International Journal for Parasitology 52 (2022) 559–567

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

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

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ARTICLE INFO

Article history:
Received 8 November 2021
Received in revised form 2 February 2022
Accepted 7 February 2022
Available online 28 March 2022

Keywords:
Monogenean
Teleost
Specificity
Metabarcoding approach
Diversity
Host-parasite interactions

ABSTRACT

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

1. Introduction

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

Among the many organisms that parasitize teleost fish, the class Monogenea mainly includes ectoparasites and is often abundant on fish skin and gills. Monogeneans are generally highly host specific compared with other parasite groups such as copepods (Poulin, 1992). With the notable exception of viviparous gyrodactylids, monogeneans are oviparous and have a direct life cycle (i.e. they only parasitize one host during their life cycle). Adult monogeneans lay eggs in the water column that generally attach to various supports before hatching into ciliated larvae, called oncomiracidia. These free-swimming larvae actively search for a specific host(s) and colonise its (their) skin (Kearn, 1967; Buchmann and Lindenstrøm, 2002). The larvae then lose their ciliature, some of them remain on the skin but most mature ones migrate from the skin to the gills of their fish host (Tinsley and Owen, 1975; Tinsley, 1983; Kearn, 1985). Several studies showed that monogenean hatching is not continuous throughout the day but displays particular egg laying and hatching rhythms that maximise opportunities to infect fish hosts (Kearn, 1973). Egg laying and hatching rhythms have been shown to respond to many environmental factors such as light, mechanical disturbances, water currents and chemical and osmotic stimuli generally generated by the host (Kearn, 1974, 1980; Kearn and Macdonald, 1976; Macdonald and Jones, 1978; Tinsley, 1978; Whittington and Kearn, 1986, 1988, 1989, 2011).

Several studies demonstrated that the attraction and specificity of monogeneans are governed by factors present within the host surface mucus (Kearn, 1974; Du Preez and Kok, 1997; Buchmann, 1999; Yoshinaga et al., 2002). Since the 1960s, it has been shown that monogenean larvae are able to distinguish their specific hosts

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https://doi.org/10.1016/j.ijpara.2022.02.001
0020-7519/

from other organisms and inorganic substrates. Kearn (1967, 1974) showed experimentally that the oncomiracidia of *Entobdella solea* were attracted by agar blocks that had been in contact with the cutaneous mucus of *Solea solea* (Soleidae, Teleostei), their specific host, more than by mucus from other fish species, suggesting the presence of specific chemicals in the host mucus that induce attraction and attachment of specific monogenean larvae. Other studies showed that some monogenean larvae, known as strictly host specific, are able to parasite other fish species under experimental conditions but only for a brief time (Whittington et al., 2000). For example, Le Brun et al. (1988) infected gills of seven different fish species (six cyprinids and one centrarchid) with eggs of *Diplozoon paradoxum*, a specific monogenean species of *Abramis brama* (Cyprinidae). They observed (by dissecting gills) that oncomiracidia of *D. paradoxum* succeeded in attaching to the gill filaments of all fish species. However, oncomiracidium development and maturation were only observed on *A. brama*, whereas their number decreased rapidly in other fish species until their complete disappearance within a few days (Le Brun et al., 1988). These observations suggest that different factors are involved in monogenean specificity and its establishment has multiple levels of regulation, including host recognition and the ability of larvae to survive and mature on their fish host (on both skin and gills). According to these studies, it can be hypothesised that the monogenean host range is large when the larvae colonise host skin and decreases with larval maturation. However, the establishment and the determinants of the specificity and attraction of monogeneans toward their fish hosts is still poorly understood (Whittington et al., 2000; Buchmann and Lindenstrøm, 2002; Simková et al., 2006). Until now, these two mechanisms have only been investigated by analysing the diversity and abundance of monogeneans based on morphological identification. The aim of this study is therefore to address the hypothesis that monogenean larvae have a wider host range than adults by using a new molecular approach.

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

The use of metabarcoding approaches using the 18S rRNA gene is booming in the field of eukaryotic diversity, allowing a more in-depth investigation of eukaryotic communities including all life stages (Leung et al., 2009; Bass et al., 2015; Huver et al., 2015; Lott et al., 2015; Aivelo and Medlar, 2018; Scheifler et al., 2019). However, when applied to parasite communities, because the 18S rRNA gene is shared between the host and its eukaryote parasites, and host tissues are present in large amounts in samples, it is critical to limit, if not avoid, the amplification of host DNA. This generally could lead to an underestimation of parasitic diversity (Leray et al., 2013; Liu et al., 2019). The use of such methods for study of host-parasite interactions remains a challenge. In the present study, in order to compare monogenean diversity between fish skin and gills, we designed a new metabarcoding protocol involving the use of specific primers for Platyhelminthes (Littlewood and Olson, 2001) and restriction enzymes to cut host DNA sequences to hamper their amplification (Flaherty et al., 2018).

We focused on a well-known fish-parasite interaction in the Mediterranean Sea, between Sparidae (Perciformes, 19 species in Mediterranean Sea, (Chiba et al., 2009) and their specific monogenean gill ectoparasites belonging to the *Lamellodiscus* genus (Diplectanidae; 28 species were recorded in the Gulf of Lion). The specificity of each *Lamellodiscus* sp. (adult individuals) within fish gills has been intensively studied in Mediterranean sparids and can thus be considered well known (Euzet and Oliver, 1966, 1967; Oliver, 1968, 1973, 1974; Euzet, 1984; Desdevises, 2001; Amine et al., 2006, 2007; Boudaya et al., 2009; Diamanka et al., 2011) (Supplementary Table S1). Two host species, *Boops boops* and *Dentex dentex*, were never found to be parasitised by *Lamellodiscus* while the host specificity of *Lamellodiscus* spp. in the other sparid species is highly variable (specific richness from one to 13 *Lamellodiscus* spp., Supplementary Table S1). Until now, there has been no report characterising the diversity of *Lamellodiscus* spp. on fish skin.

The aim of this study was to use our new metabarcoding protocol to assess the *Lamellodiscus* spp. diversity on sparid skin and gills, to shed light on the establishment process of the parasite on its specific host(s). Our objectives were to test whether this novel metabarcoding protocol was able to detect more parasite species than a standard approach. We also wanted to assess if this sequencing technique was able to identify expected *Lamellodiscus* spp. (i.e. the same species observed by morphological identification or known to parasitize a particular host fish species) associated with gills and skin mucus of teleosts and/or unexpected species, which would colonise the external surfaces for a short period.

2. Materials and methods

2.1. Ethics statement

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

2.2. Fish sampling

Fish sampling was conducted between April 2018 and July 2019 in the Bay of Banyuls-sur-Mer (northwest Mediterranean, France) (Table 1). For all fish individuals, a gill fishing net was placed over-

Table 1
DNA samples sequenced in this study

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

night between 0 and 6 m deep. Approximately 6 h later, fish were collected from the net, handled with gloves and put into individual plastic bags right after collection from the net. They were immediately brought from the vessel to the laboratory for dissection. Skin mucus and gill mucus (collected from one gill arch) were collected per fish individual with a sterile spatula and scissors. We collected the same gill arch from all individuals, while the seven other arches were used to determine *Lamellodiscus* spp. diversity and abundance. Samples were immediately put into sterile tubes and frozen at −80 °C until DNA extraction. A total of 43 fish individuals from 10 sparid species (Table 1) were sampled for their skin and gill mucus.

2.3. Characterisation of gill parasites

Lamellodiscus individuals were sampled on each fish individual under a dissecting microscope from the seven gill arches not used to perform sequencing (see below). To determine *Lamellodiscus* spp. (including *Furnestinia echeneis*, as the phylogenetic analysis by Desdevises (2001) supported that this monospecific genus is included in the *Lamellodiscus* clade), we identified each individual based on the opisthohaptor and copulatory organ morphology under an optical microscope (Euzet and Oliver, 1966).

2.4. DNA extraction and 18S rRNA amplification

DNA was extracted by using the Quick-DNA Faecal/Soil Microbe MiniPrep Kit (Zymo Research, Orange, California, USA) following the manufacturer's instructions and eluted in 50 µl of elution buffer. Samples were frozen at −80 °C. PCR amplifications were carried out in triplicate and performed using primers targeting a portion of the 18S rRNA gene (360 bp): L7 (5′-TGATTTGTCTGGTTTATTCCGAT-3′) and 18S-4 (5′-AGCGACGGGCGGTGTGTAC-3′) (primers designed for Platyhelminthes; Littlewood and Olson, 2001). The PCR mix contained 5 µl of 1× KAPA 2G Fast Ready Mix (Sigma-Aldrich, France), 0.2 µl of each primer (concentration of 0.2 µM), 3.6 µl of ultrapure water and 1 µl of DNA in a final volume of 10 µl. After 3 min of initial denaturation at 95 °C, the following conditions were applied: 30 cycles of 95 °C for 45 s (denaturation), 50 °C for 45 s (annealing) and 68 °C for 90 s (extension), with a final extension at 68 °C for 5 min. For each sample, three PCRs were performed under the same conditions, to increase the DNA quantity and to avoid bias due to each reaction. The three amplifications from the same sample were pooled. Then, in order to reduce the proportions of fish amplicons, 15 µl of each PCR product were digested with the restriction enzyme Bst*EII* (or Eco*91I*) following the manufacturer's instructions (Thermofisher, France). Samples were purified by using a ReliaPrep™ DNA Clean Up and Concentration System Kit (Promega, France) and eluted in 15 µl of water before another PCR amplification (same protocol as described above). Individual barcode sequences were added to each mix during a second PCR. The second PCR mix contained 12.5 µl of 1X KAPA 2G Fast Ready Mix (Sigma-Aldrich, France), 0.5 µl of each barcode (Nextera Index Sequences described on the Illumina website), 10.5 µl of ultrapure water and 1 µl of DNA for a final volume of 25 µl. PCR conditions were as follows: initial denaturation at 98 °C for 30 s followed by eight cycles of 98 °C for 10 s, 60 °C for 20 s, 72 °C for 30 s and a final extension at 72 °C for 2 min. Each PCR product was run on 1 % agarose gel at 100 V for 20 min in an electrophoresis chamber (Mupid®-One) to visualise the presence of high molecular weight DNA. The visualisation was carried out in a GelMaxTM photodocumenter (UVP®). When the DNA was visible in the gel, incubation (37 °C for 30 min, 85 °C for 15 min) with USB ExoSAP-IT PCR Product Cleanup (Thermofisher, France) was performed to remove any free and unligated primers/barcodes/Illumina adapters. This step is essential to reduce the levels of index hopping and avoid contamination of librairies. All libraries were stored at −20 °C. All PCR products were normalised with a 96-well SequalPrep Normalisation Plate (Thermofisher, France). Amplicons were pooled and concentrated by using the Wizard SV Gel and PCR Clean up Kit (Promega, France) and sent directly to the sequencing platform. Amplicons were sequenced using Illumina 2 × 250 bp MiSeq Nano sequencing (GENOMER Platform, Station Biologique de Roscoff, France). To increase the diversity of clusters, we added 20% PhiX to the sequencing mix (recommended by the sequencing platform). Sequence data are available in the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) database belonging to the BioProject PRJNA750891.

2.5. Processing of 18S sequences

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

3. Results

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

Fourteen *Lamellodiscus* spp. were identified from fish skin and gills (Table 2). The two generalist species *Lamellodiscus ignoratus* and *Lamellodiscus elegans* displayed the most important number of reads (2882 and 6151 respectively). Most *Lamellodiscus* spp. observed in the gills of each host species (using a dissecting microscope) were also identified in the sequence data, as expected (Table 3). It is particularly the case for fish species known to harbour only one or two *Lamellodiscus* spp. in their gills: *Oblada melanura* (*L. elegans* and *Lamellodiscus gracilis*), *Pagellus acarne* (*Lamellodiscus drummondi* and *Lamellodiscus virgula*), *Pagellus erythrinus* (*Lamellodiscus erythrini*), *Sarpa salpa* (*L. ignoratus* and *Lamellodiscus parisi*), *Sparus aurata* (*Furnestinia echeneis*) and *Spicara maena* (*Lamellodiscus knoepffleri*) (Table 3). For *Diplodus* host species, only the expected generalist *Lamellodiscus* spp., *L. elegans*, *Lamellodiscus ergensi* or *L. ignoratus,* were identified, whereas the other species observed in the gills were not (*Lamellodiscus abbreviatus*, *Lamellodiscus diplodi*, *Lamellodiscus falcus*, *Lamellodiscus tomentosus* and *Lamellodiscus neifari*) (Table 3). In addition to these

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

Lamellodiscus spp.	Number of reads in...	
	Gill mucus	Skin mucus
Furnestinia echeneis	304	466
Lamellodiscus coronatus	20	19
Lamellodiscus drummondi	463	48
Lamellodiscus elegans	4273	1878
Lamellodiscus ergensi	680	63
Lamellodiscus erythrini	72	0
Lamellodiscus fraternus	60	273
Lamellodiscus furcosus	54	0
Lamellodiscus gracilis	259	12
Lamellodiscus ignoratus	2714	168
Lamellodiscus knoepffleri	54	0
Lamellodiscus mirandus	254	0
Lamellodiscus parisi	1358	55
Lamellodiscus virgula	266	27
Unidentified species	153	4
Subtotal	10,984	3013
Total	13,997	

generally consistent observations between morphological and sequence analyses, the metabarcoding approach revealed the presence of several unexpected *Lamellodiscus* spp. within the gills of seven fish species (out of 10) (Fig. 1A-D, F-H). For example, two *Lamellodiscus* spp., *L. elegans* and *L. ignoratus*, were identified from the sequence data (in low read abundances, Fig. 1A) in the gills of *B. boops*, a sparid species in the study area known not to be parasitised by *Lamellodiscus*. Similarly, sequences assigned to *L. elegans* were found in sparid species from which this parasite has never been observed in nature: *P. acarne* (Fig. 1F), *S. aurata* (Fig. 1I), *S. maena* (Fig. 1J) and, as mentioned, *B. boops* (Fig. 1A). Note that *L. elegans* has already been observed in *S. aurata* gills under aquaculture conditions (Mladineo and Maršić-Lučić, 2007).

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

4. Discussion

Characterising symbiotic communities within hosts is of primary importance, as these interactions can affect both the ecology and evolution of the different partners and play a crucial role in the maintenance of ecosystem stability (Bush et al., 2001; Pedersen and Fenton, 2007; Ives and Carpenter, 2007; Lafferty et al., 2008; Ings et al., 2009). Traditionally, identification of the eukaryotic symbiotic diversity (in particular parasites) has been conducted using morphological approaches (Chisholm and Whittington, 1998; Öztürk and Özer, 2014). While indispensable, such methods require extensive taxonomic expertise, are very time consuming

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

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

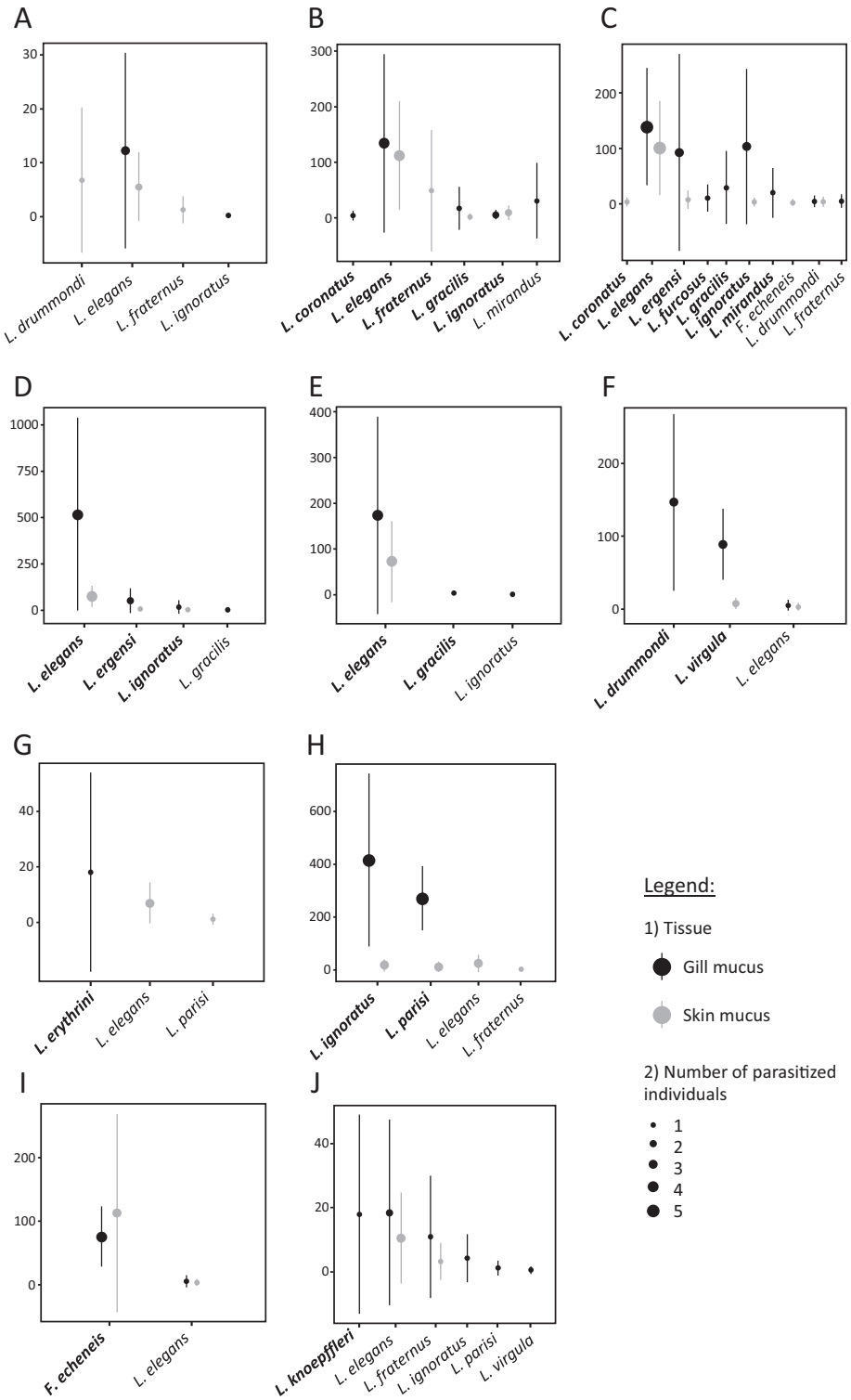

Fig. 1. *Lamellodiscus* spp. identified from *Boops boops* (A), *Diplodus annularis* (B), *Diplodus sargus* (C), *Diplodus vulgaris* (D), *Oblada melanura* (E), *Pagellus acarne* (F), *Pagellus erythrinus* (G), *Sarpa salpa* (H), *Sparus aurata* (I) and *Spicara maena* (J). Expected *Lamellodiscus* spp.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

and the existence of different life stages and/or the microscopic size of many organisms increase the difficulty in assessing the exhaustive diversity of symbionts (Cowart et al., 2015). The use of new high throughput sequencing methods is now established as powerful tools to complement traditional morphological identification, and allows a much faster assessment of species diversity in symbiotic assemblages (Tanaka et al., 2014; Cowart et al., 2015; Aivelo and Medlar, 2018). One of the objectives of this study was to improve the characterisation of monogenean communities associated with the skin and gills of teleost fish species, compared with traditional methods. First, as in Scheifler et al. (2019), we identified a large number of taxa that can potentially establish symbioses with teleost hosts, such as fungi, arthropods (copepods), cnidarians, nematodes or platyhelminths. In Scheifler et al. (2019),

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

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

the authors used universal primers for partial rRNA that favoured the amplification of bacteria over eukaryotes (76% of all reads corresponded to bacteria) and where most eukaryotic sequences (94%) were teleost sequences. The present characterisation of ectosymbiotic communities within teleost fish species relies on an improvement of this protocol in order to focus on eukaryotic symbionts and especially platyhelminths. Recently, two methods have been applied to inhibit the amplification of host DNA: designing blocking primers to hamper the amplification of host DNA (Vestheim and Jarman, 2008) or using restriction enzymes that require a unique cutting site in the host sequence (which can be difficult to find) (Flaherty et al., 2018). These two methods enhance the detection of eukaryotic symbionts by avoiding, as much as possible, amplification of host sequences (Leray et al., 2013; Clerissi et al., 2018; Flaherty et al., 2018). In our study, we used both more specific primers for Platyhelminthes (Littlewood and Olson, 2001) and restriction enzymes that reduced considerably the number of teleost sequences, by more than half (94% to 36.6%), as reported in previous studies (Flaherty et al., 2018). This method, involving the use of both specific primers and restriction enzymes, allowed us to improve characterisation of the diversity and composition of monogenean assemblages. The application of restriction enzymes or blocking primers in marine animals has been so far limited to a few studies (Clerissi et al., 2018), and most focused on gut content (Vestheim and Jarman, 2008; Leray et al., 2013; Liu et al., 2019).

As already mentioned, monogenean ciliated larvae are attracted to the mucus of teleost fish (Kearn, 1967; Buchmann and Lindenstrøm, 2002). The larvae then lose their ciliature, migrate to the gills and develop into adults. It should then be expected that adult *Lamellodiscus* spp. identified in the gills of each fish species are also present on the skin (as the larval stage). This is generally confirmed by the sequence data. For example, *F. echeneis*, the specific parasite of *S. aurata* and identified in the host gills, was also found on its skin. Similarly, *L. ignoratus* and *L. parisi*, parasites of *S. salpa*, are both observed on the host's skin and gills. The comparison of *Lamellodiscus* diversity between skin and gills of several fish species allowed us to propose new hypotheses on the establishment of monogenean host specificity, that is, oncomiracidia are not specifically attracted only to the host species where adults are found, but selection steps take place on hosts that eliminate some larvae, to reach a more stringent specificity in adult monogeneans. The monogenean life cycle appeared to be more complex than initially described and the process from egg expulsion in the water column to the establishment of the adult in fish gills seems to be regulated at multiple levels: (i) host recognition, colonisation and attachment of monogenean larvae on fish skin, (ii) their survival on fish skin, (iii) their ability to mature (deciliation of larvae) and migrate to gills, and finally (iv) their capacity to survive and attach to the gills (Fig. 2). First of all, we highlighted that each fish species harbours a high diversity of *Lamellodiscus* spp. on their skin. Then, even if monogenean larvae are able to distinguish fish species, colonise and attach to the skin of particular fish species, as previously reported (Kearn, 1967), (i) host recognition, colonisation and attachment do not seem to be highly restricted at this stage (i.e. compared with the pattern known for adults in the gills) as multiple unexpected *Lamellodiscus* spp. were identified on fish skin. Moreover, the survival of monogenean larvae both on (ii) skin and (iii) gills has been shown to be influenced by different factors, which seem partly related to the fish immune system. For example, Ohashi et al. (2007) demonstrated experimentally that larvae of the monogenean *Heterobothrium okamotoi*, highly specific for *Takifugu rubripes*, could only survive for a few days on skin and gills of other teleost fish species and finally detached. Similarly, Bakke et al. (1991) showed that infection of *Gyrodactylus salaris* on a non-specific host, the European eel *Anguilla Anguilla*, is 75% shorter than its average life-span on salmon. This detachment could be linked to (i) the inability of parasites to feed on certain hosts and they therefore starve to death or to (ii) immunological factors that can prevent long-term attachment of monogeneans to fish skin or gill filaments (Ohashi et al., 2007). More recently, it has been shown that immune system molecules produced by *T. rubripes* induce the deciliation and oncomiracidium development, and attachment on gills of its specific parasite, *H. okamotoi* (Igarashi et al., 2017; Matsui et al., 2020). This suggests that some parasitic species are able to use host molecules as a receptor for gill infection and can escape from the host immune system (Buchmann and Lindenstrøm, 2002).

Contrarily to other host species, few *Lamellodiscus* spp. known to parasitize the genus *Diplodus* were identified by using the metabarcoding approach, especially from *D. sargus* and *D. vulgaris*. Indeed, *L. abbreviatus*, *L. diplodi*, *L. falcus*, *L. tomentosus*, and *L. neifari* were not identified from sequence data in this study. Two hypotheses may explain these observations. First, in order to use a high throughput sequencing technique, we decided to target a portion of the 18S rRNA gene of approximately 360 bp long, which may not be resolutive enough to discriminate *Lamellodiscus* spp. Indeed, these species have been characterised from longer 18S sequences (approximately 500 bp) and the complete Internal Transcriber Spacer 1 (ITS1) (between 480 and 700 bp) (Desdevises et al., 2000; Desdevises, 2001). Second, since the 2000s, a high number of new *Lamellodiscus* spp. were described based on morphological variations of previously described species (especially *L. ergensi* and *L. ignoratus*). These descriptions were based only on the observation of very small morphological changes (in sclerotised parts of the opisthohaptor and the male copulatory organ) without performing any molecular analysis to support these new descriptions. *Lamellodiscus abbreviatus*, *L. neifari*, *L. diplodi* and *L. falcus* were described on morphological variations of the generalist species *L. ignoratus*, whereas *L. tomentosus* is derived from *L. ergensi* (Amine et al., 2006, 2007; Boudaya et al., 2009; Diamanka et al., 2011). Poisot et al. (2011) sequenced for the first time these new *Lamellodiscus* spp. and reported that molecular and morphological evolution are weakly correlated. Indeed, these authors showed that the genetic variation between *L. ignoratus*, *L. neifari*, *L. diplodi* and *L. falcus* and between *L. ergensi* and *L. tomentosus* was too low to support these new species and concluded that most of these new *Lamellodiscus* spp., despite their morphological differences, did not represent valid species based on a phylogenetic reconstruction from molecular data. Poisot et al. (2011) proposed that monogeneans (at least *Lamellodiscus*) display a high level of intraspecific phenotypic variation, especially within generalist species (see also Kaci-Chaouch et al., 2008). The absence of *L. abbreviatus*, *L. neifari*, *L. diploidi, L. falcus and L. tomentosus* from the sequence data of the

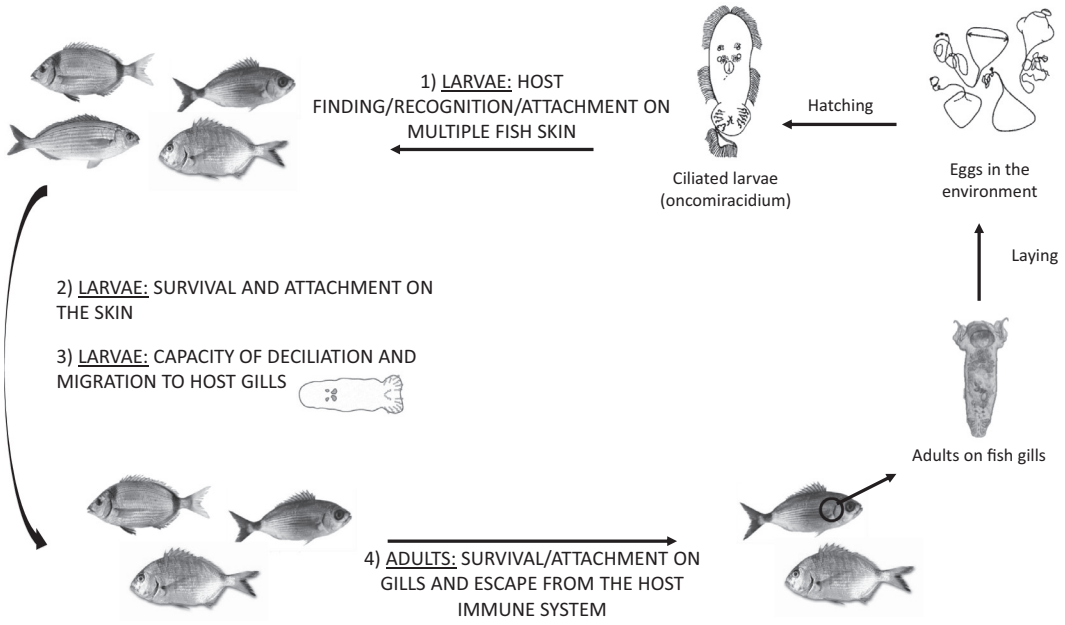

Fig. 2. Establishment of the host specificity of monogeneans during their life cycle (modified from Llewellyn, 1963; Roubal, 1994; Sanchez-García et al., 2015).

present paper support their hypothesis that they do not represent valid species.

The metabarcoding approach applied to parasitic communities remains nevertheless subject to biases and limitations. First, it is difficult to consider the abundances (i.e. number of reads) obtained in this study (and more generally with sequencing approaches) as real quantitative data (Jorge et al., 2013), as numerous technical factors (DNA extractions, PCR primers that can amplify the DNA of some species at the expense of others) and biological factors (amount of DNA that varies according to the species, size or developmental stage of the individuals) may influence the abundances of reads obtained (Bittleston et al., 2016; Fouhy et al., 2016). These methods are reliable, useful and very fast in detecting the presence of parasitic species, but we cannot interpret these results quantitatively in the same way as data generated by traditional methods based on morphological identification. Morphological methods are obviously also partly biased, as not all parasitic individuals are systematically collected and may also be difficult to identify. Furthermore, this metabarcoding method does not distinguish between sequences obtained from larval or adult stages. The monogenean life cycle is quite simple and we can assume (as in this study) that the teleost skin is mainly parasitised by larvae, whereas gills are parasitised by monogenean adults, as *Lamellodiscus* are only known as gill parasites. However, for more complex life cycles involving several larval stages, for example digeneans such as *Schistosoma*, this method cannot distinguish between eggs, miracidium larvae and cercaria larvae, as all are present in the aquatic environment (Sengupta et al., 2019). Finally, when studying environmental DNA, we cannot exclude that one part of parasite sequences are obtained from dead organisms or organisms that were found near the tissue by chance (e.g. caused by water currents). These organisms may have left DNA traces on the hosts, which may be degraded more or less rapidly depending on the biotic and abiotic conditions of the environment (Dejean et al., 2011; Pilliod et al., 2013; Barnes et al., 2014; Barnes and Turner, 2016), and which may therefore not represent the full reality of the infection dynamics.

Monogeneans are common ectoparasites of a wide diversity of marine and freswater fish species (Buchmann and Bresciani, 2006). For many years, monogeneans have been recognised as causing serious diseases, considerable production loss and mortality in sea-caged aquaculture (Ernst et al., 2002; Whittington, 2004; Whittington et al., 2012). Captive fish usually grow under more crowded conditions than in the natural environnement. As monogeneans are highly fecund, have a short generation time, and are characterised by a direct life cycle, their presence in dense fish populations can result in exponential multiplication and growth. Captivity causes stress such as aggressive behaviour between individuals and poor water quality, that generally inhibit the fish immune system response to infection. Monogenean infestations usually result in severe damages to fish skin and gills. Multiple clinical signs have been reported such as (i) skin damages (clamped fins; grey patches, open wounds and colour change), (ii) behavioural modifications (lethargy, swimming near the surface, seeking and rubbing the corners or the sides of the tank, loss of appetite) and (iii) metabolic issues (severe respiratory distress) (Reed et al., 2012). Secondary infections with bacteria are common in tissues damaged by monogeneans. Until now, to avoid introduction and/or multiplication of monogeneans, parasitological surveys have been performed by host dissection to recover parasites, followed by morphological identification. Exhaustive surveys of parasitic communities are generally time-consuming and difficult to perform, considering the high diversity of parasite life stages, most of which cannot be detected or identified using morphological criteria. The metabarcoding approach we developed for the present study therefore may be very helpful in identifying monogenean species and prevent infections in aquaculture. While in the present study samples were obtained from dead fish, skin and gill mucus collection can easily be performed on living individuals in a non-invasive way (Aivelo et al., 2018). Our metabarcoding approach is also fast and has great potential to detect early monogenean life stages, helping to prevent their multiplication more rapidly in aquaculture.

In this study, we analysed *Lamellodiscus* 18S rRNA sequence data generated from a specifically designed metabarcoding approach in order to investigate the *Lamellodiscus* monogenean species diversity on their sparid fish hosts in an unprecedented way. This study is the first to highlight the strong potential of

molecular approaches to investigate how host specificity establishes in monogeneans, that should be applicable to either parasite taxa. Both morphological and molecular identification are useful to study these processes. The present data uncover a large number of monogeneans species from teleost hosts with the presence of both expected and unexpected *Lamellodiscus* spp. in all fish host species in skin and gills, suggesting that even in nature monogenean larvae are less host-specific than adults, which adds a level of complexity to the monogenean life cycle.

Acknowledgements

We thank the Marine Biodiversity and Biotechnology platform (Bio2Mar) (http://bio2mar.obs-banyuls.fr) for providing access to instrumentation, as well as the "Service des Moyens à la Mer (FR3724) de l'Observatoire Océanologique de Banyuls/Mer" for fish sampling. This work was funded by the Sorbonne University, France, programme Emergence 2016 (project SU-16-R-EMR-22-MICROFISH).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2022.02.001.

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