176 Research, Orange, California, USA) following the manufacturer's instructions and eluted in 50 µl of
177 elution buffer. Samples were frozen at -80°C. PCR amplifications were carried out in triplicate and

178 performed using primers targeting a portion of the 18S rRNA gene (360 bp): L7 (5'-
179 TGATTTGTCTGGTTTATTCCGAT-3') and 18S-4 (5'-AGCGACGGGCGGTGTGTAC-3') (primers designed
180 for Platyhelminthes; Littlewood and Olson, 2001). The PCR mix contained 5 µl of 1X KAPA 2G Fast
181 Ready Mix (Sigma-Aldrich, France), 0.2 µl of each primer (concentration of 0.2 µM), 3.6 µl of
182 ultrapure water and 1 µl of DNA in a final volume of 10 µl. After 3 min of initial denaturation at
183 95°C, the following conditions were applied: 30 cycles of 95°C for 45 s (denaturation), 50°C for 45 s
184 (annealing) and 68°C for 90 s (extension), with a final extension at 68°C for 5 min. For each sample,
185 three PCRs were performed under the same conditions, to increase the DNA quantity and to avoid
186 bias due to each reaction. The three amplifications from the same sample were pooled. Then, in
187 order to reduce the proportions of fish amplicons, 15 µl of each PCR product were digested with
188 the restriction enzyme *BstEII* (or *Eco91I*) following the manufacturer's instructions (Thermofisher,
189 France). Samples were purified by using a ReliaPrep™ DNA Clean Up and Concentration System Kit
190 (Promega, France) and eluted in 15 µl of water before another PCR amplification (same protocol as
191 described above). Individual barcode sequences were added to each mix during a second PCR. The
192 second PCR mix contained 12.5 µl of 1X KAPA 2G Fast Ready Mix (Sigma-Aldrich, France), 0.5 µl of
193 each barcode (Nextera Index Sequences described on the Illumina website), 10.5 µl of ultrapure
194 water and 1 µl of DNA for a final volume of 25 µl. PCR conditions were as follows: initial
195 denaturation at 98°C for 30s followed by eight cycles of 98°C for 10 s, 60°C for 20 s, 72°C for 30 s
196 and a final extension at 72°C for 2 min. Each PCR product was run on 1 % agarose gel at 100V for
197 20 min in an electrophoresis chamber (Mupid®-One) to visualize the presence of high molecular
198 weight DNA. The visualization was carried out in a GelMax™ photodocumenter (UVP®). When
199 the DNA was visible in the gel, incubation (37°C for 30 min, 85°C for 15 min) with USB ExoSAP-IT
200 PCR Product Cleanup (Thermofisher, France) was performed to remove any free and unligated
201 primers/barcodes/Illumina adapters. This step is essential to reduce the levels of index hopping
202 and avoid contamination of libraries. All libraries were stored at -20°C. All PCR products were

203 normalized with a 96-well SequelPrep Normalization Plate (Thermofisher, France). Amplicons
204 were pooled and concentrated by using the Wizard SV Gel and PCR Clean up Kit (Promega,
205 France) and sent directly to the sequencing platform. Amplicons were sequenced using Illumina
206 2×250 bp MiSeq Nano sequencing (GENOMER Platform, Station Biologique de Roscoff, France). To
207 increase the diversity of clusters, we added 20% PhiX to the sequencing mix (recommended by the
208 sequencing platform). Sequence data are available in the NCBI Sequence Read Archive (SRA,
209 <https://www.ncbi.nlm.nih.gov/sra>) database belonging to the BioProject PRJNA750891.

210

211 2.5. Processing of 18S sequences

212 The analysis of the raw sequences was done using QIIME2 software and the standard
213 pipeline of DADA2 v.1.16 (Caporaso et al., 2010; Callahan et al., 2017; Hall and Beiko, 2018).
214 Briefly, raw reads were demultiplexed, quality checked and trimmed to remove primer regions,
215 paired ends were assembled, chimeric sequences were discarded, and reads were denoised.
216 DADA2 infers a list of Amplicon Sequence Variants (ASVs). Sequences were aligned against the
217 SILVA 138 reference database (using *qiime feature-classifier classify-sklearn*) distributed by the
218 Silva project (Quast et al., 2013; Yilmaz et al., 2014). In order to achieve a finer taxonomical
219 resolution, especially for monogenean species, we performed an additional BLAST search (95%
220 minimum similarity) (Altschul et al., 1990). Based on the classification, all unassigned sequences
221 were removed. By analyzing the rarefaction plot, the data were rarefied to 2011 sequences. Three
222 samples were discarded (one from skin mucus (*Boops boops*) and two from gill mucus (*B. boops*
223 and *Pagellus acarne*) samples). The remaining sequences were used to assess the diversity of
224 monogeneans among fish species and tissues. All raw data and the 18S sequences processing
225 steps described above are available on MendeleyData (doi:10.17632/rhdf6bjpyn.1).

226

227 3. Results

228 The present study is focused on monogenean diversity, but it should be noted that in spite
229 of the specific primers used, a much larger taxonomic diversity of putative symbionts was
230 obtained (as in Scheifler et al., 2019, see Supplementary Table S2 for more details). Monogeneans
231 accounted for 16.2% of reads obtained (17,125 out of 105,000, after teleost sequences have been
232 removed) out of which 81.7% were assigned to *Lamellodiscus* (13,997) (Supplementary Table S2).

233 Fourteen *Lamellodiscus* spp. were identified from fish skin and gills (Table 2). The two
234 generalist species *Lamellodiscus ignoratus* and *Lamellodiscus elegans* displayed the most
235 important number of reads (2,882 and 6,151 respectively). Most *Lamellodiscus* spp. observed in
236 the gills of each host species (using a dissecting microscope) were also identified in the sequence
237 data, as expected (Table 3). It is particularly the case for fish species known to harbor only one or
238 two *Lamellodiscus* spp. in their gills: *Oblada melanura* (*L. elegans* and *Lamellodiscus gracilis*),
239 *Pagellus acarne* (*Lamellodiscus drummondi* and *Lamellodiscus virgula*), *Pagellus erythrinus*
240 (*Lamellodiscus erythrini*), *Sarpa salpa* (*L. ignoratus* and *Lamellodiscus parisi*), *Sparus aurata*
241 (*Furnestinia echeneis*) and *Spicara maena* (*Lamellodiscus knoepffleri*) (Table 3). For *Diplodus* host
242 species, only the expected generalist *Lamellodiscus* spp., *L. elegans*, *Lamellodiscus ergensi* or
243 *L. ignoratus*, were identified, whereas the other species observed in the gills were not
244 (*Lamellodiscus abbreviatus*, *Lamellodiscus diplodi*, *Lamellodiscus falcus*, *Lamellodiscus tomentosus*
245 and *Lamellodiscus neifari*) (Table 3). In addition to these generally consistent observations
246 between morphological and sequence analyses, the metabarcoding approach revealed the
247 presence of several unexpected *Lamellodiscus* spp. within the gills of seven fish species (out of 10)
248 (Fig. 1A-D, F-H). For example, two *Lamellodiscus* spp., *L. elegans* and *L. ignoratus*, were identified
249 from the sequence data (in low read abundances, Fig. 1A) in the gills of *B. boops*, a sparid species
250 in the study area known not to be parasitized by *Lamellodiscus*. Similarly, sequences assigned to *L.*
251 *elegans* were found in sparid species from which this parasite has never been observed in nature:
252 *P. acarne* (Fig. 1F), *S. aurata* (Fig. 1I), *S. maena* (Fig. 1J) and, as mentioned, *B. boops* (Fig. 1A). Note

253 that *L. elegans* has already been observed in *S. aurata* gills under aquaculture conditions
254 (Mladineo and Maršić-Lučić, 2007).

255 Out of the 14 species identified in the sequence data, 10 were found on fish skin (all
256 species except *L. erythrini*, *Lamellodiscus furcosus*, *L. knoepffleri* and *Lamellodiscus mirandus*) (Fig.
257 1). All fish species harbored *Lamellodiscus* parasites on their skin with a different species diversity
258 according to the host species, between two (*O. melanura*, *P. acarne*, *P. erythrinus*, *S. aurata* or *S.*
259 *maena*, Fig. 1E-G, I-J) and six species (*Diplodus sargus*, Fig. 1C). As noticed for gills, unexpected
260 *Lamellodiscus* spp. were also identified on fish skin. For example, *L. drummondi* was found on the
261 skin of *B. boops* and *Diplodus annularis* (Fig. 1A-B), but is not known to parasitize these species.
262 Similarly, sequence data suggest that the skin of *B. boops*, *S. salpa* and *S. maena* was parasitized
263 by *Lamellodiscus fraternus* (Fig. 1A, H, J), a species known from *D. annularis* and *Diplodus vulgaris*.
264 There was no difference in *Lamellodiscus* spp. richness between skin and gills: some host species
265 showed a higher parasitic richness in gills, such as *D. annularis* or *S. maena*, while others, such as
266 *B. boops* or *D. sargus*, hosted more *Lamellodiscus* spp. on their skin (Table 4).

267

268 4. Discussion

269 Characterizing symbiotic communities within hosts is of primary importance, as these
270 interactions can affect both the ecology and evolution of the different partners and play a crucial
271 role in the maintenance of ecosystem stability (Bush et al., 2001; Pedersen and Fenton, 2007; Ives
272 and Carpenter, 2007; Lafferty et al., 2008; Ings et al., 2009). Traditionally, identification of the
273 eukaryotic symbiotic diversity (in particular parasites) has been conducted using morphological
274 approaches (Chisholm et al., 1998; Öztürk and Özer, 2014). While indispensable, such methods
275 require extensive taxonomic expertise, are very time consuming and the existence of different life
276 stages and/or the microscopic size of many organisms increase the difficulty in assessing the
277 exhaustive diversity of symbionts (Cowart et al., 2015). The use of new high throughput

278 sequencing methods is now established as powerful tools to complement traditional
279 morphological identification, and allows a much faster assessment of species diversity in symbiotic
280 assemblages (Tanaka et al., 2014; Cowart et al., 2015; Avelo and Medlar, 2018). One of the
281 objectives of this study was to improve the characterization of monogenean communities
282 associated with the skin and gills of teleost fish species, compared with traditional methods. First,
283 as in Scheifler et al. (2019), we identified a large number of taxa that can potentially establish
284 symbioses with teleost hosts, such as fungi, arthropods (copepods), cnidarians, nematodes or
285 platyhelminths. In Scheifler et al. (2019), the authors used universal primers for partial rRNA that
286 favored the amplification of bacteria over eukaryotes (76% of all reads corresponded to bacteria)
287 and where most eukaryotic sequences (94%) were teleost sequences. The present characterization
288 of ectosymbiotic communities within teleost fish species relies on an improvement of this protocol
289 in order to focus on eukaryotic symbionts and especially platyhelminths. Recently, two methods
290 have been applied to inhibit the amplification of host DNA: designing blocking primers to hamper
291 the amplification of host DNA (Vestheim and Jarman, 2008) or using restriction enzymes that
292 require a unique cutting site in the host sequence (which can be difficult to find) (Flaherty et al.,
293 2018). These two methods enhance the detection of eukaryotic symbionts by avoiding, as much as
294 possible, amplification of host sequences (Leray et al., 2013; Clerissi et al., 2018; Flaherty et al.,
295 2018). In our study, we used both more specific primers for Platyhelminthes (Littlewood and
296 Olson, 2001) and restriction enzymes that reduced considerably the number of teleost sequences,
297 by more than half (94% to 36.6%), as reported in previous studies (Flaherty et al., 2018). This
298 method, involving the use of both specific primers and restriction enzymes, allowed us to improve
299 characterization of the diversity and composition of monogenean assemblages. The application of
300 restriction enzymes or blocking primers in marine animals has been so far limited to a few studies
301 (Clerissi et al., 2018), and most focused on gut content (Vestheim and Jarman, 2008; Leray et al.,
302 2013; Liu et al., 2019).

303 As already mentioned, monogenean ciliated larvae are attracted to the mucus of teleost
304 fish (Kearn, 1967; Buchmann and Lindenstrøm, 2002). The larvae then lose their ciliature, migrate
305 to the gills and develop into adults. It should then be expected that adult *Lamellodiscus* spp.
306 identified in the gills of each fish species are also present on the skin (as the larval stage). This is
307 generally confirmed by the sequence data. For example, *F. echeneis*, the specific parasite of *S.*
308 *aurata* and identified in the host gills, was also found on its skin. Similarly, *L. ignoratus* and *L.*
309 *parisi*, parasites of *S. salpa*, are both observed on the host's skin and gills. The comparison of
310 *Lamellodiscus* diversity between skin and gills of several fish species allowed us to propose new
311 hypotheses on the establishment of monogenean host specificity, that is, oncomiracidia are not
312 specifically attracted only to the host species where adults are found, but selection steps take
313 place on hosts that eliminate some larvae, to reach a more stringent specificity in adult
314 monogeneans. The monogenean life cycle appeared to be more complex than initially described
315 and the process from egg expulsion in the water column to the establishment of the adult in fish
316 gills seems to be regulated at multiple levels: i) host recognition, colonization and attachment of
317 monogenean larvae on fish skin, ii) their survival on fish skin, iii) their ability to mature (deciliation
318 of larvae) and migrate to gills, and finally iv) their capacity to survive and attach to the gills (Fig. 2).
319 First of all, we highlighted that each fish species harbors a high diversity of *Lamellodiscus* spp. on
320 their skin. Then, even if monogenean larvae are able to distinguish fish species, colonize and
321 attach to the skin of particular fish species, as previously reported (Kearn et al., 1967), i) host
322 recognition, colonization and attachment do not seem to be highly restricted at this stage (i.e.
323 compared with the pattern known for adults in the gills) as multiple unexpected *Lamellodiscus*
324 spp. were identified on fish skin. Moreover, the survival of monogenean larvae both on ii) skin and
325 iii) gills has been shown to be influenced by different factors, which seem partly related to the fish
326 immune system. For example, Ohashi et al. (2007) demonstrated experimentally that larvae of the
327 monogenean *Heterobothrium okamotoi*, highly specific for *Takifugu rubripes*, could only survive

328 for a few days on skin and gills of other teleost fish species and finally detached. Similarly, Bakke et
329 al. (1991) showed that infection of *Gyrodactylus salaris* on a non-specific host, the European eel
330 *Anguilla Anguilla*, is 75% shorter than its average life-span on salmon. This detachment could be
331 linked to i) the inability of parasites to feed on certain hosts and they therefore starve to death or
332 to ii) immunological factors that can prevent long-term attachment of monogeneans to fish skin or
333 gill filaments (Ohashi et al., 2007). More recently, it has been shown that immune system
334 molecules produced by *T. rubripes* induce the deciliation and oncomiracidium development, and
335 attachment on gills of its specific parasite, *H. okamotoi* (Igarashi et al., 2017; Matsui et al., 2020).
336 This suggests that some parasitic species are able to use host molecules as a receptor for gill
337 infection and can escape from the host immune system (Buchmann and Lindenstrøm, 2002).

338 Contrarily to other host species, few *Lamellodiscus* spp. known to parasitize the genus
339 *Diplodus* were identified by using the metabarcoding approach, especially from *D. sargus* and *D.*
340 *vulgaris*. Indeed, *L. abbreviatus*, *L. diplodi*, *L. falcus*, *L. tomentosus*, and *L. neifari* were not
341 identified from sequence data in this study. Two hypotheses may explain these observations. First,
342 in order to use a high throughput sequencing technique, we decided to target a portion of the 18S
343 rRNA gene of approximately 360 bp long, which may not be resolute enough to discriminate
344 *Lamellodiscus* spp. Indeed, these species have been characterized from longer 18S sequences
345 (approximately 500 bp) and the complete Internal Transcriber Spacer 1 (ITS1) (between 480 and
346 700 bp) (Desdevises et al., 2000, 2001). Second, since the 2000s, a high number of new
347 *Lamellodiscus* spp. were described based on morphological variations of previously described
348 species (especially *L. ergensi* and *L. ignoratus*). These descriptions were based only on the
349 observation of very small morphological changes (in sclerotized parts of the opisthohaptor and the
350 male copulatory organ) without performing any molecular analysis to support these new
351 descriptions. *Lamellodiscus abbreviatus*, *L. neifari*, *L. diplodi* and *L. falcus* were described on
352 morphological variations of the generalist species *L. ignoratus*, whereas *L. tomentosus* is derived

353 from *L. ergensi* (Amine et al., 2006, 2007; Boudaya et al., 2009; Diamanka et al., 2011). Poisot et al.
354 (2011) sequenced for the first time these new *Lamellodiscus* spp. and reported that molecular and
355 morphological evolution are weakly correlated. Indeed, these authors showed that the genetic
356 variation between *L. ignoratus*, *L. neifari*, *L. diploidi* and *L. falcus* and between *L. ergensi* and *L.*
357 *tomentosus* was too low to support these new species and concluded that most of these new
358 *Lamellodiscus* spp., despite their morphological differences, did not represent valid species based
359 on a phylogenetic reconstruction from molecular data. Poisot et al. (2011) proposed that
360 monogeneans (at least *Lamellodiscus*) display a high level of intraspecific phenotypic variation,
361 especially within generalist species (see also Kaci-Chaouch et al., 2008). The absence of
362 *L. abbreviatus*, *L. neifari*, *L. diploidi*, *L. falcus* and *L. tomentosus* from the sequence data of the
363 present paper support their hypothesis that they do not represent valid species.

364 The metabarcoding approach applied to parasitic communities remains nevertheless
365 subject to biases and limitations. First, it is difficult to consider the abundances (i.e. number of
366 reads) obtained in this study (and more generally with sequencing approaches) as real quantitative
367 data (Jorge et al., 2013), as numerous technical factors (DNA extractions, PCR primers that can
368 amplify the DNA of some species at the expense of others) and biological factors (amount of DNA
369 that varies according to the species, size or developmental stage of the individuals) may influence
370 the abundances of reads obtained (Bittleston et al., 2016; Fouhy et al., 2016). These methods are
371 reliable, useful and very fast in detecting the presence of parasitic species, but we cannot interpret
372 these results quantitatively in the same way as data generated by traditional methods based on
373 morphological identification. Morphological methods are obviously also partly biased, as not all
374 parasitic individuals are systematically collected and may also be difficult to identify. Furthermore,
375 this metabarcoding method does not distinguish between sequences obtained from larval or adult
376 stages. The monogenean life cycle is quite simple and we can assume (as in this study) that the
377 teleost skin is mainly parasitized by larvae, whereas gills are parasitized by monogenean adults, as

378 *Lamellodiscus* are only known as gill parasites. However, for more complex life cycles involving
379 several larval stages, for example digeneans such as *Schistosoma*, this method cannot distinguish
380 between eggs, miracidium larvae and cercaria larvae, as all are present in the aquatic environment
381 (Sengupta et al., 2019). Finally, when studying environmental DNA, we cannot exclude that one
382 part of parasite sequences are obtained from dead organisms or organisms that were found near
383 the tissue by chance (e.g. caused by water currents). These organisms may have left DNA traces on
384 the hosts, which may be degraded more or less rapidly depending on the biotic and abiotic
385 conditions of the environment (Dejean et al., 2011; Pilliod et al., 2014; Barnes et al., 2014; Barnes
386 and Turner, 2016), and which may therefore not represent the full reality of the infection
387 dynamics.

388 Monogeneans are common ectoparasites of a wide diversity of marine and freshwater fish
389 species (Buchmann and Bresciani, 2006). For many years, monogeneans have been recognized as
390 causing serious diseases, considerable production loss and mortality in sea-caged aquaculture
391 (Ernst et al., 2002; Whittington, 2004; Whittington et al., 2012). Captive fishes usually grow under
392 more crowded conditions than in the natural environment. As monogeneans are highly fecund,
393 have a short generation time, and are characterized by a direct life cycle, their presence in dense
394 fish populations can result in exponential multiplication and growth. Captivity causes stress such
395 as aggressive behavior between individuals and poor water quality, that generally inhibit the fish
396 immune system response to infection. Monogenean infestations usually result in severe damages
397 to fish skin and gills. Multiple clinical signs have been reported such as i) skin damages (clamped
398 fins; gray patches, open wounds and color change), ii) behavioral modifications (lethargy,
399 swimming near the surface, seeking and rubbing the corners or the sides of the tank, loss of
400 appetite) and iii) metabolic issues (severe respiratory distress) (Reed et al., 2012). Secondary
401 infections with bacteria are common in tissues damaged by monogeneans. Until now, to avoid
402 introduction and/or multiplication of monogeneans, parasitological surveys have been performed

403 by host dissection to recover parasites, followed by morphological identification. Exhaustive
404 surveys of parasitic communities are generally time-consuming and difficult to perform,
405 considering the high diversity of parasite life stages, most of which cannot be detected or
406 identified using morphological criteria. The metabarcoding approach we developed for the
407 present study therefore may be very helpful in identifying monogenean species and prevent
408 infections in aquaculture. While in the present study samples were obtained from dead fish, skin
409 and gill mucus collection can easily be performed on living individuals in a non-invasive way
410 (Aivelo et al., 2017). Our metabarcoding approach is also fast and has great potential to detect
411 early monogenean life stages, helping to prevent their multiplication more rapidly in aquaculture.

412 In this study, we analyzed *Lamellodiscus* 18S rRNA sequence data generated from a
413 specifically designed metabarcoding approach in order to investigate the *Lamellodiscus*
414 monogenean species diversity on their sparid fish hosts in an unprecedented way. This study is the
415 first to highlight the strong potential of molecular approaches to investigate how host specificity
416 establishes in monogeneans, that should be applicable to either parasite taxa. Both morphological
417 and molecular identification are useful to study these processes. The present data uncover a large
418 number of monogeneans species from teleost hosts with the presence of both expected and
419 unexpected *Lamellodiscus* spp. in all fish host species in skin and gills, suggesting that even in
420 nature monogenean larvae are less host-specific than adults, which adds a level of complexity to
421 the monogenean life cycle.

422

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430

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669 Legend to Figures

670

671 Fig. 1. *Lamellodiscus* spp. identified from *Boops boops* (A), *Diplodus annularis* (B), *Diplodus sargus*
672 (C), *Diplodus vulgaris* (D), *Oblada melanura* (E), *Pagellus acarne* (F), *Pagellus erythrinus* (G), *Sarpa*
673 *salpa* (H), *Sparus aurata* (I) and *Spicara maena* (J). Expected *Lamellodiscus* spp. (i.e. same
674 *Lamellodiscus* spp. observed by morphological identification or known to parasitize the host
675 species) within each fish species are in bold. The y-axis represents the number of reads obtained
676 for each *Lamellodiscus* sp. in each fish species.

677

678 Fig. 2. Establishment of the host specificity of monogeneans during their life cycle (modified from
679 Llewellyn et al., 1963; Roubal, 1994; Sánchez-García et al., 2015).

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683

684 Table 1. DNA samples sequenced in this study.

Fish species	Number of samples	
	Gill mucus	Skin mucus
<i>Boops boops</i>	5	5
<i>Diplodus annularis</i>	5	5
<i>Diplodus sargus</i>	5	5
<i>Diplodus vulgaris</i>	4	4
<i>Oblada melanura</i>	4	4
<i>Pagellus acarne</i>	4	4
<i>Pagellus erythrinus</i>	4	4
<i>Sarpa salpa</i>	5	5
<i>Sparus aurata</i>	4	4
<i>Spicara maena</i>	3	3
Total	86	

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688

689 **Table 2.** *Lamellodiscus* spp. associated with fish skin and gill mucus and number of reads identified
690 for each species.

<i>Lamellodiscus</i> spp.	Number of reads in...	
	Gill mucus	Skin mucus
<i>Furnestinia echeneis</i>	304	466
<i>Lamellodiscus coronatus</i>	20	19
<i>Lamellodiscus drummondi</i>	463	48
<i>Lamellodiscus elegans</i>	4273	1878
<i>Lamellodiscus ergensi</i>	680	63
<i>Lamellodiscus erythrini</i>	72	0
<i>Lamellodiscus fraternus</i>	60	273
<i>Lamellodiscus furcosus</i>	54	0
<i>Lamellodiscus gracilis</i>	259	12
<i>Lamellodiscus ignoratus</i>	2714	168
<i>Lamellodiscus knoepffleri</i>	54	0
<i>Lamellodiscus mirandus</i>	254	0
<i>Lamellodiscus parisi</i>	1358	55
<i>Lamellodiscus virgula</i>	266	27
Unidentified species	153	4
Subtotal	10984	3013
Total		13997

691

692 **Table 3.** Comparison of *Lamellodiscus* spp. diversity between morphological observation and sequencing within gills. *Lamellodiscus* spp. identified
 693 through both methods are in bold.

Fish species	Known richness	Observed richness	<i>Lamellodiscus</i> spp. identified by morphological observation	Sequencing richness	<i>Lamellodiscus</i> spp. identified by sequencing
<i>Boops boops</i>	0	0		2	<i>L. elegans</i> / <i>L. ignoratus</i>
<i>Diplodus annularis</i>	7	4	<i>L. elegans</i> / <i>L. gracilis</i> / <i>L. ignoratus</i> / <i>L. ergensi</i>	5	<i>L. coronatus</i> / <i>L. elegans</i> / <i>L. gracilis</i> / <i>L. ignoratus</i> / <i>L. mirandus</i>
<i>Diplodus sargus</i>	13	12	<i>L. abbreviatus</i> / <i>L. coronatus</i> / <i>L. diplodi</i> / <i>L. elegans</i> / <i>L. ergensi</i> / <i>L. falcus</i> / <i>L. furcosus</i> / <i>L. gracilis</i> / <i>L. ignoratus</i> / <i>L. mirandus</i> / <i>L. tomentosus</i> / <i>L. neifari</i> / <i>L. diplodi</i> / <i>L. elegans</i> / <i>L. ergensi</i> / <i>L. falcus</i> / <i>L. fraternus</i> / <i>L. ignoratus</i> / <i>L. neifari</i> / <i>L. tomentosus</i>	8	<i>L. drummondi</i> / <i>L. elegans</i> / <i>L. ergensi</i> / <i>L. fraternus</i> / <i>L. furcosus</i> / <i>L. gracilis</i> / <i>L. ignoratus</i> / <i>L. mirandus</i>
<i>Diplodus vulgaris</i>	9	8	<i>L. falcus</i> / <i>L. fraternus</i> / <i>L. ignoratus</i> / <i>L. neifari</i> / <i>L. tomentosus</i>	4	<i>L. elegans</i> / <i>L. ergensi</i> / <i>L. gracilis</i> / <i>L. ignoratus</i>
<i>Oblada melanura</i>	2	2	<i>L. elegans</i> / <i>L. gracilis</i>	2	<i>L. elegans</i> / <i>L. gracilis</i>
<i>Pagellus acarne</i>	2	2	<i>L. drummondi</i> / <i>L. virgula</i>	3	<i>L. drummondi</i> / <i>L. elegans</i> / <i>L. virgula</i>
<i>Pagellus erythrinus</i>	1	1	<i>L. erythrini</i>	1	<i>L. erythrini</i>
<i>Sarpa salpa</i>	2	2	<i>L. ignoratus</i> / <i>L. parisi</i>	2	<i>L. ignoratus</i> / <i>L. parisi</i>
<i>Sparus aurata</i>	1	1	<i>Furnestinia echeneis</i>	2	<i>F. echeneis</i> / <i>L. elegans</i>
<i>Spicara maena</i>	1	1	<i>L. knoepffleri</i>	6	<i>L. knoepffleri</i> / <i>L. elegans</i> / <i>L. fraternus</i> / <i>L. ignoratus</i> / <i>L. parisi</i> / <i>L. virgula</i>

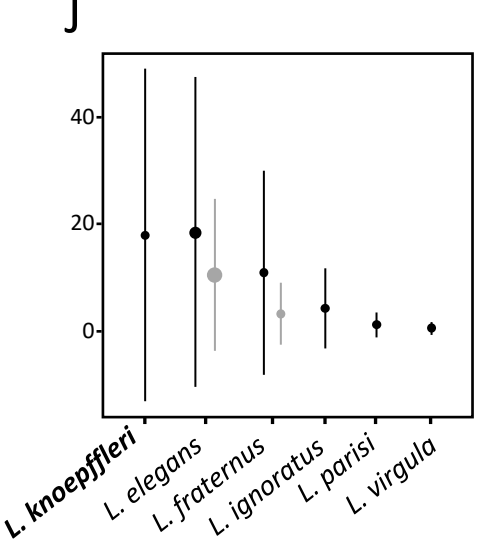
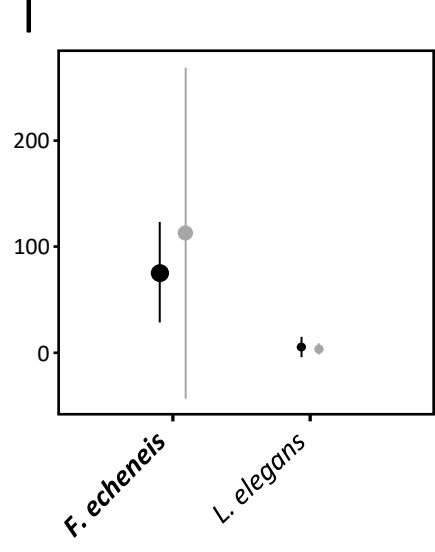
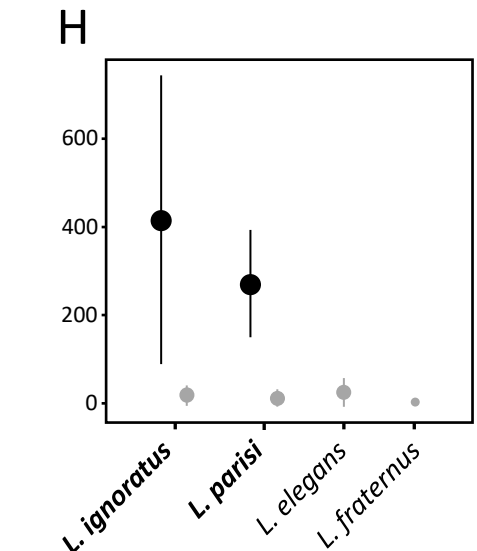
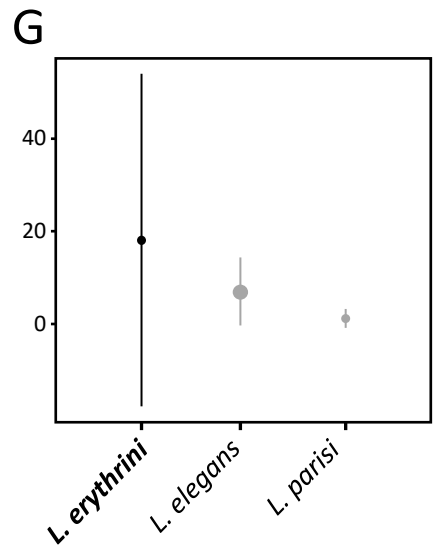
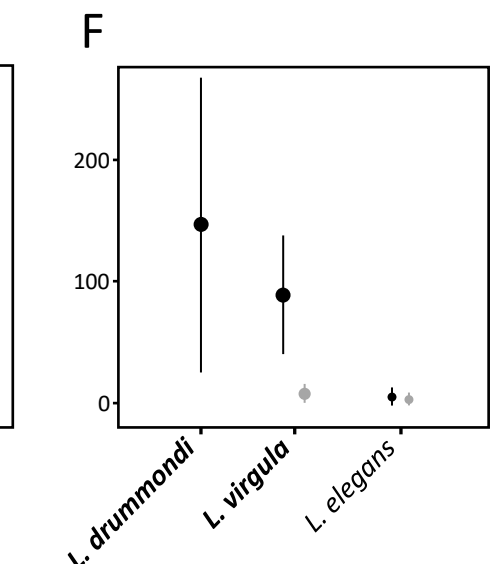
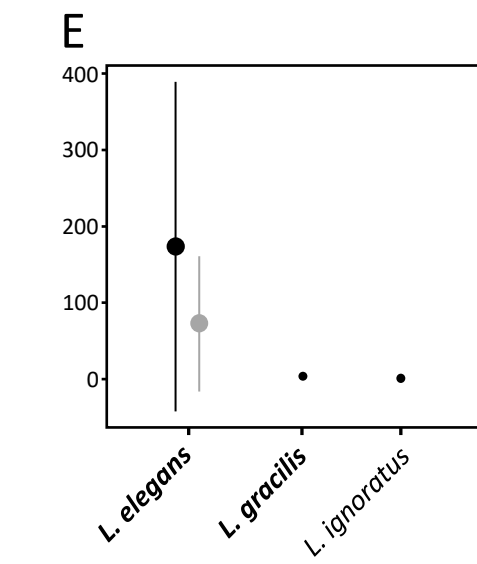
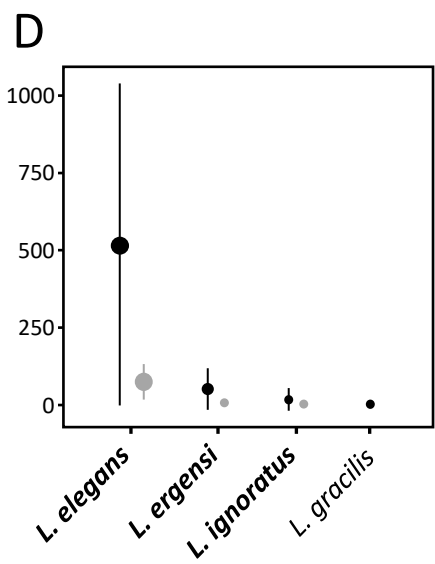
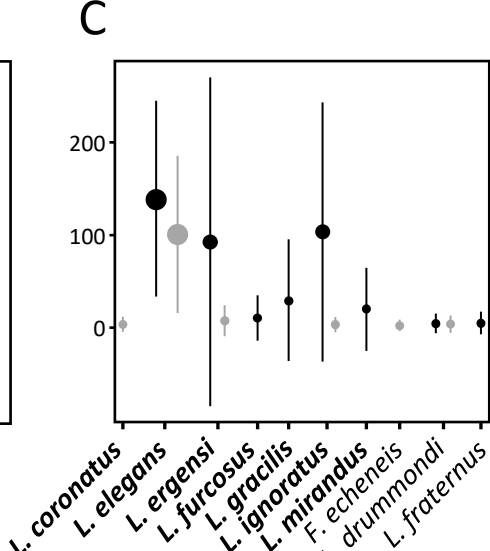
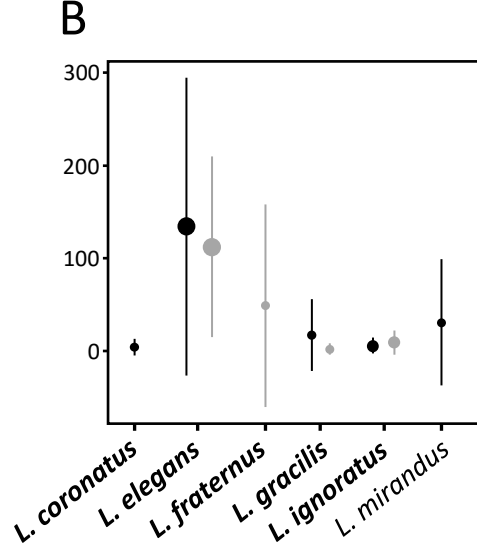
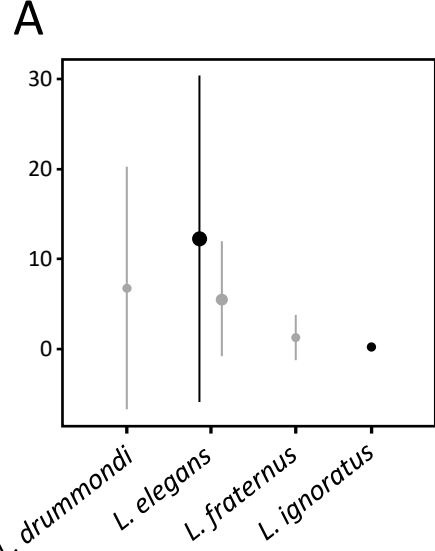
694 **Table 4.** Comparison of species richness (number of *Lamellodiscus* spp. found in gill or skin mucus
695 samples from each fish species) between gill mucus and skin mucus in each teleost host species.
696 The highest richness in each host species is in bold.

Fish species	<i>Lamellodiscus</i> richness within...	
	Gill mucus	Skin mucus
<i>Boops boops</i>	2	3
<i>Diplodus annularis</i>	5	4
<i>Diplodus sargus</i>	8	6
<i>Diplodus vulgaris</i>	4	3
<i>Oblada melanura</i>	2	2
<i>Pagellus acarne</i>	3	2
<i>Pagellus erythrinus</i>	1	2
<i>Sarpa salpa</i>	2	4
<i>Sparus aurata</i>	2	2
<i>Spicara maena</i>	6	2

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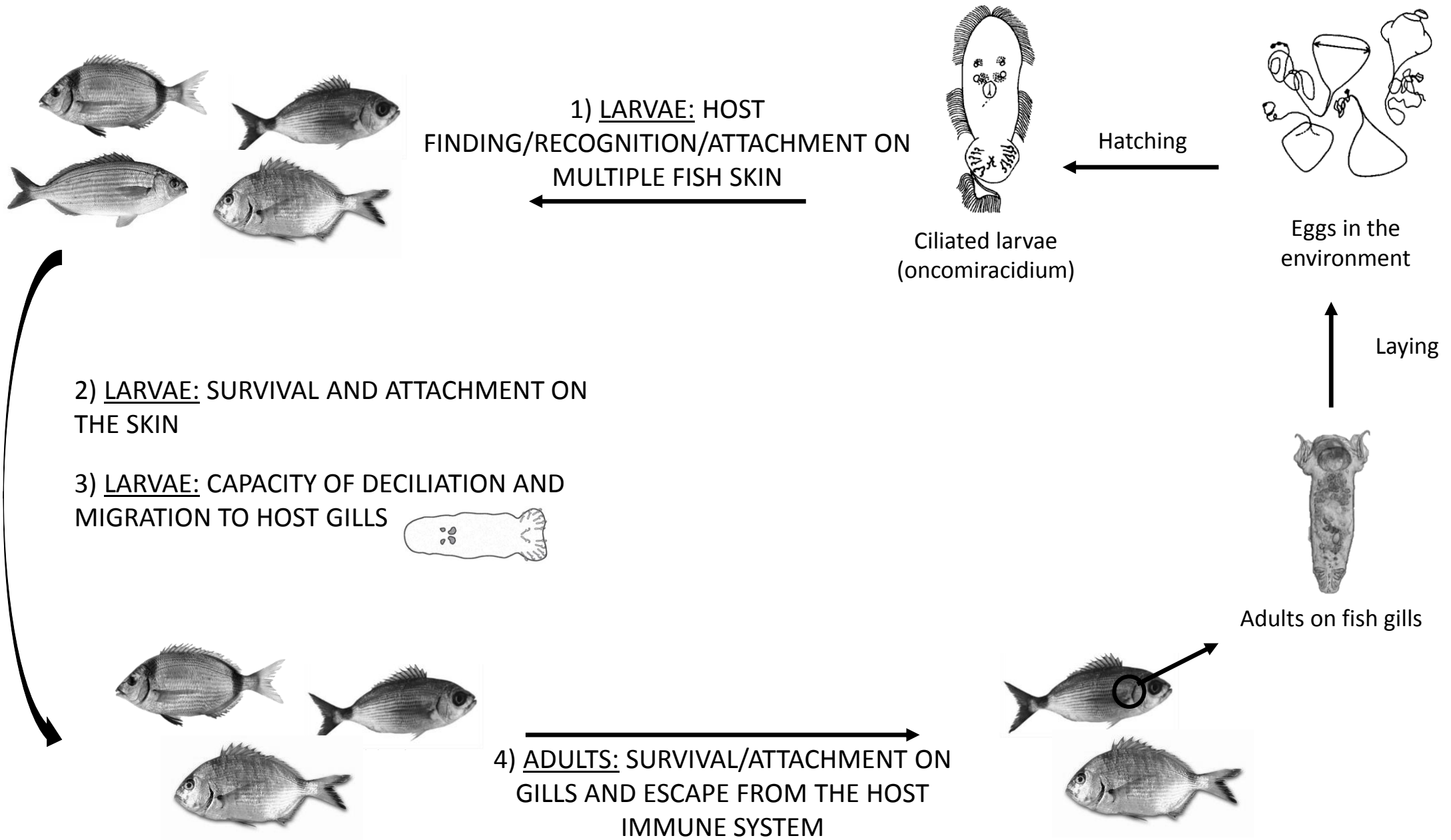
Legend:

1) Tissue

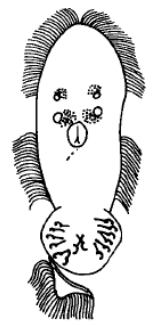
- Gill mucus
- Skin mucus

2) Number of parasitized individuals

- 1
- 2
- 3
- 4
- 5



1) LARVAE: HOST FINDING/RECOGNITION/ATTACHMENT ON MULTIPLE FISH SKIN



Ciliated larvae (oncomiracidium)

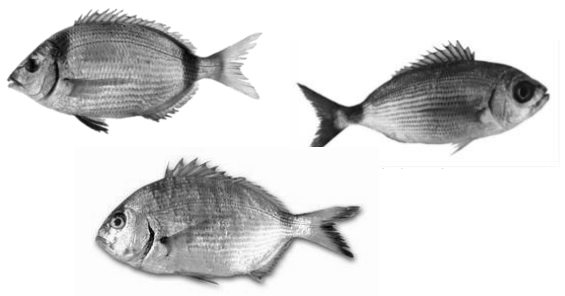
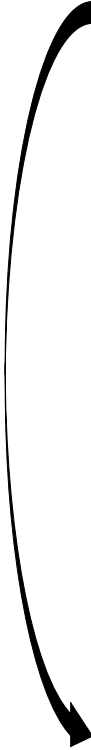
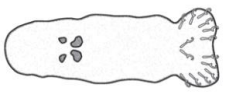


Eggs in the environment

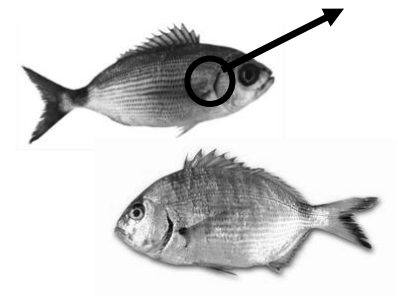
Hatching

2) LARVAE: SURVIVAL AND ATTACHMENT ON THE SKIN

3) LARVAE: CAPACITY OF DECILIATION AND MIGRATION TO HOST GILLS



4) ADULTS: SURVIVAL/ATTACHMENT ON GILLS AND ESCAPE FROM THE HOST IMMUNE SYSTEM



Laying

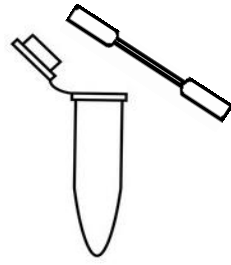


Adults on fish gills



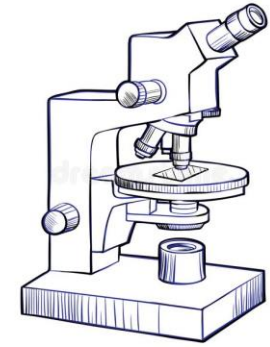
Skin mucus

Gill mucus and
gill arches



Specific primers and
restriction enzymes

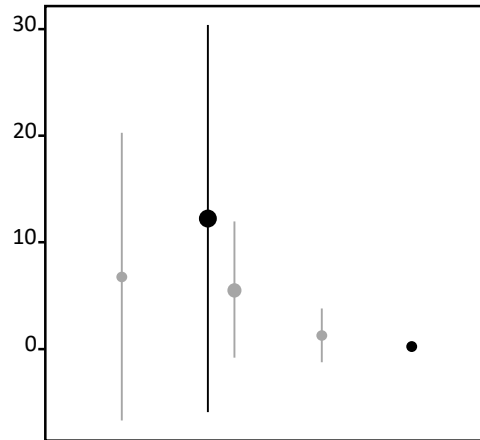
High throughput sequencing



Morphological identifications

Expected and **unexpected** *Lamellodiscus* species

Boops boops



Diplodus annularis

