Host specificity is linked to intraspecific variability in the genus *Lamellodiscus* (Monogenea)

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SUMMARY

We investigated whether host specificity is linked to variability within species of *Lamellodiscus* monogeneans, which are gill ectoparasites of the Sparidae. We sampled fish parasites in the northeastern part of the Mediterranean Sea: 4 specialist species, using 1 single host species, and 3 generalist species, using 2 distinct host species. Intraspecific variability was assessed from 2 different datasets. Morphometric variability of the attachment organ, called the haptor, was estimated first from measurements of several sclerified haptoral parts on 102 individuals. Genetic variability was calculated based on comparisons of sequences derived from the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA of 62 individuals. Morphometric variances in the specialist *versus* generalist species were compared *via* principal component analysis and *F*-tests, and uncorrected genetic distances (p-distances) were estimated within each species. We showed that the inter-individual variance of morphometric characters, as well as p-distances, are clearly greater within generalist species than specialist ones. These findings suggest that a relative increase in morphological and molecular variability enhances the possibility to colonize new host species in monogeneans, and supports the hypothesis that intraspecific variability could be a potential determinant of host specificity.

Key words: Monogenea, *Lamellodiscus*, Sparidae, host specificity, morphometry, first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA.

INTRODUCTION

Parasites are often considered as good models to study the specificity of organisms with respect to a given resource (De Meeûs *et al*. 1998), because the resource is easily defined as a host(s) or organ(s) used (Lymbery, 1989; Timms and Read, 1999). While parasite taxa exhibit various ranges of host specificity (Sasal *et al*. 1998; Bush *et al*. 2001), monogeneans are known to be highly host-specific (e.g. Rohde, 1994) and have a direct life-cycle that makes them practical for studies of factors linked to host specificity. Although studied extensively during the last decade, few things are known about these determinants (see discussion in Caro *et al*. 1997; Desdevises *et al*. 2002*b*). Ecological factors may 'drive' host specificity, which could be a way for parasites to aggregate on hosts for reproduction (Rohde, 1994). It is also acknowledged that host size is linked

to host specificity in monogeneans (Sasal *et al*. 1999; Desdevises *et al*. 2002*b*; Morand *et al*. 2002), and that host immunity plays a critical role (Buchmann, 1999). Early studies focused mainly on host factors, such as habitat characteristics favouring parasite specialization, but only a few studies have investigated intrinsic parasite factors that may act on host specificity (e.g. Gemmill *et al*. 2000).

Recently, Jarkovsky *et al*. (2004) and Simkova *et al*. (2006) showed a relationship between morphological features of parasites and some components of their ecological niche within hosts (see also Morand *et al*. 2000). Monogeneans attach to their host *via* their opisthohaptor, which is composed of sclerified parts whose sizes and morphologies are characteristic for each parasite species. However, while size and shape of the different haptoral pieces involved in gill attachment could be under strong selection pressure (Rohde, 1979, 1989; Rohde and Hobbs, 1986; Brooks and McLennan, 1991; Simkova *et al*. 2001*a*; Poulin, 2007), the role of morphological variability in host specificity, which was suggested as important in a previous study (Desdevises *et al*. 2002*b*), has not been fully investigated.

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Table 1. Pattern of host-parasite associations between sparid fishes and their gill monogenean parasites from the genus *Lamellodiscus*

	Parasites: *Lamellodiscus* spp.	Number of hosts	Hosts: Sparid fish species
Specialists	*L. baeri*	1	*Pagrus pagrus*
	L. drummondi	1	*Pagellus acarne*
	L. erythrini	1	*Pagellus erythrinus*
	L. virgula	1	*Pagellus acarne*
Generalists	*L. elegans*	5	*Diplodus annularis, D. sargus, D. vulgaris, Oblada melanura, Spondyliosoma cantharus*
	L. ergensi	4	*Diplodus annularis, D. puntazzo, D. sargus, D. vulgaris*
	L. ignoratus	6	*Diplodus annularis, D. puntazzo, D. sargus, D. vulgaris, Lithognathus mormyrus, Sarpa salpa*

To limit the effects of phylogenetic inertia in the present investigation, we compared generalist and specialist species in a well-defined taxonomic group: monogeneans of the genus *Lamellodiscus*, which are gill parasites of sparid fishes. The pattern of host specificity within *Lamellodiscus* is well known, because this host-parasite system has been intensively studied in the Mediterranean Sea (Euzet and Oliver, 1966, 1967; Oliver, 1968, 1973, 1974, 1987; Euzet, 1984; Desdevises *et al*. 2000, 2001, 2002*a,b*; Neifar *et al*. 2004; Amine and Euzet, 2005). This genus contains strict specialist species that use a single host species, and generalist species, parasitizing several distinct host species (up to 6 in the study area).

We investigated whether size variability in haptoral parts is different between specialist and generalist monogenean species. It was hypothesized that specialist species exhibit lower haptoral variability (related to the 'low' variability encountered in gill size of their unique host) than generalist species that attach to a variety of different host species. To test this hypothesis, we selected strict specialists *versus* clear generalists using at least 4 host species, and conducted morphometric and molecular analyses. Morphometric studies of parasites are commonly conducted (e.g. Almeida *et al*. 2007; Kuchta *et al*. 2007), particularly for monogeneans (e.g. Geets *et al*. 1999). However, thus far, they have mainly addressed taxonomic (Shinn *et al*. 2001; Mariniello *et al*. 2004; Rubtsova *et al*. 2006) and ecological (Simkova *et al*. 2001*b*) questions. While interindividual morphometric variability within monogeneans is often mentioned (Mariniello *et al*. 2004; Rubtsova *et al*. 2006), and can be relatively high (Huyse and Volckaert, 2002; Mariniello *et al*. 2004; Davidova *et al*. 2005), it has never been used for investigating determinants of host specificity. As the same pattern of variability may also be encountered at the molecular level and would be independent of putative phenotypic plasticity, we also studied a 'variable' molecular marker (the first internal transcribed spacer of nuclear ribosomal DNA,

ITS-1) in order to compare levels of morphometric and genetic variability within specialist and generalist species.

MATERIALS AND METHODS

Sampling

Host specimens were sampled in the Gulf of Lion, in the northeastern part of the Mediterranean Sea. Gills were isolated, and gill arches were separated and placed in containers of seawater. Monogeneans were removed under a dissecting microscope and identified based on morphological characteristics of the haptor and copulatory organ under a light microscope. After identification and measurements (see next section), individual parasites were fixed in 70% ethanol for subsequent molecular analyses. Parasite specialist species *Lamellodiscus virgula* and *L. drummondi* were both collected from *Pagellus acarne*, whereas *L. erythrini* and *L. baeri* were sampled from *Pagellus erythrinus* and *Pagrus pagrus*, respectively (Table 1). Parasite generalist species, *L. elegans*, *L. ergensi* and *L. ignoratus*, were sampled exclusively from 2 different host species, i.e. *Diplodus vulgaris* and *D. sargus* (Table 1), in order to minimize a possible host influence on parasite morphology. The parasite species selected were all 'scattered across' the phylogenetic tree of *Lamellodiscus* (see Desdevises *et al*. 2002*b*; Fig. 1B), thus avoiding the comparison of 2 groups of closely related species and generating confounding phylogenetic effects.

Morphometric data

Morphometric data were measured microscopically with an optical micrometer at 400× magnification. Haptoral parts (Fig. 1) were measured according to Amine and Euzet (2005). Only sclerified pieces were assessed because of the absence of deformation during dissection or measurement. Eleven variables (designated a, a′, b, b′, c, c′, d, f, g, Bd, Bv) were measured on the 102 parasites collected. A correlation
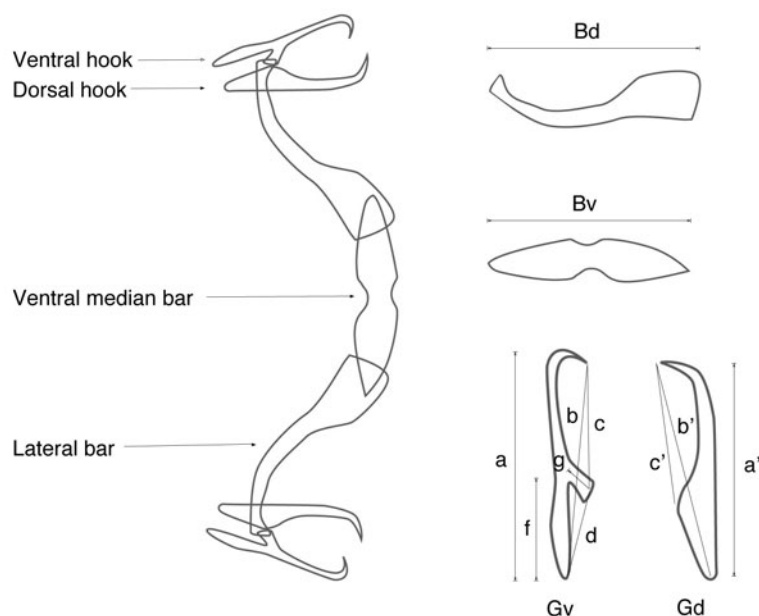
Fig. 1. Morphometric variables measured on the sclerified parts of the opisthohaptor of *Lamellodiscus* species:
a, a′ = total length of dorsal and ventral hook; b, b′ = point length of dorsal and ventral hook; c, c′ = blade opening
of dorsal and ventral hook; d = distance between grip and hilt; f = grip length; g = hilt length; Bd = length of dorsal
lateral bar length; Bv = length of median ventral bar.

matrix was then computed between all morphometric variables, in order to eliminate redundant variables for Pearson correlation coefficients of >0.9 (the kept variable was assumed to represent both). The remaining morphometric variables were assessed using a principal component analysis (PCA), to determine whether individual dispersions were different between specialist and generalist species. PCA and ordination plots were conducted using the program R Package 4.0 (Casgrain and Legendre, 2000).

Variances of each morphometric variable were compared among 2 groups: specialists and generalists. We computed weighted average variances for each variable within each group that were compared using an F-test (see Sokal and Rohlf, 1995) using the program XLSTAT 2007 (Data Analysis and Statistics Software for Microsoft Excel, Addinsoft, Paris, France).

Molecular data

Previously, it was shown that the ITS-1 was relatively variable in sequence among species of *Lamellodiscus*, but relatively conserved within species, with some inter-individual sequence variability (Desdevises *et al.* 2000). Therefore, the ITS-1 region was employed herein to ascertain genetic variability within species. Genomic DNA was extracted from whole individual monogeneans using Bio-Rad Chelex 100™ and proteinase K (10 mg/ml), according to the protocol of Hillis *et al.* (1996). Whenever possible, the same individuals were used for morphometric and molecular analyses. Genomic DNAs were isolated from 58 individuals and each dissolved in 100 μl of water.

For generalist species, sampling was by host species; 6 specimens of *L. ignoratus* were sampled from *Diplodus sargus* and 5 from *D. vulgaris*; for *L. elegans*, 4 individuals were sampled from *D. vulgaris* and 4 from *D. sargus*; for *L. ergensi*, 3 specimens were sampled from *D. sargus* and 4 from *D. vulgaris*. For specialist species, 19 individuals of *L. drummondi* and 5 *L. virgula* were sampled from *Pagellus acarne*, 8 specimens of *L. erythrini* were sampled from *P. erythrinus*, and 4 *L. baeri* individuals from *Pagrus pagrus*.

The entire ITS-1 region was amplified using the primers L7 (5′-TGATTTGTCTGGTT-TATTCCGAT-3′) (Verneau *et al.* 1997) and IR8 (5′-GCTAGCTGCGTTCTTCATCGA-3′) that anneal to the 18S and 5·8S rRNA genes, respectively. PCR was carried out using standard reagents (GoTaq Flexi DNA Polymerase, Promega Corp., USA) in a volume of 25 μl containing 5 μl of genomic DNA ($\sim$25 ng), under the following cycling conditions: 5 min at 95 °C, followed by 34 cycles of 1 min at 48 °C, 1 min 30 at 72 °C, 1 min at 95 °C, and a final elongation of 10 min at 72 °C. Subsequently, PCR products were resolved by agarose electrophoresis and detected following ethidium bromide staining and ultraviolet transillumination (Transilluminator 312 nm Ultraviolet, Model TC-312A, Spectroline, USA). The band of interest ($\sim$1 kb) was excised, purified using the Nucleospin Extract II kit (Macherey-Nagel GmbH & Co, Germany) and subjected to automated sequencing (in both directions) using the same primers as used for the PCR
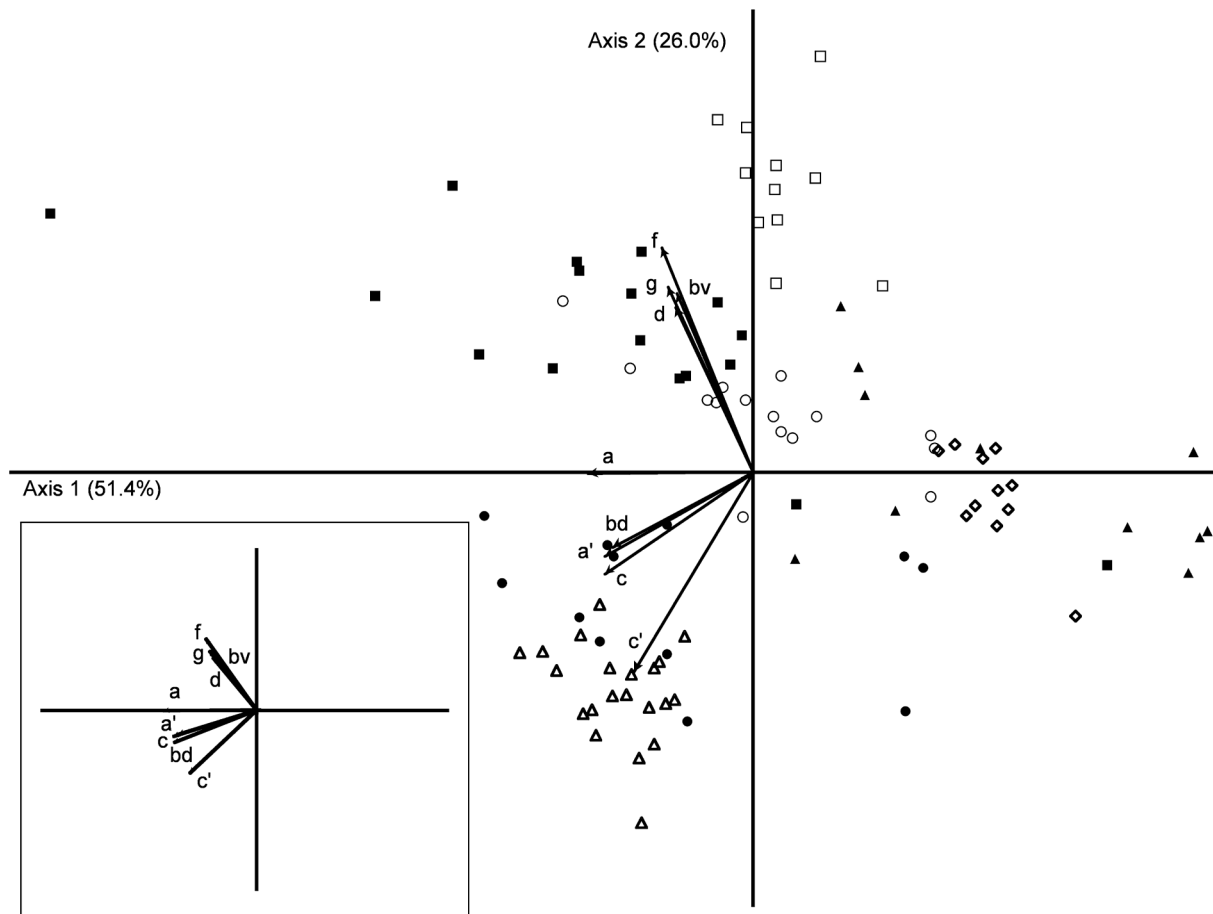
Fig. 2. Ordination diagrams computed from the Principal Component Analysis performed on the morphometric variables described in Fig. 1, on 102 *Lamellodiscus* individuals. The main diagram is a distance bi-plot (preserving the original distances among individuals), the small diagram is a correlation bi-plot (angles indicate corrrelations between variables). Open symbols: specialists (□ : *L. baeri*, △ : *L. drummondi*, ○ : *L. erythrini*, ◇ : *L. virgula*), filled symbols: generalists (● : *L. elegans*, ■ : *L. ergensi*, ▲ : *L. ignoratus*).

amplification (L7 and IR8). A total of 62 sequences (i.e. 58 new ones and 4 reference sequences with GenBank Accession numbers AJ276444–AJ276447; Desdevises *et al.* 2000) were aligned using the program MAFFT v.5 (Katoh *et al.* 2002, 2005) and the alignment adjusted by eye using the program Se-Al v.2.0a11 software (Rambaut, A., 1996. Se-Al: Sequence Alignment Editor, available at http:// evolve.zoo.ox.ac.uk/). Regions in which sequence heterogeneity was detected (and could not be aligned with confidence) were removed from the alignment before computing (with PAUP* 4.0b10 (Swofford, 2003)) uncorrected genetic distances within specialist and generalist species.

RESULTS

Morphometry

Based on the correlation matrix, variables b and b′ were removed from the subsequent numerical analysis, because they were highly collinear with variables a and b. The PCA was then conducted on

the 9 remaining morphometric variables and the resultant ordination diagram is presented in Fig. 2. The two main axes represent 77·4% of the original variance (51·4% for Axis 1 and 26·0% for Axis 2). The most important variables, shaping the distribution of individuals in the morphospace, are ventral hook length (variable a, Pearson correlation coefficient (r) = 0·98 with Axis 1) and grip size (variable f, r = 0·76 with Axis 2). These variables were strongly correlated to several other variables and broadly define 2 groups of quite independent variables (see correlation matrix in Table 2). One variable is linked to hook size (correlated to variable a) and one to the haptoral muscles insertion (Kearn, 1971), then mechanical strength of attachment (variables correlated to grip size (f): g, d, and Bv). On the ordination plot (Fig. 2), all but 1 specialist species, *L. erythrini*, clustered in small and well-defined areas, whereas the distribution of generalist species was more scattered. Variances and variance comparisons between specialists and generalists are shown in Tables 3 and 4, respectively. For each character, variance of the haptoral part within generalists was higher than

Table 2. Correlation matrix (Pearson correlation coefficient) between morphometric variables measured on the haptor of *Lamellodiscus* monogeneans

(See Fig. 1 for nomenclature.)

	a	c	d	f	g	a′	c′	bv	Bd
a	1·00	0·86	0·48	0·55	0·47	0·89	0·69	0·36	0·75
c	0·86	1·00	0·09	0·22	0·33	0·82	0·82	0·16	0·75
d	0·48	0·09	1·00	0·65	0·26	0·28	−0·03	0·42	0·26
f	0·55	0·22	0·65	1·00	0·74	0·28	−0·12	0·52	0·17
g	0·47	0·33	0·26	0·74	1·00	0·24	−0·08	0·52	0·19
a′	0·89	0·82	0·28	0·28	0·24	1·00	0·79	0·16	0·70
c′	0·69	0·82	−0·03	−0·12	−0·08	0·79	1·00	−0·08	0·75
bv	0·36	0·16	0·42	0·52	0·52	0·16	−0·08	1·00	0·38
bd	0·75	0·75	0·26	0·17	0·19	0·70	0·75	0·38	1·00

Table 3. Variances of morphometric variables measured on the haptor of *Lamellodiscus* monogeneans

(See Fig. 1 for nomenclature.)

Lamellodiscus species	Morphometric variables								
	a	c	d	f	g	a′	c′	bv	bd
erythrini	25·86	23·21	10·49	13·40	2·19	9·73	7·50	56·99	66·63
baeri	6·13	16·36	20·55	6·30	7·95	2·08	11·48	88·65	71·83
drummondi	7·08	6·94	3·26	6·28	3·18	5·99	2·91	38·13	30·69
virgula	7·98	11·32	7·29	2·70	2·28	6·31	7·09	15·52	26·29
elegans	100·08	57·53	13·39	18·42	3·44	79·42	23·86	85·76	229·38
ergensi	87·35	97·24	14·48	27·61	25·07	41·82	19·44	318·21	351·49
ignoratus	74·41	34·43	11·52	27·54	3·88	77·12	24·45	399·12	165·26

Table 4. Comparison of variances between specialists (4 species, $n = 61$ individuals) and generalists (3 species, $n = 41$) for morphometric variables described on Fig. 1

(Significantly higher variances are in bold (F-test, $P < 0.05$).)

		a	c	d	F	g	a′	c′	bv	bd
Weighted variances	Specialists	11·68	13·60	9·15	7·34	3·68	6·21	6·50	48·19	46·61
	Generalists	**87·96**	**68·17**	13·36	**24·69**	**12·66**	**62·98**	**22·16**	**270·97**	**232·64**

that within specialists. All but 1 (d: distance between grip and hilt) of these variance differences were statistically significant.

Molecular data

The 58 sequences determined (deposited in the GenBank database under Accession numbers EU259002 to EU259059 were aligned and compared with the reference sequences (Accession numbers AJ276444–AJ276447; Desdevises *et al*. 2000). The length of the ITS-1 region varied from 340 to 729 bp. Length variation has already been reported among species within the genera *Lamellodiscus* (see Desdevises *et al*. 2000), *Gyrodactylus* (see Cable *et al*. 1999; Huyse and Volckaert, 2002; Zietara and Lumme, 2002) and *Polystoma* (see Bentz *et al*. 2006). ITS-1 sequences from individuals of the same species were aligned, as the sequence differences among species are too extensive to achieve an accurate alignment (precluding the reconstruction of a phylogenetic tree and comparison with an outgroup). Intraspecific variations (%) computed from these alignments are given in Table 5; variation is greater within the generalist species than within specialist species. Obviously, the >8-fold variation between specialists and generalists suggests a clear trend, although it cannot be tested for significance (i.e. distances are not independent data).

DISCUSSION

Variances of all morphometric variables investigated, and genetic distances, are clearly greater within species of generalist *Lamellodiscus* than within those of specialists. These results support the hypothesis

Table 5. Mean inter-individual uncorrected genetic distances (%) on ITS1 DNA sequences, with standard deviations

(Codes for *Lamellodiscus* species: ery: *erythrini*; bae: *baeri*; dru: *drummondi*; vir: *virgula*; ele: *elegans*; erg: *ergensi*; ign: *ignoratus*. n: number of individuals. The two last lines show the mean inter-individual uncorrected genetic distances in generalist species sampled from individual hosts (Ds: *Diplodus sargus*; Dv: *D. vulgaris*).)

Lamellodiscus species	Specialists				Generalists					
	ery	bae	dru	vir	ele		erg		ign	
n	8	4	19	5	8		7		11	
Mean (S.D.)	6·77 (6·50)	0 (0)	0 (0)	0·11 (0·15)	13·43 (9·86)		15·42 (8·15)		13·14 (5·69)	
Grand mean					14·00					
					Ds 12·93	Dv 12·98	Ds 8·95	Dv 14·61	Ds 13·85	Dv 11·89

that the use of a narrowly delimited resource by specialists is linked to a lower range of phenotypic and genotypic variation. Furthermore, this use strengthens the adaptive role of the attachment organ (Adamson and Caira, 1994) and its function towards host specificity (Simkova *et al.* 2006). The correlation found between host specificity and intrinsic parasite factors is in accordance with earlier results showing that host specificity in *Lamellodiscus* exhibits a significant level of phylogenetic inertia (Desdevises *et al.* 2002*b*), in that parasites with the same level of host specificity tend to be in the same clades.

Morphometric differences in attachment apparatus between specialist and generalist monogeneans have been reported previously for *Dactylogyrus* (see Jarkovsky *et al.* 2004), where specialist species are more similar than generalists. This latter finding was interpreted to be an effect of a specialization with respect to the host. Similarly, Simkova *et al.* (2001*a*) showed that, within the same genus, a positive correlation between host size and haptor size exists for specialist species (while no significant correlation was found within generalists), suggesting a tighter adaptation of parasite specialists to their resource (i.e. the host species). Nevertheless, only the means of haptoral characters, not their variances, were considered in these studies, which may explain why some authors have failed to observe a link between host size and haptor-related variables within generalists, while this link holds for specialists alone (Morand *et al.* 2002). The intraspecific differences in variances that we found within *Lamellodiscus* are consistent with the results of previous studies of species of *Dactylogyrus*, and furthermore, extend to the individual level. Only mean values for individual species were compared in these previous studies and not individuals. In addition, because variability in attachment organ and genotype could point to

a putative process determining specificity, the present results represent an important advance: more variable species could colonize a larger host spectrum.

However, an important question remains: is the intraspecific variability observed within *Lamellodiscus* specialists and generalists a cause or a consequence of host specificity? Generalist parasites could exhibit a higher variance simply because the different host species used (representing various niches) exert different pressures on parasite morphology, thus increasing morphometric variances as well as genetic distances. In this case, intraspecific variability would be a consequence of host specificity, i.e. the more a parasite species uses different host species, the more its intraspecific variance increases. Because we sampled generalists from only 2 different host species among their broad host spectrum, a putative influence of host species on phenotypic development of parasites has been controlled for. Indeed, if this influence were real, we should have observed the same level of intraspecific variability within specialist and generalist species infesting the same host species. However, this was not the case; intraspecific genetic distance computed between individuals from generalist species sampled from the same host species were comparable to distances computed between all individuals, and clearly higher than distances measured within specialist species (see Table 5). It is still possible that the host species from which generalist species were sampled possess intrinsic characteristics different from host species harbouring the specialist species selected. Specialists were collected on *Pagellus* spp. and *Pagrus pagrus*, whereas generalist species were sampled from *Diplodus sargus* and *D. vulgaris*. If *Diplodus* species present a higher variability in gill morphology or immune defence than *Pagellus* and *Pagrus*, or very different genetic structures, this may select for higher variability in

their parasites, whatever their level of specificity. Presently, there are no data supporting this hypothesis, but it cannot be refuted. Specialist parasite species from these hosts would be required to test this hypothesis, but there is only 1 specialist species of *Lamellodiscus* known from 1 of these 2 hosts, namely *L. mirandus* from *Diplodus sargus* (Euzet and Oliver, 1966), and it is a very rare species.

The alternative hypothesis is that an increase of morphometric variability in the attachment organ allows parasites to colonize more host species. This increase in variability would offer the parasites more opportunities to settle on different host species, and would then be a cause of host specificity. The case of *L. erythrini*, a clear specialist, then becomes quite interesting. This species exhibits a high morphometric and molecular variability compared with other specialist species, although the variation is lower than within generalists. However, if variability were driven by specificity, whatever its cause, *L. erythrini* should have either a narrower intraspecific variance or a larger host spectrum. It could be assumed that lack of host switching opportunities preclude it from capturing new host species, which is consistent with the observation that *L. erythrini* and *L. baeri* form a clade for which a co-speciation event with their ancestral host species has been hypothesized (Desdevises *et al.* 2002*a*). Cospeciation is a process which is probably 'driven' mostly by the absence of host switching opportunities (see Poulin, 2007), and within monogeneans parasites is illustrated by the capture of new host species which seems to be clearly dependent on ecological constraints (Klassen and Beverley-Burton, 1987, 1988; Desdevises *et al.* 2002*a*; Simkova *et al.* 2004; Huyse and Volckaert, 2005). Subsequent speciation may or may not follow, leading to a new specialist species or simply increases the host range of the former switcher.

Finally, it has been suggested that some monogeneans display different haptoral morphologies, depending on the infected hosts, *via* phenotypic plasticity (Mo, 1991*a*, *b*). This plasticity could be an alternative explanation for the greater morphological variability observed within generalist species. However, it is always coupled to a greater genetic variability (in ITS-1), as is the case for the specialist species *L. erythrini*. This observation suggests that, if host-induced phenotypic plasticity exists, it is not the only cause of the variability measured in the attachment organ.

The high level of genetic variation within generalist species may also be explained by the occurrence of cryptic species infesting distinct host species. All of the species of *Lamellodiscus* investigated are clearly defined from a morphological viewpoint (Oliver, 1987; Amine and Euzet, 2005). However, the ITS-1 sequences derived from different individuals herein can be aligned within generalist species while this is not possible between distinct species (Desdevises *et al.* 2000). Similarly, we did not find any evidence in the present study that individuals within the 3 generalist species form clades that would be specific of the host species from which they were collected. If cryptic species exist, each of the new 'molecular' species should also be elevated to the status of generalist species. Another argument against the possibility of cryptic species is that all *L. elegans* individuals sequenced were different in their ITS-1 sequence, whereas this parasite is one of the most clearly defined species morphologically, with its male genital organ having a very specific and unique shape (Euzet and Oliver, 1966; Oliver, 1968). As we cannot rule out the hypothesis of co-occurrence of cryptic parasite species within host species, the ITS-1 region and mitochondrial genes (Vilas *et al.* 2005), such as cytochrome *c* oxidase 1, should be analysed for additional generalist individuals sampled from their entire host spectrum.

In conclusion, the present findings suggest that host switching is more likely from parasite species exhibiting more intraspecific variability. Such findings have consequences for aquaculture, if more variable species do indeed have more opportunities to colonize new host species. For example, the generalist *L. elegans* enlarges its range to a new host, *Sparus aurata*, under aquaculture conditions (Mladineo and Marsic-Lucic, 2007). Nonetheless, other factors do contribute to host specificity within monogeneans, such as host responses, particularly *via* host mucus (Buchmann and Lindenstrøm, 2002); therefore, immunological factors active during a host switch should be investigated. Similarly, because specificity involves host-parasite compatibility, the variability between host individuals within a species should also be explored in future investigations.

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