



## Neither *Diplectanum* nor specific: a dramatic twist to the taxonomic framework of *Diplectanum* (Monogenea: Diplectanidae)

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### ABSTRACT

The taxonomy of the genus *Diplectanum* has been exclusively based on morphological features, with 28 nominal species parasitic on perciform fishes recognised. We used molecular data, to our knowledge for the first time, to evaluate the taxonomic framework of *Diplectanum*, infer the relationships amongst species attributed to this genus, re-assess the degree of host specificity and explore the population genetic structure of *Diplectanum* spp. parasitising Mediterranean sciaenids, which are potential target fish species for aquaculture diversification in the region. A minimum of 10 specimens of *Diplectanum* spp. were sequenced per host species (*Argyrosomus regius*, *Sciaena umbra*, *Umbrina canariensis* and *Umbrina cirrosa*) and locality (Burriana, Sant Carles de la Ràpita and Santa Pola (Spain)) together with five individuals of the type species *Diplectanum aequans*. Sequences of partial 28S rDNA and internal transcribed spacer region of *Diplectanum* spp. were analysed together with those from other Diplectanidae spp. in GenBank using Bayesian inference and maximum likelihood phylogenetic methods. Population genetic analyses were performed using cytochrome *c* oxidase subunit I gene sequences for a diplectanid species with a wide distribution across host species and localities. Results showed that *Diplectanum* was not monophyletic, nor were the specimens infecting sciaenids. Instead they formed two separate clades, 26.1–28.6% divergent for the internal transcribed spacer and 13.2–16.9% for the 28S region from *D. aequans*. Altogether, our results suggest that these specimens represent two distinct new genera from *Diplectanum* and five putative species with low host specificity. It is likely that morphological variability has led to the description of more species than were detected by molecular methods. In contrast to other monogeneans, Diplectaninae gen. spp. are chiefly generalists. Nonetheless, intraspecific genetic divergence in the internal transcribed spacer region of Diplectaninae gen. spp., and population genetic analyses of one presumed generalist species, Diplectaninae gen. sp. 1.2, showed significant variation between subpopulations living on different hosts. The intraspecific genetic structure by host also suggests different cross-infection potential amongst sciaenid species.

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### 1. Introduction

The Mediterranean Sea is one of the oceanic regions with the greatest potential for the development of the aquaculture industry (Schmidt et al., 2011; Massa et al., 2017), which already provides 25% of the seafood for human consumption in this region (Rosa et al., 2012). However, Mediterranean aquaculture is currently limited to a small number of species (Massa et al., 2017). One of the main challenges for the industry is the diversification of farmed

species in order to avoid market saturation and overproduction (Rigos and Katharios, 2010; Schmidt et al., 2011; Harvey, B., Soto, D., Carolsfeld, J., Beveridge, M., Bartley, D.M., 2017. Planning for aquaculture diversification: the importance of climate change and other drivers. FAO Technical Workshop, 23–25 June 2016, FAO Rome. FAO Fisheries and Aquaculture Proceedings No. 47. Rome, FAO). Sciaenid fishes meet the criteria to be considered new candidates for Mediterranean aquaculture (Mañanós et al., 2008; Rigos and Katharios, 2010; Duncan et al., 2013). Identifying parasites and their putative impact on target fish species is crucial to assess the viability of these new cultures (Rigos and Katharios, 2010; Shinn et al., 2015). Ectoparasites such as monogeneans may constrain farm production since their direct life cycle favours

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their proliferation under cultured conditions (Nowak, 2007). Monogenean species of the genus *Diplectanum* Diesing, 1858 are commonly found amongst Mediterranean sciaenids (*Argyrosomus regius* (Asso, 1801), *Sciaena umbra* Linnaeus, 1758, *Umbrina cirrosa* (Linnaeus, 1758) and *Umbrina canariensis* Valenciennes, 1843), with every sciaenid harbouring at least two different *Diplectanum* spp. (Oliver, 1968, 1980; Oliver and Paperna, 1984). In aquaculture, infections caused by species of *Diplectanum* have been related to severe histopathological damage and fish mortality in the moronid *Dicentrarchus labrax* (Linnaeus, 1758) (Dezfuli et al., 2007; Sánchez-García et al., 2011) and in the sciaenid *A. regius* after being subjected to management stress (Andree et al., 2015). A detailed evaluation of the occurrence of *Diplectanum* spp. in wild and cultured sciaenids from the western Mediterranean is therefore timely and relevant.

The genus *Diplectanum* has a world-wide distribution, and infects the gills of Siluriformes, Scorpaeniformes and Perciformes (Pillai and Pillai, 1976; Oliver, 1980; Oliver and Paperna 1984; Wu and Li, 2003; Domingues and Boeger, 2008; Justine, 2008; Pandey and Agrawal, 2008; Justine and Henry, 2010). Domingues and Boeger (2008) included a total of 14 valid species within this genus, although up to 28 are named and listed in the World Register of Marine Species (WoRMS) ([www.marinespecies.org](http://www.marinespecies.org)). Species differentiation in this group has been based solely on morphological features, in particular on the morphology of haptor and copulatory structures (Oliver, 1980; Domingues and Boeger, 2008). However, incorporating molecular information into monogenean systematics has revealed incongruities between molecular phylogenies and the morphological classification of some taxa (Perkins et al., 2009; Boeger et al., 2014). In order to re-evaluate the current classification of *Diplectanum* based on morphology, the gathering of molecular data on *Diplectanum* spp. would help to assess the validity of the species, elucidate the relationships amongst them and clarify the systematics of the Diplectaninae, as has been the case for other platyhelminths (Olson and Littlewood, 2002; Blasco-Costa et al., 2016). To date, the only molecular information available is for the partial 18S rDNA of the type species of the genus (*Diplectanum aequans* (Wagener, 1857)) and the partial 28S rDNA of three *Diplectanum* spp. considered incertae sedis by Domingues and Boeger (2008) (i.e., *Diplectanum penangi* Liang and Leong, 1991, *Diplectanum umbrinum* Tripathi, 1959 and *Diplectanum veropolynemi* Nagibina, 1976). Consequently, the validity of morphologically distinguishable species and the phylogenetic relationships within this genus remain largely unexplored.

*Diplectanum* spp. which are parasitic on Mediterranean sciaenids exhibit a high host specificity, following a trend commonly reported for monogeneans (Whittington et al., 2000), although our knowledge on these host-parasite associations is still restricted to taxa differentiated by morphological criteria. Nine of the 11 diplectanids parasitising sciaenid fishes from the Mediterranean region are specific to a single sciaenid species (*Diplectanum banyulense* Oliver, 1968, *Diplectanum bocqueti* Oliver, 1980, *Diplectanum chaubaudi* Oliver, 1980, *Diplectanum dollfusi* Oliver, 1980, *Diplectanum grassei sensu lato* (s.l.) Oliver, 1974, *Diplectanum labourgi* Oliver, 1973, *Diplectanum melvillei* s.l. Oliver and Paperna, 1984, *Diplectanum nagibinae* s.l. Oliver and Paperna, 1984 and *Diplectanum sciaenidae* Van Beneden and Hesse, 1863). Only two *Diplectanum* spp. (*Diplectanum aculeatum* Parona and Perugia, 1889 and *Diplectanum simile* Bychowsky, 1957) show relatively low host specificity, both found on two sciaenid genera (Oliver, 1968, 1980, 1993; Domingues and Boeger, 2008). The integration of molecular data is required to assess whether the current knowledge on host specificity is accurate or whether polymorphism or phenotypic plasticity in diplectanids parasitising distinct hosts has been wrongly interpreted as species level differences. Moreover, literature shows that populations of parasitic platyhelminths can be

genetically structured by host species (e.g., Wang et al., 2006; Karlsson et al., 2014) or show geographical variation (e.g., Lagrue et al., 2016; Lumme et al., 2016). Such studies on monogeneans are still scarce and mainly restricted to species of *Gyrodactylus* von Nordmann, 1832 (Meinilä et al., 2004; Bueno-Silva et al., 2011; Lumme et al., 2016) and *Cichlidogyrus* Paperna, 1960 (Kmentová et al., 2016). Population-level genetic studies offer great potential to evaluate fine scale host-parasite associations since hosts can be considered isolated habitat patches for parasite species such as the presumably generalist *Diplectanum* spp.

This study aimed to (i) investigate the number of distinct molecular lineages and phylogenetic relationships amongst *Diplectanum* spp. that infect Mediterranean sciaenids (11 out of 28 listed in WoRMS), (ii) update the phylogeny of the Diplectanidae Monticelli, 1903 and discuss its congruence with previous phylogenetic analyses using morphological data, (iii) assess the degree of specificity of sciaenid-*Diplectanum* interactions in order to improve our understanding of their diversity and evolutionary associations with their hosts, and (iv) examine the role of the host and locality as source of intraspecific genetic variation within generalist *Diplectanum* spp.

## 2. Materials and methods

### 2.1. Parasite sampling

A total of 30 sciaenid individuals (*A. regius*, *S. umbra*, *U. canariensis* and *U. cirrosa*) were obtained from three Spanish Western Mediterranean localities during winter 2017: Sant Carles de la Ràpita (40°37'N 0°35'E), Burriana (39°51'N 0°4'W) and Santa Pola (38°11'N 0°33'W). While cultured specimens of *A. regius* were exclusively collected from Burriana, wild *S. umbra*, *U. canariensis* and *U. cirrosa* were sampled from Sant Carles de la Ràpita and Santa Pola. Unfortunately, no *A. regius* could be provided from the wild. Fishes were dissected and the gills were carefully examined for diplectanid monogeneans under a Leica MZ APO (8×–100×) stereomicroscope with a transmitted light source. The specimens of *Diplectanum* spp. were divided into two parts: a small piece of the anterior part of the body was fixed in 100% ethanol for molecular analysis, while the rest (hologenophore) was mounted on a slide with Kaiser's glycerol-gelatin (Sigma-Aldrich, USA) to be used for subsequent identification by morphological characters. A minimum of 10 individuals from each host and locality were used for the molecular analysis. In addition, five specimens of *D. aequans* were collected from a European seabass (*D. labrax*) caught off Sant Carles de la Ràpita in order to obtain additional molecular markers to those available in GenBank and for inclusion of the type species of *Diplectanum* in the phylogenetic analyses.

### 2.2. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from ethanol-fixed fragments of individual specimens of *Diplectanum* spp. in 200 µl of a 5% suspension of Chelex™ in deionized water containing 0.1 mg/ml of proteinase K, followed by incubation at 56 °C for 3 h, boiling and centrifugation at 15,000g for 10 min. Extracted DNA was amplified using three different markers; internal transcribed spacer region (ITS), 28S rRNA gene and the mitochondrial cytochrome c oxidase subunit I gene (COI). Each PCR amplification reaction was performed as 15 µl reactions containing 3 µl of extraction supernatant, 2x MyFi™ Mix (Bioline, USA) and 0.4 µM of each PCR primer. The complete ITS cluster (ITS1-5.8-ITS2) of the rRNA gene was amplified using primers BD1 (5'-GTCGTAACAAGGTTCCGTA-3') and BD2 (5'-TATGCTTAAATTCAGCGGT-3') (Luton et al.,

1992; Morgan and Blair, 1995). The thermocycling profile for the ITS region amplification was as follows: initial denaturation at 95 °C for 3 min, followed by 38 cycles of amplification: 40 s denaturation at 94 °C, 40 s primer annealing at three different temperatures (48 °C, 52 °C and 56 °C, three replicate PCRs with a varying annealing temperature for each sample), and 60 s at 72 °C for primer extension; and a final extension of 5 min at 72 °C. Partial 28S rDNA fragments (C1–D2) were amplified using the universal primers C1 (5'-ACCCGCTGAATTAAGCAT-3') and D2 (5'-TGGTCCGTGTTCAAGAC-3') (Hassouna et al., 1984) with the same thermocycling conditions described above but different annealing temperatures (54 °C, 56 °C and 58 °C). Finally, the COI fragment was amplified with the specific primers COI-ASmit1 (5'-TTTTTGGGCATCTGAGGTTTAT-3') and COI-ASmit2 (5'-TAAAGAAAGACATAATGAAAATG-3') (Littlewood et al., 1997) and the thermocycling conditions were: 95 °C for 3 min followed by 38 cycles; 30 s denaturation at 94 °C, 40 s primer annealing at 49 °C and 40 s at 72 °C for extension; and a final extension of 5 min at 72 °C. Prior to sequencing, PCR amplicons were directly purified with exonuclease I and shrimp alkaline phosphatase enzymes (Werle et al., 1994) or gel-excised using High Pure PCR product purification kit™ (Roche, Mannheim, Germany). Purified amplicons were cycle sequenced using the same primers as for PCR. Sequencing of both strands of amplicons was performed at the commercial facility of Macrogen Europe (Amsterdam, The Netherlands). Sequences of complete ITS (731–1151 bp) as well as partial 28S rDNA (712–965 bp) and COI (144–450 bp) were assembled using Geneious® (v. 8.1 Biomatters Ltd, Auckland, New Zealand) and submitted to GenBank (see Tables 1, 2 for GenBank accession codes).

### 2.3. Phylogenetic analyses

Phylogenetic relationships within Diplectaninae were explored using the newly generated ITS (ITS1-5.8S-ITS2 gene cluster) and 28S rDNA sequences of *Diplectanum* spp. in two independent datasets. Forty-seven new ITS sequences of *Diplectanum* spp. from Mediterranean sciaenids were aligned together with two sequences of *D. aequans*. Ninety-five new 28S rDNA sequences were aligned with those previously published for species belonging to the Diplectanidae (32 sequences for 28S). Sequences of species of Dactylogyridae Bychowsky, 1933 (*Cichlidogyrus* sp. Paperna, 1960, *Euryhaliotrema chrysoaeniae* (Young, 1968), *Ligophorus mugilinus* (Hargis, 1955) and *Tetrancistrum* sp. Goto and Kikuchi, 1917) were included as outgroups. Subsequently, a new dataset containing only one representative sequence of each newly sequenced diplectanid lineage (i.e., putative species; sequences differing more than 1% in the 28S region) was individually compiled for the 28S rDNA to establish the phylogenetic relationships within Diplectanidae. Sequence alignment was performed using MAFFT (Katoh and Standley, 2013) implemented in GUIDANCE (Penn et al., 2010) and the extremities of the alignment were trimmed to match the shortest sequences. Two alignments were retained for analysis: a full dataset including all nucleotide positions (1372 bp for ITS and 1121 bp for 28S) as obtained from MAFFT and a stringent alignment (1032 bp for ITS and 985 bp for 28S) in which nucleotide positions with an alignment score below 0.5 were excluded. Genetic divergence within and amongst *Diplectanum* spp./lineages for the ITS and 28S rDNA regions was calculated as the percentage of pairwise nucleotide differences between trimmed aligned sequences from the full alignments. Additionally, intra- and intergeneric genetic distances were also obtained for *Diplectanum* and those Diplectanidae genera for which 28S sequences were available for two or more species. Full alignments are available at Mendeley Data (DOI: <https://doi.org/10.17632/gjr-j67wbty.2>; DOI: <https://doi.org/10.17632/cp4243xbzs.1>; DOI: <https://doi.org/10.17632/dmfvfmjysy.1>).

The ITS and 28S rDNA datasets were analysed independently via maximum likelihood (ML) and Bayesian inference (BI) methods. The best nucleotide substitution model, GTR +  $\Gamma$  (general-time-reversible model with gamma distribution among-site rate variation) and GTR + I +  $\Gamma$  (GTR +  $\Gamma$  with invariant sites), were selected according to the Akaike information criterion (AIC) score for the ITS and 28S rDNA datasets, respectively, using jModelTest 2.1.4 (Guindon and Gascuel, 2003; Darriba et al., 2012). ML analyses were performed using RAxML v.8 (Stamatakis, 2014), searches for the best scoring ML tree were simultaneously performed with rapid bootstrapping, letting the program halt bootstrapping automatically, and repeated at least three times to ensure consistent results. BI trees were constructed using MrBayes v. 3.2 (Ronquist et al., 2012), running two independent Markov Chain Monte Carlo (MCMC) runs of four MCMC chains for 10 million generations with a sampling frequency of 1000 generations. Burn-in periods were automatically set to the first 2500 trees to ensure the inclusion of generations for which the value of standard deviation of split frequency had reached <0.01. BI and ML analyses were performed on the computational resource CIPRES (Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In Proceedings of the Gateway Computing Environments Workshop (GCE): 14 November 2010; New Orleans, USA, pp 1–8).

### 2.4. Population analyses

In order to investigate the intraspecific variation within a generalist *Diplectanum* sp., partial sequences of the mitochondrial COI gene were obtained for this putative species (based on the phylogenetic analyses above) from as many hosts (at least three sciaenids) and localities (three sampling sites) as possible. Sequences were aligned following the same procedure described above and were collapsed into unique haplotypes using DnaSP v. 5.10 (Librado and Rozas, 2009). Statistical parsimony haplotype networks (Clement et al., 2000) were constructed using PopART v1.7 (Leigh and Bryant, 2015). The same software was used to perform a non-hierarchical analysis of molecular variance (AMOVA) in order to investigate whether genetic variation of *Diplectanum* sp. was structured by host (the number of available samples only allowed for a comparison between *U. cirrosa* and *S. umbra*) or by locality (Sant Carles de la Ràpita and Santa Pola). Pairwise  $F_{ST}$  values between pairs of hosts and localities were calculated in Arlequin v3.5 (Excoffier et al., 2005) using the Tamura and Nei model of nucleotide substitution (Tamura and Nei, 1993). Significance of  $F_{ST}$  values was estimated by 1000 random permutations of haplotypes amongst populations.

## 3. Results

### 3.1. Phylogenetic relationships within the Diplectanidae

A total of 144 sequences of nuclear ribosomal gene fragments (49 sequences of ITS and 95 of 28S) of six supposed *Diplectanum* spp., parasitising five perciform species (four sciaenids and one moronid) in the western Mediterranean, were obtained in this study. Phylogenetic analyses, ML and BI, based on both the full and stringent alignments (see Section 2.3), of the partial 28S rDNA region yielded congruent consensus trees with similar branch topologies. These phylogenetic trees revealed a ladder-like structure with three well-supported clades within the Diplectanidae (labelled A–C in Fig. 1). However, internal nodes showed low support in both ML and BI analyses, leading to a polytomy. Clade A encompassed species of *Calydiscoides* and *Lamellodiscus* (Lamellodiscinae), parasites of nemipterid and sparid fishes (Perciformes).

**Table 1**  
List of monogenean species used in the phylogenetic analyses. Host family, host order, GenBank accession codes and references providing molecular data are also included.

GenBank ID		Monogenean species <sup>a</sup>	Host family	Host order	References
28S	ITS				
EF100557		<i>Calydiscoides indianus</i> <sup>b</sup> ( <i>Calydiscoides flexuosus</i> )	Nemipteridae	Perciformes	Wu et al. (unpublished)
EF100559		<i>Calydiscoides</i> sp.			Wu et al. (unpublished)
AF218124		<i>Cichlidogyrus</i> sp. <sup>c</sup>	Cichlidae	Perciformes	Mollaret et al. (2000)
JN254760		<i>Diplectanocotyla gracilis</i>	Megalopidae	Elopiformes	Freeman and Shinn (2011)
MK203833	MK208301	<i>Diplectanum aequans</i> <sup>d</sup>	Moronidae	Perciformes	Present study
AY553627		<i>Diplectanum blairense</i> <sup>b</sup> ( <i>Paradiplectanum blairense</i> )	Sillaginidae	Perciformes	Wu et al. (2005b)
DQ054820		<i>Diplectanum grouperi</i> <sup>b</sup> ( <i>Pseudorhabdosynochus grouperi</i> )	Serranidae	Perciformes	Tingbao et al. (2006)
DQ054821		<i>Diplectanum penangi</i> inc. sed.	Latidae	Perciformes	Tingbao et al. (2006)
AY553626		<i>Diplectanum sillagonum</i> <sup>b</sup> ( <i>Paradiplectanum sillagonum</i> )	Sillaginidae	Perciformes	Wu et al. (2005b)
MK203834		<i>Diplectaninae</i> gen. sp. 1.1	Sciaenidae	Perciformes	Present study
MK203835	MK208302-MK208303	<i>Diplectaninae</i> gen. sp. 1.2	Sciaenidae	Perciformes	Present study
MK203837	MK208304-MK208307	<i>Diplectaninae</i> gen. sp. 1.3	Sciaenidae	Perciformes	Present study
MK203836	MK208308	<i>Diplectaninae</i> gen. sp. 1.4	Sciaenidae	Perciformes	Present study
MK203838	MK208301- MK208310	<i>Diplectaninae</i> gen. sp. 2.1	Sciaenidae	Perciformes	Present study
EF100560		<i>Diplectanum umbrinum</i> inc. sed.	Sciaenidae	Perciformes	Wu et al. (unpublished)
AY553625		<i>Diplectanum veropolynemi</i> inc. sed.	Polynemidae	Perciformes	Wu et al. (2005b)
FJ882609		<i>Echinoplectanum leopardi</i>	Serranidae	Perciformes	Dang et al. (unpublished)
AF026115		<i>Euryhaliotrema chrysotaeniae</i> <sup>c</sup>	Lutjanidae	Perciformes	Mollaret et al. (1997)
DQ054822		<i>Lamellodiscus acanthopagri</i>	Sparidae	Perciformes	Tingbao et al. (2006)
AF131711		<i>Furnestinia echenei</i> <sup>b</sup> ( <i>Lamellodiscus echeneis</i> )	Sparidae	Perciformes	Mollaret et al. (2000)
FJ767865		<i>Lamellodiscus japonicus</i> <sup>b</sup> ( <i>Calydiscoides japonicus</i> )	Sparidae	Perciformes	Su (unpublished)
EF100562		<i>Lamellodiscus pagrosomi</i>	Sparidae	Perciformes	Wu et al. (unpublished)
DQ054823		<i>Lamellodiscus spari</i>	Sparidae	Perciformes	Wu et al. (unpublished)
DQ054826		<i>Laticola paralatesi</i>	Latidae	Perciformes	Tingbao et al. (2006)
DQ054827		<i>Lepidotrema longipenis</i>	Terapontidae	Perciformes	Tingbao et al. (2006)
AF131710		<i>Ligophorus mugilinus</i> <sup>c</sup>	Mugilidae	Perciformes	Mollaret et al. (2000)
EF100556		<i>Lobotrema sciaenae</i>	Sciaenidae	Perciformes	Wu et al. (unpublished)
DQ157672		<i>Murraytrema pricei</i> <sup>b</sup> ( <i>Pseudomurraytremaoides pricei</i> )	Muraenesocidae	Anguilliformes	Wu et al. (2006)
JN712915		<i>Murraytremaoides</i> sp.	Muraenesocidae	Anguilliformes	Xiong et al. (unpublished)
AY553623		<i>Pseudorhabdosynochus coioideis</i>	Serranidae	Perciformes	Wu et al. (2005a)
FJ882608		<i>Pseudorhabdosynochus cupatus</i>	Serranidae	Perciformes	Dang et al. (unpublished)
AY553622		<i>Pseudorhabdosynochus epinepheli</i>	Serranidae	Perciformes	Wu et al. (2005a)
AY553624		<i>Pseudorhabdosynochus lantauensis</i>	Serranidae	Perciformes	Wu et al. (2005a)
AY553621		<i>Pseudorhabdosynochus latesi</i> <sup>b</sup> ( <i>Laticola latesi</i> )	Latidae	Perciformes	Wu et al. (2005b)
FJ882607		<i>Pseudorhabdosynochus melanesiensis</i>	Serranidae	Perciformes	Dang et al. (unpublished)
AY553620		<i>Pseudorhabdosynochus seabassi</i> <sup>b</sup> ( <i>Laticola seabassi</i> )	Latidae	Perciformes	Wu et al. (2005b)
DQ054830		<i>Pseudorhabdosynochus shenzhenensis</i>	Serranidae	Perciformes	Tingbao et al. (2006)
GQ495270		<i>Pseudorhabdosynochus</i> sp. 2			Dang et al. (unpublished)
FJ882604		<i>Pseudorhabdosynochus summanoides</i>	Serranidae	Perciformes	Dang et al. (unpublished)
DQ157673		<i>Sinodiplectanotrema argyromus</i>	Sciaenidae	Perciformes	Wu et al. (2006)
GU573891		<i>Sinodiplectanotrema malayanum</i>	Sciaenidae	Perciformes	Lim et al. (2010)
AF026114		<i>Tetrancistrum</i> sp. <sup>c</sup>	Siganidae	Perciformes	Mollaret et al. (1997)

<sup>a</sup> The valid scientific names for species taxonomically reassigned after their sequences were deposited in GenBank are shown in brackets.

<sup>b</sup> Unaccepted species based on the World Register of Marine Species.

<sup>c</sup> Species used as outgroups in the 28S phylogenetic analysis.

<sup>d</sup> Species used as outgroups in the internal transcribed spacer region (ITS) phylogenetic analysis.

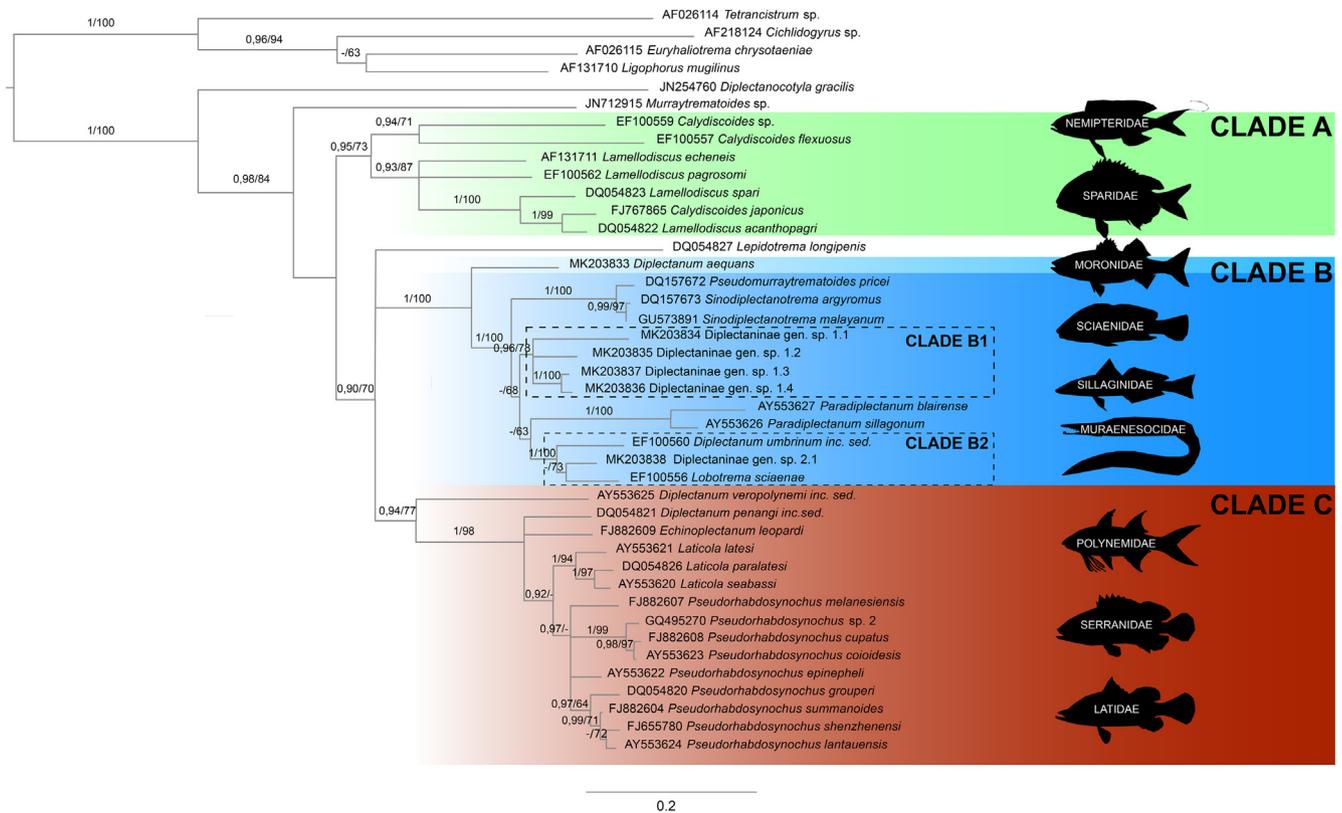
**Table 2**  
List of the mitochondrial cytochrome c oxidase subunit I gene haplotypes of *Diplectaninae* gen. sp. 1.2 with GenBank accession codes, number of sequences (N) by haplotype, host and locality.

GenBank ID	Haplotype	Host	Locality (Spain)	N	
MK208311	Hap 1	<i>A. regius</i>	Burriana	1	
			Sant Carles de la Ràpita	9	
		<i>S. umbra</i>	Santa Pola	12	
			<i>U. cirrosa</i>	Sant Carles de la Ràpita	1
			Santa Pola	2	
MK208312	Hap 2	<i>A. regius</i>	Burriana	2	
			Sant Carles de la Ràpita	1	
		<i>S. umbra</i>	Santa Pola	1	
			<i>U. cirrosa</i>	Sant Carles de la Ràpita	20
			Santa Pola	3	
MK208313	Hap 3	<i>S. umbra</i>	Santa Pola	2	

$N_{\text{total}} \textit{Argyrosomus regius} = 3$ ;  $N_{\text{total}} \textit{Sciaena umbra} = 25$ ;  $N_{\text{total}} \textit{Umbrina cirrosa} = 26$ ;  $N_{\text{total}} \textit{Burriana} = 3$ ;  $N_{\text{total}} \textit{Sant Carles de la Ràpita} = 31$ ;  $N_{\text{total}} \textit{Santa Pola} = 20$ .

Clade B comprised representative sequences of *Diplectanum* spp., *Lobotrema sciaenae* (Bychowsky & Nagibina, 1977), *Paradiplectanum* spp. and *Sinodiplectanotrema* spp., members of the Diplectaninae parasitising moronids, sciaenids and sillaginids (Perciformes); as well as *Pseudomurraytremaoides pricei* (Caballero, Bravo-Hollis

and Grocott, 1955) (*Murraytremaoidinae*) parasite of muraenesocids (Anguilliformes). Clade C included sequences of *Echinoplectanum leopardi* Justine & Euzet, 2006, *Diplectanum* spp., *Laticola* spp. and *Pseudorhabdosynochus* spp., members of the Diplectaninae infecting latids, polynemids and serranids



**Fig. 1.** Phylogram of the Diplectanidae estimated by maximum likelihood using partial sequences of the 28S rRNA gene (full alignment, 800 nucleotides long). Species belonging to the Dactylogyridae were used as outgroups. Posterior probabilities and bootstrap support values are indicated above the branches (posterior probabilities <0.90 and bootstrap values <60 are not reported). Fish silhouettes represent the host families from which the parasite species in each clade have been found.

(Perciformes). *Diplectanocotyla gracilis* Yamaguti, 1953, *Murraytrematoides* sp. and *Lepidotrema longipenis* (Yamaguti, 1934) formed three separate branches with low bootstrap support (Fig. 1).

### 3.2. Interrelationships amongst *Diplectanum* spp. from Mediterranean sciaenids

Sequences of species assigned to *Diplectanum* using morphological criteria did not form a monophyletic clade, nor did the sequences of *Diplectanum* spp. parasitising exclusively sciaenid fishes from the western Mediterranean region (hereafter referred to as Diplectaninae gen. spp.). The sequence of the type species of the genus, *D. aequans*, parasitic on Atlantic and Mediterranean moronid fish, was placed at the base of clade B. No sequence of the other *Diplectanum* spp. was found as sister to the type species. Clade B included Diplectaninae gen. spp. from sciaenids. Four of them clustered together (Clade B1), whereas Diplectaninae gen. sp. 2.1, only found in *Umbrina* spp., was recovered as sister taxa to *L. sciaenae* and *D. umbrinum* (inc. sed.), Indo-Pacific parasites of sciaenid fishes (Clade B2). Diplectaninae gen. sp. 1.3 appeared as sister species to Diplectaninae gen. sp. 1.4, both being host generalist parasites of Mediterranean sciaenid fishes. They formed a clade together with Diplectaninae gen. sp. 1.2 and Diplectaninae gen. sp. 1.1, a host generalist and host specialist respectively, but the relationships within clade B1 were unresolved (Fig. 1).

Genetic divergence between Diplectaninae clade B1 and Diplectaninae gen. sp. 2.1 (in clade B2) ranged from 19.5% to 24.1% for the ITS and 9.6% to 12.8% for the 28S region. Genetic distances between species within clade B1 were 8.8–21.9% for the ITS and 1.8–11.0% for the 28S region. The closest species pair was Diplectaninae gen. sp. 1.3–Diplectaninae gen. sp. 1.4, and the fur-

thest Diplectaninae gen. sp. 1.2–Diplectaninae gen. sp. 1.4 for ITS and Diplectaninae gen. sp. 1.1–Diplectaninae gen. sp. 1.2 for 28S. Diplectaninae gen. sp. 2.1 diverged 7.4–9.1% from *Lobotrema* and *D. umbrinum* for the 28S region, similar to the interspecific divergence estimated amongst Diplectaninae gen. spp. within clade B1. Genetic divergence was much higher between any of these clades and the type species, *D. aequans*, ranging from 26.1% to 28.6% for the ITS region and 13.2–16.9% for the 28S region. The lack of available ITS sequences for closely related taxa in GenBank renders the divergence analysis of the ITS sequences comparable only amongst the species sequenced for this study. Interspecific genetic divergences for those Diplectanidae genera for which 28S sequences were available for more than two species ranged from 3.4% to 6.3% in *Laticola*, 6.6% to 16.5% in *Lamellogadus* and 0.8% to 10.7% in *Pseudorhabdosynochus*. Overall, the range of interspecific divergence in these genera was comparable with the variation detected between species within clades B1 and B2.

Genetic divergence between Diplectaninae gen. spp. and the type species *D. aequans* was similar to that recorded between these diplectanids and closely related genera. Pairwise distances between Diplectaninae gen. spp. parasitic on sciaenids and other genera within Diplectanidae varied between 22.8–24.6% for Diplectaninae gen. spp.-*Laticola*, 15.0–27.1% for Diplectaninae gen. spp.-*Lamellogadus*, 14.8–19.0% for Diplectaninae gen. spp.-*Paradiplectanum*, 19.8–25.1% for Diplectaninae gen. spp.-*Pseudorhabdosynochus* and 13.3–16.7% for Diplectaninae gen. spp.-*Sinodiplectanotrema*.

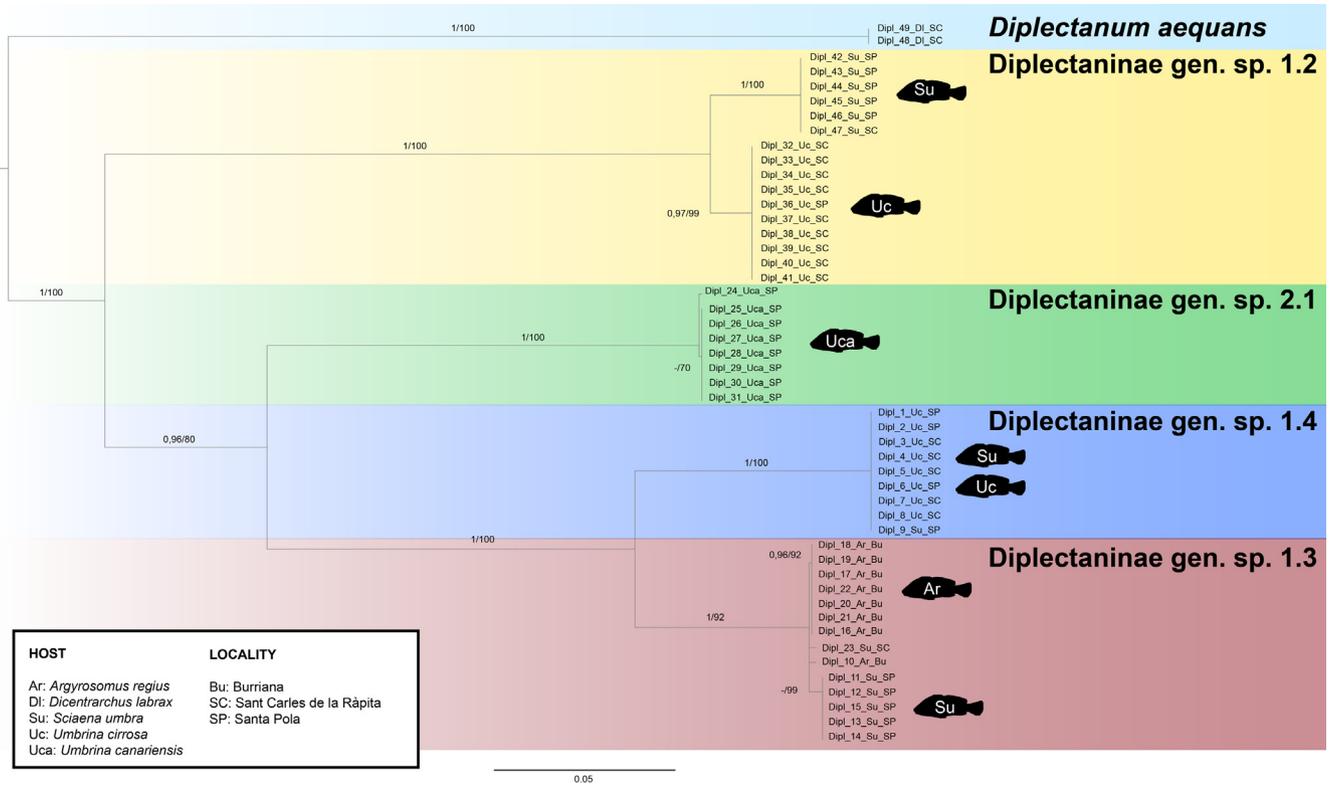
### 3.3. Intraspecific variability of generalist *Diplectaninae* gen. Spp

The phylogenetic tree inferred from the ITS sequences of Diplectaninae gen. spp. featured four well-supported clades

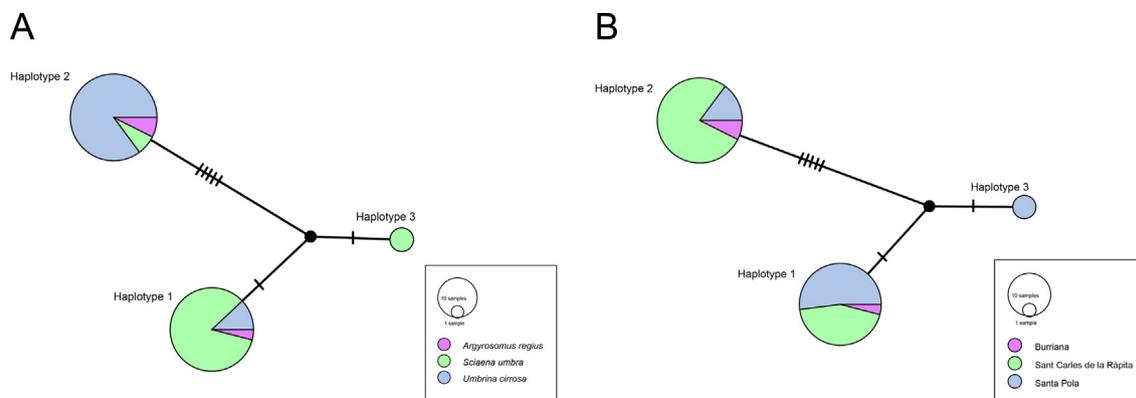
consistent with those inferred from 28S rDNA sequences for four out of the five species characterized based on molecular data (Fig. 2). Unfortunately, representative ITS sequences of *Diplectaninae* gen. sp. 1.1 could not be obtained. Two subclades were found within the clades of the generalists *Diplectaninae* gen. sp. 1.2 and 1.3, each subclade including specimens from a particular host species (Fig. 2). No intraspecific divergence in the ITS was detected for *Diplectaninae* gen. sp. 1.4, but some was observed for *Diplectaninae* gen. sp. 1.2 (0–3.6%), *Diplectaninae* gen. sp. 1.3 (0–1.1%) and *Diplectaninae* gen. sp. 2.1 (0–0.1%).

Intraspecific variability within *Diplectaninae* gen. sp. 1.2, a generalist on Mediterranean sciaenids, was also estimated using 54

COI sequences (144–449 bp). This species was selected for the population genetic analyses on the basis of its low host specificity (detected in every sciaenid genus analysed), and widespread distribution across our sampled localities. A total of three unique haplotypes was detected amongst all COI sequences containing 19 polymorphic sites (0.9–12.6% genetic divergence) (Table 2). Haplotype network analysis revealed two dominant haplotypes: Haplotype 1, at a frequency of 46.3% parasitising mainly *S. umbra* in two different localities and Haplotype 2 at a frequency of 50.0% chiefly parasitic on *U. cirrosa* from Sant Carles de la Ràpita (Fig. 3A, B). *Diplectaninae* gen. sp. 1.2 showed strong population genetic structure across hosts species ( $\Phi_{ST} = 0.56, P < 0.001$ ) and



**Fig. 2.** Phylogram based on maximum likelihood analysis of the internal transcribed spacer region (ITS1-5.8S-ITS2) gene cluster of specimens morphologically assigned to *Diplectanum* spp. Posterior probabilities and bootstrap support values are indicated above the branches (posterior probabilities <0.90 and bootstrap values <60 are not reported). Isolate names represent the individual number followed by two/three letter codes of the host species and the Spanish locality where they were collected. Fish silhouettes indicate Mediterranean sciaenid species from which the specimens included in each clade were collected.



**Fig. 3.** Median Joining haplotype network of the cytochrome c oxidase subunit I of *Diplectaninae* gen. sp. 1.2. Colours indicate different host species (A) and Spanish localities of the origins of the isolates (B). Circle sizes are proportional to the frequency of the haplotypes in the samples. Perpendicular bars along the branches indicate the numbers of substitutions between observed haplotypes.

slightly less across localities ( $\Phi_{ST} = 0.16$ ,  $P = 0.002$ ). Pairwise  $\Phi_{ST}$  comparisons indicated significant genetic differentiation amongst Diplectaninae gen. sp. 1.2 populations from *S. umbra* and *U. cirrosa* (pairwise  $\Phi_{ST} = 0.77$ ,  $P < 0.001$ ). Significant genetic differences were also found between parasite populations of Sant Carles de la Ràpita and Santa Pola (pairwise  $\Phi_{ST} = 0.29$ ,  $P < 0.001$ ).

#### 4. Discussion

This study is to our knowledge the first attempt to examine the systematic and taxonomic frameworks of the genus *Diplectanum* using molecular data for a number of species currently listed as valid in WoRMS, including the type species of the genus, *D. aequans*. The phylogenetic position and genetic divergence of newly and previously sequenced specimens of presumed *Diplectanum* spp. revealed that they do not form a monophyletic group and therefore do not belong to a single genus. Indeed, they may represent at least three different genera: *Diplectanum sensu stricto*, (i.e. *D. aequans*), Diplectaninae gen. clade B1 and Diplectaninae gen. clade B2. In contrast to previous reports of Mediterranean *Diplectanum* spp. (e.g., Oliver, 1968, 1980, 1993), host specificity of the monogeneans included in these putative new genera is relatively low since most species parasitise at least two host genera. However, detailed population genetic analysis of Diplectaninae gen. sp. 1.2 revealed that when this generalist species is examined with variable markers, populations on different hosts appear to be genetically distinct.

The monophyly of *Diplectanum* spp. had already been rejected in previous phylogenetic analyses using morphological data (Domingues and Boeger, 2008), something attributed to the absence of morphological boundaries to delimit the species included in this genus (Kritsky et al., 2000). Consequently, several authors have assigned new species to *Diplectanum* “provisionally” (Justine, 2008; Justine and Henry, 2010). Domingues and Boeger (2008) considered that only *D. aequans* belonged to the genus after the morphological re-examination of the five *Diplectanum* spp. included in their analysis; the other species were regarded as incertae sedis or transferred to other genera. Our phylogenetic results fully support the view of Domingues and Boeger (2008) as no other *Diplectanum* spp. sequenced to date appear sister to the type species. Altogether, we can only agree with the conclusion of Kritsky et al. (2000) that *Diplectanum* represents an unnatural and catch-all group. Our results call into question the species richness of the genus *Diplectanum* and highlight the need to incorporate molecular data to determine the systematic position of species morphologically attributed to this controversial genus.

Of particular interest is the fact that the phylogenetic analyses retrieved species traditionally assigned to the genus *Diplectanum* in separate clades and as sister taxa to other genera recently described using both morphological (Domingues and Boeger, 2008) and molecular criteria (Lim et al., 2010). Additionally, genetic distances between species from Mediterranean sciaenids formerly placed in *Diplectanum* and *D. aequans* were higher than those reported within monophyletic genera of the Diplectaninae ranging from 0.9% to 10% (Wu et al., 2005b; Lim et al., 2010), but similar to divergences recorded between closely related genera such as *Paradiplectanum* and *Sinodiplectanotrema*. Given that genetic distances amongst the taxa newly sequenced herein and *D. aequans* are comparable with intergeneric distances between diplectanids, we suggest that diplectanids from Mediterranean sciaenids may constitute at least two genera (Diplectaninae gen. clade B1 and Diplectaninae gen. clade B2) different from *Diplectanum*. Our findings lead to the transfer of 1/3 of *Diplectanum* spp. previously considered valid to new genera, and thus, to a drastic reduction in the species richness previously known for *Diplectanum*.

Additionally, our results also suggest a reduction in the number of diplectanid species morphologically described from Mediterranean sciaenids and of their host specificity since most of the Diplectaninae gen. spp were found to be generalists. Notably, three out of five species parasitise at least two different fish genera and only Diplectaninae gen. sp. 1.1 (restricted to *A. regius*) and Diplectaninae gen. sp. 2.1 (restricted to *Umbrina* spp.) are apparently host-specific. Conversely, most of the morphologically defined species of *Diplectanum* parasitising Mediterranean sciaenids (up to 11 species in total) were each exclusively found on a single sciaenid species (i.e. *D. bocqueti*, *D. dollfusi* and *D. sciaenidae* from *A. regius*, *D. banyulense* and *D. labourgi* from *U. canariensis* and *D. chabaudi*, *D. grassei* s.l., *D. melvillei* s.l and *D. nagibinae* s.l., from *U. cirrosa*; Oliver, 1968; 1980; Oliver and Paperna, 1984). Based on morphological data, only *D. aculeatum* and *D. simile* were considered generalists as both parasitise two different genera: *Argyrosomus-Sciaena* and *Sciaena-Umbrina*, respectively (Oliver, 1968, 1993; Domingues and Boeger, 2008). The incongruence between the number of molecular lineages, host associations and previously recognised *Diplectanum* spp. parasitising Mediterranean sciaenids make it difficult to attribute the five Diplectaninae gen. spp. sequenced herein to a morphologically defined species name. We propose to refer to the molecular lineages detected in this study according to their terminal clade (B1 or B2) as follows: Diplectaninae gen. sp. 1.1, Diplectaninae gen. sp. 1.2, Diplectaninae gen. sp. 1.3, Diplectaninae gen. sp. 1.4 (clade B1) and Diplectaninae gen. sp. 2.1 (Clade B2). Reports on incongruities between molecular and morphological data in other diplectanids suggest an overestimation of species richness and host specificity in this group (Desdevises et al., 2000) that could be attributable to the phenotypic variability of some diagnostic characters (i.e. haptor sclerites) related to the environment and host (Olstad et al., 2009; Poisot et al., 2011; Rodríguez-González et al., 2015; Brazenor et al., 2017). Therefore, it is likely that phenotypic variability or plasticity in the morphological structures of Diplectaninae gen. spp. from different sciaenid hosts or localities has been interpreted as species-specific diagnostic features, leading to an overestimation of species richness and host specificity, as suggested for other monogeneans (Morand et al., 2002). Further analyses must be conducted to morphologically characterise the molecular lineages reported herein and provide a new taxonomic framework taking into account the available names of the morphologically defined species of *Diplectanum* from sciaenids.

Based on the phylogenetic reconstruction of the Diplectanidae, an association exists between diplectanid clades and specificity to the host at the family level, with taxa parasitising the same fish family clustering together. The diplectanid species in Clade A parasitise sparids, those in Clade B infect mainly sciaenids and sillaginids, and those in Clade C latids and serranids. Our phylogenetic results generally agree with those published by Lim et al. (2010) as well as with previous phylogenetic analyses using morphological data (Desdevises et al., 2001; Domingues and Boeger, 2008). However, some inconsistencies were found with respect to the evolutionary origin and position of some taxa. Genetic data revealed that *Lamellodiscus japonicus*, which was reassigned by Thoney (1989) to *Calydiscooides* Young, 1969, should be considered a member of *Lamellodiscus*, as this taxon appears sister to *Lamellodiscus acanthopagri* Roubal, 1981 (Lim et al., 2010; present study). The recent synonymy of *Furnestinia* (Wagener, 1857) and *Lamellodiscus* (Desdevises, 2001; Domingues and Boeger, 2008) as well as the new combination of *C. japonicus* onto *L. japonicus* would allow *Lamellodiscus* to become monophyletic (as previously reported by Lim et al. (2010)). However, sequences of *Telegamatrix* Ramalingum, 1955 are required to verify the monophyly of *Lamellodiscus*, since both genera formed a polytomy in phylogenetic analyses based on morphological data (Desdevises

et al., 2001; Domingues and Boeger, 2008). *Murraytrematoides* Yamaguti, 1958 appeared isolated and basal to the clade including *L. sciaenidae* and *P. pricei*. The present results on the Pseudomurraytrematoidinae are in line with Domingues and Boeger (2008), but *Murraytrematoides* and *Pseudomurraytrematoides* swapped their positions. As in previous genetic analyses (Wu et al., 2005b; Lim et al., 2010), two main clades were found within Diplectaninae, but clear differences from previous phylogenetic studies using morphological data were detected regarding the earliest divergent taxon within this subfamily. Whereas *D. aequans* appeared as a basal taxon in clade B, the previous analyses based on morphology suggested *Paradiplectanum* as the most basal taxon within Diplectaninae (Domingues and Boeger, 2008). The early divergent position of *D. aequans* suggests the existence of a common ancestor for diplectanids of sciaenids and sillaginids. Additionally, present genetic data suggest the monophyletic origin of *Laticola*. The monophyly of *Pseudorhabdosynochus*, previously hypothesised by Wu et al. (2005b), could not be confirmed due to the low support for the internal nodes. It is important to note that molecular information is still lacking for 16 of the 29 valid diplectanid genera, which are missing from the current molecular analyses. Hence, molecular data should be obtained from a larger representation of species and genera in order to assess the evolutionary relationships within the Diplectanidae thoroughly. Moreover, most of the sequences available to date belong to species from the Pacific Ocean; the inclusion of sequences from other geographic regions is therefore required to avoid spurious inferences caused by the potential for the geographic proximity of the samples to mask the 'true' evolutionary relationships within the Diplectanidae.

While conserved genetic markers (28S rDNA) allowed recognition of Diplectaninae gen. sp. 1.2 (and other Diplectaninae gen. spp.) as generalist of western Mediterranean sciaenids, ITS (to some extent) and mitochondrial data detected noticeable genetic structures amongst subpopulations in relation to both the hosts and localities where this lineage was found. In other words, different genetic haplotypes tend to be associated with specific hosts and localities. We cannot rule out the possibility that the genetic differentiation detected for locality is an artifact of the uneven sampling. Unfortunately, the number of samples analysed were not equally represented amongst the different localities sampled due to fish availability or infection levels and this could have potentially influenced the results. Furthermore, the natural distribution and migratory habits of the sciaenid hosts studied (Chao, 1986; <http://www.fishbase.se>) and the geographical proximity and similarity in abiotic conditions amongst the three sampling locations (Hofrichter, 2004; <http://www.puertos.es/en-us/oceanografía/Pages/portus.aspx>) make it unlikely that populations of Diplectaninae gen. sp. 1.2 are meaningfully genetically structured by locality. In contrast to the geographic comparison, the sampling design across hosts (*S. umbra*  $n = 25$  and *U. cirrosa*  $n = 26$ , see Table 2) was balanced, and therefore the statistical results are reliable. The intraspecific segregation of monogeneans by host species has been suggested for many other species (e.g., Meinilä et al., 2004; Bueno-Silva et al., 2011; Kmentová et al., 2016) and can be reflected in differential infection levels and fitness of the parasites on potential hosts (Poulin, 2006; Bielby et al., 2015; Forbes et al., 2017). Thus, despite the apparent low host specificity of Diplectaninae gen. sp. 1.2, the intraspecific genetic differentiation detected across hosts suggests that distinct parasite genotypes have a preference or perform better in particular sciaenid species. This finding is relevant for sciaenid aquaculture because *U. cirrosa* and *S. umbra* are considered suitable candidates for the diversification of fish farming in the Mediterranean region (Mylonas et al., 2004; Chatzifotis et al., 2006). Despite parasites from the currently cultured sciaenid, *A. regius*, having to be left out of the statistical analysis due to the limited sample size ( $n = 3$ ), the haplotype networks

revealed that parasite specimens infecting *A. regius* share parasitic haplotypes with specimens from both wild sciaenids (*S. umbra* and *U. cirrosa*). This leads us to consider Diplectaninae gen. sp. 1.2 cross-infection quite likely between cultured and non-cultured host species. However, cross infection would be less frequent between the wild sciaenids analysed, since each host species is mainly parasitized by different dominant parasite haplotypes even when they are sympatric in a locality. Therefore, in order to determine the infective potential of a particular Diplectaninae gen. spp. in sciaenid cultures, we advise considering not just the parasite identity and host specificity, but also its population variability across sympatric hosts.

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