Mediterranean-wide transfer of the polyopisthocotylean *Sparicotyle chrysophrii* between wild sparids and farmed gilthead seabream (*Sparus aurata*) inferred by ddRAD loci

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26 ABSTRACT

27 The polyopisthocotylean *Sparicotyle chrysophrii* is considered one of the most harmful
28 pathogens in Mediterranean farmed gilthead seabream (*Sparus aurata*). For the first time
29 recorded in aquaculture in the mid-eighties, it has rapidly spread through facilities in the
30 whole Mediterranean basin, causing mortalities due the anemia, the mechanical damage of
31 the host's gill tissues and the consequent secondary bacterial infections. Even though one of
32 the recognized risk factors for spreading of sparicotylosis is the transfer of the parasite from
33 wild fish, there is a need to infer the extent of this event over a wider geographic area, as well
34 as using more informative multilocus genotyping, such as typing based on the discovery of
35 SNPs. Therefore, we sampled a total of 330 *S. chryspohrii* specimens from Italian, Croatian,
36 and Greek wild and farmed gilthead seabream, as well as from Spanish farmed fish. After a
37 double digest restriction-site associated DNA sequencing (ddRADseq), a total 173 samples
38 were used to infer the genetic structure of the parasite and draw conclusions on its migratory
39 patterns in the Mediterranean. Our results confirm a panmictic distribution of the
40 polyopisthocotylean in the Mediterranean basin, with no clear genetic structure. However,
41 three discrete genetic pools or clusters, probably driven by geographic constraints, have been
42 observed within the parasite populations of origins. We speculate that the dispersion of
43 polyopisthocotylean eggs via the circulation of dominant currents and fronts, as well as
44 anthropogenic influence, facilitates the gene flow within the genetic clusters. In addition,
45 gene flow between polyopisthocotyleans parasitizing wild and farmed gilthead seabream is
46 evident, but other wild sparids (*i.e.*, the auxiliary and annular seabream, blotched pickerel,
47 bogue, and common pandora) likely play a role in the event. This indicates that *S. chrysophrii*
48 should be considered a family-specific rather than a host-specific parasite.

49

Keywords: ectoparasite, gilthead seabream, ddRAD, *Sparicotyle chrysophrii*, *Sparus aurata*, transfer, wild fish

1. Introduction

Sparicotyle chrysophrii is a hematophagous polyopisthocotylean with a direct life cycle that parasitizes the gills of the gilthead seabream (GSB) (*Sparus aurata*). The host range encompasses only a couple of other representatives of the family Sparidae, *i.e*., the sharpsnout seabream (*Diplodus puntazzo*) (Sánchez-García et al., 2015) and the bogue (*Boops boops*) (Mladineo et al., 2009).

The polyopisthocotylean causes endemic sparicotylosis almost throughout the year in intensive aquaculture facilities located in the Mediterranean basin. Fast infection rate within reared fish is conditioned by a higher fish density, admixture of different fish age categories, elevated seawater temperature, low water flow through cages (caused by biofouling overgrowth on the nets), and the presence of wild sparid populations as a natural source of the parasite (Fioravanti et al., 2020; Mladineo et al., 2023). Other gill-infecting species, such as monopisthocotyleans *Lamellodiscus echeneis* (Mladineo and Maršić Lučić, 2007; Antonelli et al., 2010) and *Gyrodactylus* spp. (Paladini et al., 2009; 2011) are present throughout the Mediterranean in the farmed GSB, sometimes in co-infection with *S. chrysophrii*, but show rather a patchy distribution and rarely cause mortality. In contrast, *S. chrysophrii* pathogenicity is more pronounced, similar to detrimental infections of *Zeuxapta seriolae* in the farmed greater amberjack (*Seriola dumerili*) (Montero et al., 2004). Sparicotylosis is ubiquitous throughout the Mediterranean and is reported in the northwest part of the Mediterranean in facilities along the Spanish and French coasts, in the northcentral part in Italy and Croatia, and in the eastern and southeastern Mediterranean in the aquaculture

74 facilities in the Aegean Sea, and Red Sea (Mladineo et al., 2023). Unfortunately, there are no
75 epidemiological data on its presence in Middle Eastern countries.

76 Sparicotylosis manifests with systemic and general clinical signs in the farmed GSB,
77 including lethargy, sluggish movements, body discoloration, an increased frequency of
78 opercular movements that exacerbates permanently dilated opercula, anorexia, emaciation and
79 cachexia, gill paleness, and lastly, general anemia (Vagianou et al., 2006; Sitjà-Bobadilla et al.,
80 2009). Pathogenesis is due to the specificity of the monogenean attaching apparatus, which is
81 formed by a large number of clamps that are added daily into the opisthaptor throughout
82 parasite lifetime (Repullés-Albelda et al., 2011). The co-morbidity with epitheliocystis (Padros
83 and Crespo, 1995; Toxqui-Rodríguez et al., 2023) and *Tenacibaculum* sp. (Padros, personal
84 observations), as well as blood feeding, estimated to amount to 4.31 µl of blood per hour (Riera-
85 Ferrer et al., 2022; 2023) further aggravate the infection outcome. Although much effort has
86 been invested in combating the disease through good disease management (Fioravanti et al.,
87 2020), an efficient treatment for the industry is still pending (Sitjà-Bobadilla et al., 2006; Rigos
88 et al., 2013; 2023; Firmino et al., 2020; Mladineo et al., 2021; Kogiannou et al., 2022).

89 Prompted by the aggregation of wild GSB infected with *S. chrysophrii* (irrespective of their
90 origin, *i.e.*, natural fish populations or farmed escapees) and potentially also other wild sparids
91 at the Mediterranean aquaculture sites, the objective of the study was to infer: i)
92 polyopisthocotylean's transfer between wild and farmed host populations, and ii) *S.*
93 *chrysophrii* genetic structure in the Mediterranean Sea using restriction site-associated DNA
94 (RAD) markers.

95

96 2. Material and Methods

97 *2.1. Fish sampling and isolation of Sparicotyle chrysophrii*

98 Polyopisthocotyleans were collected from gilthead seabream (GSB) (*Sparus aurata*)
99 farmed in four locations in the Mediterranean Sea (Spain, Italy, Croatia, and Greece) and
100 from wild GSB and other sparids inhabiting the same aquaculture sites. Off-shore open
101 waters were only sampled in the case of Italy (Figure 1). Depending on the parasite intensity,
102 either multiple specimens or a single specimen was collected from a single fish
103 (Supplementary Table 1).

104 Due to the disproportionate effort needed to collect enough wild hosts from the open
105 waters infected by the monogenean, the study was limited to collections of specimens
106 aggregating at aquaculture sites.

107 Spain: Four heavily infected farmed GSB (70 g) from net cages (Province of
108 Castellón; 39°59'02.7"N 0°08'09.3"E) were provided in July 2018. Fishing operation for wild
109 sparids was organized on one occasion in July 2018 through the engagement of professional
110 fishermen, resulting in the collection of six specimens. However, the extracted DNA was not
111 suitable for ddRAD libraries and was therefore excluded.

112 Italy: Farmed GSB (n=65; 200-300 g) infected with *S. chrysophrii* were provided by
113 three Italian farms through September and December 2016, located in different areas:
114 Spezzina Itticoltura (Italian farm 1; 44° 4’23.57"N 9°50'28.33"E) in the Ligurian Sea,
115 Piscicoltura del Golfo di Gaeta (Italian farm 2; 41°13’48.24"N 13°35'56.83"E) in the
116 Tyrrhenian Sea, and Ca’ Zuliani (Italian farm 3; 45°45’56.64"N 13°35'28.87"E) in the
117 northern Adriatic Sea. In 2018, 126 wild GSB (200-300 g) were collected by fishermen: 50
118 fished from the Porto Buso area and 76 from the Marano Lagoon (45°42’1.57"N
119 13°15'27.97"E). It is worth noting that in contrast to other wild GSB specimens that were
120 caught from the farming sites, these fish were not farm-associated, and thus represent a truly
121 wild population.

122 Croatia: GSB (n=100; 200-220 g) farmed in a facility in middle Adriatic
123 (44°01'57.7"N 15°13'28.5"E) were sampled in July and November 2016, and March and June
124 2017. From the same farming site, different wild farm-associated fish (n=444); blotched
125 picarel (*Spicara maena*), picarel (*S. smaris*), bogue (*Boops boops*), GSB, annular seabream
126 (*D. annularis*), sharpsnout seabream (*D. puntazzo*), common two-banded seabream (*D.
127 vulgaris*), golden grey mullet (*Liza aurata*), common pandora (*Pagellus erythrinus*),
128 blackspot seabream (*P. bogaraveo*), axillary seabream (*P. acarne*), European seabass (*D.
129 labrax*), saddled seabream (*Oblada melanura*) were sampled by hook and line or trammel
130 net. *S. chrysophrii* were isolated from the following wild sparids in November 2016, May,
131 July, and October 2017: annular and axillary seabream, bogue, blotched picarel and common
132 pandora.
133 Greece: Two hundred farmed GSB (70-100 g) were transferred from Andromeda SA
134 (cage site, Astakos, Ionian Sea; 38° 32' 00" N, 21° 05' 00" E) to the facilities of the Hellenic
135 Centre for Marine Research in Athens in November 2015. By July 2016, 100 fish were
136 sampled to collect monogeneans. Three fishing missions (November 2015, July 2016, and
137 April 2018) were carried out to sample wild fish inhabiting the selected farming site,
138 deploying nets suspended around the cages. On the first fishing mission, collected fish (n=36)
139 of various species were caught: garrick (*Lichia amia*), saddled seabream (*O. melanura*),
140 striped mullet (*Mugil cephalus*), swallowtail seaperch (*Anthias anthias*), white seabream (*D.
141 sargus*), salema porgy (*Sarpa salpa*), crevalle jack (*Caranx hippos*) and GSB, all non-
142 infected with *S. chrysophrii*. On the second mission, only GSB was targeted (n=7, 350 g) and
143 all were found to be infected. On the third trial (38° 32' 00" N, 21° 05' 00" E), fish (n=63) of
144 various species were caught (red seabream *Pagrus major*, grouper *Epinephelus* sp., Atlantic
145 cod *Gadus morhua*, white seabream, GSB, scorpion fish *Scorpaena* sp., two-banded
146 seabream, stargazer *Uranoscopus scaber*, painted comber *Serranus scriba*, European

147 barracuda *Sphyraena sphyraena*, saddled seabream, meagre *Argyrosomus regius*, and greater
148 forkbeard *Phycis blennoides*), and *S. chrysophrii* was isolated only from bogue and a single
149 common pandora.

150 The polyopisthocotyleans were isolated according to a standardized protocol. The fish
151 were examined for the presence of the polyopisthocotyleans the same day after arrival at the
152 laboratory (up to 6 hours, transportation on ice). The gill arches were cut, the lamellae
153 carefully dissected in a Petri dish filled with seawater, with the number and side of each gill
154 arch labeled, and subsequently examined under a stereomicroscope. Each specimen was
155 identified as *S. chrysophrii*, following Mamaev (1984). Parasites (adults and juveniles, while
156 the presence of eggs was qualitatively noted) were collected and fixed in ethyl alcohol
157 (100%) and stored at -20 ºC before being sent to shipment to Croatia for DNA isolation. A
158 total of 330 parasite specimens were collected, and categorized into the following populations
159 prior to DNA extraction: Spanish farmed (n=78), Italian farmed 1 (n=24), Italian farmed 2
160 (n=10), Italian farmed 3 (n=24), Italian wild (n=22), Croatian farmed (n=54), Croatian wild
161 (n=39), Greek farmed (n=70), and Greek wild (n=9) (Supplementary Table 1).

162

163 *2.2.DNA extraction, and construction and sequencing of double digest Restriction-Site Associated*
164 *DNA (ddRAD) libraries*

165 High molecular weight (HMW) DNA from *S. chrysophrii* specimens was extracted
166 using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's
167 instructions. After DNA quantification using NanoDrop spectrophotometer (Thermo Fisher
168 Scientific, Waltham, MA, USA) and agarose gel electrophoresis visualization, ddRAD library
169 preparation followed Peterson et al. (2012) with few modifications. Three µl of each sample
170 (21 ng HMW DNA in total) were processed in one of the three libraries to achieve adequate
171 coverage, resulting in 330 *Sparicotyle* DNA used for the library preparations. Samples were

172 simultaneously digested by two high-fidelity restriction enzymes (RE); 8-cutter *SbfI*
173 (CCTGCA|GG recognition site) and 6-cutter *SphI* (GCATG|C recognition site) (New England
174 Biolabs, Ipswich, Massachusetts, USA) at 37ºC for 90 min. Barcoded adapters were
175 individually ligated to the RE sites, and the ligated samples were combined into a single pool,
176 which was then column-purified (MinElute PCR Purification Kit, Qiagen, Hilden, Germany),
177 and eluted in 70 µl of EB buffer (Qiagen, Hilden, Germany). Fragments between 400–700 bp
178 were size-selected by gel electrophoresis, gel-purified (MinElute Gel Extraction Kit, Qiagen,
179 Hilden, Germany), and eluted. The size-selected template DNA (68 µl in EB buffer) was PCR
180 amplified (15 cycles of PCR; 36 separate 12.5 µl reactions, each with 1 µl of template DNA)
181 using a Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA,
182 USA). The PCR reactions were pooled (450 µl), column-purified (MinElute PCR Purification
183 Kit, Qiagen, Hilden, Germany) and eluted into ~55 µl per library. Removal of any small
184 leftover fragments (<200 bp) was achieved by an additional clean-up with an equal volume of
185 AMPure XP Beads (Beckman Coulter, Brea, CA, USA) and eluted in the final ~22 µl of EB
186 buffer. ddRAD libraries were quantified by Qubit (Thermo Fisher Scientific, Waltham, MA,
187 USA) and sequenced on an Illumina MiSeq Next Generation Sequencer (Illumina, San Diego,
188 CA, USA) using the Reagent kit v2 (300-cycle kit, 162 bp paired-end reads) (Illumina, San
189 Diego, CA, USA) at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC)
190 of HCMR in Crete. Two MiSeq runs were performed for each library mix.

191

192 *2.3. de novo assembly and SNP calling*

193 The process_radtags function in Stacks v.2.5 was used to filter and demultiplex low-
194 quality or unidentified raw reads (Rochette et al., 2019). This step involved removing cut sites,
195 barcodes, and adapters from the ddRAD results. FastQC (Andrews, 2010) was then used to
196 assess the initial quality of the raw sequencing data. A de novo assembly of RAD-seq data was

197 performed using the Stacks software v2.64 suite to identify and genotype SNPs across all
198 paired-end read samples. To optimize the assembly parameters and enhance the quality of the
199 loci, the RADstackhelpR (De Raad, 2021) and vcfR (Knaus and Grunwald, 2017) packages
200 were employed. These tools were used to determine the optimal values for the -M, -m, and -N
201 parameters in Stacks, which are critical for balancing sensitivity and specificity in the assembly
202 (Paris et al., 2017). The -M parameter defines the maximum allowable nucleotide difference
203 between stacks within an individual, -m specifies the minimum depth of coverage required to
204 form a stack, and -N sets the maximum distance permitted when merging stacks across
205 individuals (Rochette et al., 2019). Data were assembled multiple times, varying one parameter
206 at a time (-m = 3–6, -M = 2–8, -N = 0–11), while keeping the other parameters fixed at their
207 default values (m3M2N1). For each run, the number of assembled loci, polymorphic loci, and
208 SNPs generated were recorded (Supplementary Figure 1). Optimal settings were finally
209 identified as -m 6, -M 1 and -N 2.

210 Loci were built, catalogued, SNPs called and individuals genotyped using the
211 denovo_map.pl wrapper in Stacks. Multiple filters within the Stacks populations module were
212 used to obtain loci present in 70% of all populations (p), in at least 80% of individuals in each
213 population (r=0.80), and with a maximum observed heterozygosity of 80%
214 (max_obs_het=0.80).

215 PCA, phylogenetic analysis, and F_{ST}-based analysis were performed after eliminating
216 SNPs in linkage disequilibrium with a squared coefficient of correlation of 0.5 in 50-SNP
217 sliding windows. In addition, vcftools v0.1.16 was used to exclude variants with either low
218 (<5x) or extremely high (800x) coverage depth (Danecek et al., 2011). Finally, all loci with
219 more than 25% missingness were eliminated and two SNP matrices were created in Variant
220 Call Format (VCF), consisting of: 1) 2,920 SNPs with an average locus coverage of

221 approximately 20x; and 2) a second dataset with 1,780 unlinked SNPs with an average locus
222 coverage of approximately 14x. The final dataset included a total of 173 specimens.
223
224 *2.4. Genetic diversity*
225 The R package SambaR was used on the first ddRAD dataset (2,920 SNPs) to assess
226 the expected (HE) and observed (HO) heterozygosity, private alleles, Tajima's D and
227 inbreeding coefficients among the parasite populations (De Jong et al., 2021). This was due to
228 the algorithms' assumption that the input data have not been subjected to LD filtering. Plink V
229 1.90 was used to convert the VCF file to bed format.
230
231 *2.5. Population genetic structure*
232 To assess the presence of structure within the population, we calculated pairwise FST
233 values using ddRAD sequence data, by grouping individuals based on their country of origin
234 and distinguishing between those from farm and wild populations, treating the latter as separate
235 geographic units. This analysis was carried out in Arlequin 3.5 (Excoffier and Lischer, 2010),
236 where the SNP data, initially in Plink format, were converted to the Arlequin format using
237 PGDSpider 2.1.1.5 (Lischer and Excoffier, 2011). Subsequently, Fst and p values were
238 determined by performing 1000 permutations in Arlequin 3.5.
239 A series of cluster analyzes were performed to elucidate the neutral genetic structure
240 within the parasite populations. First, we performed a discriminant analysis of principal
241 components (DAPC) (Jombart et al. 2015) to reveal the group structure without being affected
242 by within-group variance, using the adegenet package in R v4.0.5 (R Core Team, 2020. To
243 determine the most appropriate number of genetic clusters, the K-means clustering algorithm
244 was applied, selecting the configuration with the lowest Bayesian Information Criterion (BIC)
245 value as the optimal solution. Individuals were then assigned to these clusters using DAPC,

246 with the number of principal components to be retained determined by the optim.a.score
247 function. To visualize the genetic clusters among the parasite populations and the placement of
248 individuals within these clusters, diagrams were created that combined the results of DAPC
249 with PCA.

250 Subsequently, the model-based evolutionary clustering method was applied in the
251 ADMIXTURE software to calculate the genetic differentiation among the populations, and the
252 optimal number of population clusters was inferred using AdmixPiPe (Alexander et al., 2009;
253 Mussmann et al., 2020). Using the second VCF file described above, a default thin parameter
254 (t=100) was chosen, and ADMIXTURE was run with 20 replicates for each value of K ranging
255 between 1 and 20. Minimization of the cross-validation error in ADMIXTURE was used to
256 find the most likely K values in the replicate runs (Alexander and Lange, 2011). The CLUMPK
257 server (http://clumpak.tau.ac.il/) was used to visualize the clustering of individuals into
258 populations.

259 Finally, fineRADstructure and RADpainter v.0.2 were used to reveal the population
260 genetic structure based on nearest neighbor haplotypes (Malinsky et al., 2018). The script
261 Stacks2fineRAD.py within the fineRADstructure package was initiated to compute the allele
262 distribution, SNPs per locus, and individual missing data. The maximum number of SNPs per
263 locus was set to 10 and capped individual missingness to 25% when converting the haplotype
264 file to RADpainter format. Due to the susceptibility of ddRAD to batch effects caused by minor
265 variations between libraries in the size selection stage, the potential influence of missing data
266 on each library-based structure was scrutinized. The fineRADstructure pipeline was run with
267 default settings, but with extending the burn-in iterations to 200,000 and sampling 1,000,000
268 iterations at intervals of 1000. To verify consistent convergence of Bayesian posterior
269 distributions, convergence was assessed by assigning individuals to populations in multiple
270 independent runs, examining the plots for MCMC output of parameter values, and achieving

271 sufficient parameter sample size (over 100) by extending the duration of each chain. The
272 "FinestructureLibrary.R" function from the fineRADstructure package was used to display the
273 co-ancestry heatmap.

274

275 *2.6. Isolation by distance (IBD)*

276 The Mantel test in Adegenet 1.3. was used to determine whether the genetic differentiation
277 Pattern was consistent with an Isolation by Distance (IBD) model. The analysis calculated the
278 correlation between genetic distances (F_{ST}) and geographic distances (km) based on 10,000
279 permutations. For the geographic distances, the straight-line distances between the sample
280 locations, resulting from their geographic coordinates were considered (Jombart, 2011).

281

282 *2.7. Gene flow*

283 TreeMix was applied to an unlinked SNP dataset to determine the phylogenetic
284 relationships and historical admixture events among populations (Pickrell and Pritchard, 2012).
285 Initially, the tool was used to determine the connections among populations and then to
286 construct a maximum likelihood population tree to account for ancestral admixture or
287 migration events occurring among groups. Plots were created that allowed for 0-10 admixture
288 events, as indicated by the m parameter, and then the m-model was selected that had the
289 smallest m value at which the log-likelihood values stabilized while having the least residual
290 variance. The significance of the migration events was assessed by a jackknife procedure
291 within TreeMix.

292

293 *2.8. Data Deposition*

294 Obtained sequences have been deposited in the NCBI, submission ID SUB13956223,
295 BioProject PRJNA1038783.

296

297 ## 3. Results

298

299 ### *3.1. Spricotyle chrysophrii sequencing data*

300 In total 330 samples were sequenced out of the three ddRAD library mixes prepared,
301 yielding 57,300,206 high quality reads assigned to the individuals. On average, 342,180 reads
302 were obtained per individual. Approximately 10% of the sequenced parasites (36 samples)
303 yielded very few reads to build even a single locus due to low DNA quality and/or integrity,
304 leaving 294 specimens for downstream population genetics analysis. Unfortunately, among the
305 excluded samples were all six wild Spanish specimens, therefore they were not included in the
306 downstream analyses. In total, 25,550 unique RAD loci were identified in all parasites. For
307 these loci, the effective per-sample coverage had a mean coverage of 49.3x with SD=58.2x,
308 min=5.0x, max=499.1x and 203.7 number of sites per locus on average. Individuals had a range
309 of 1 up to 4,057 loci (median 395.5) including from 5 up to 758,491 reads (median 19,913.5)
310 used for building them. Following filtering, stacks populations software kept 514 RAD loci,
311 composed of 135,642 sites; 4,761 of those sites were filtered keeping 246 variant sites (SNPs).

312

313 ### *3.2. Genetic diversity*

314 Genetic diversity estimated through nucleotide diversity, expected, observed, and
315 multilocus heterozygosity, and observed homozygosity of 173 specimens obtained after
316 filtering of sequenced ddRAD libraries is shown in Table 1. The Greek farmed population had
317 the highest overall heterozygosity and Italian farm on the Adriatic side had the lowest Italian
318 farmed populations had generally lower values compared to the wild parasite population. The
319 observed heterozygosity was lower than expected in all populations. Nucleotide diversity was
320 highest in the Croatian farm parasites and lowest in the Italian wild *S. chrysophrii*.

321 All populations showed negative Tajima's D values, but these were not statistically
322 significant (Table 1). The highest negative D value was observed in the Italian farm 3
323 population (-0.1527), while the lowest was in the Greek farm (-0.0893).
324 The total number of private alleles observed across all wild populations was 119, while
325 farmed populations had a total of 1,101 private alleles. When analyzed separately, the Spanish
326 farmed *S. chrysophrii* population had the highest frequency of private alleles and the Italian
327 wild population the lowest. Similarly, the inbreeding coefficient was highest in the Spanish
328 farmed polyopisthocotylean population, and lowest in the Italian wild population (Table 1).

329 *3.3. Population Genetic Structure*

330 The genetic distance (F_{ST}) among populations was low, ranging from the smallest 0.008
331 (between the Croatian farmed and the Italian farmed 3 parasites, not significant) to the highest
332 of 0.076 (between the Italian farmed 1 and Spanish farmed in respect to the Greek farmed
333 parasites, significant) (Table 2).

334 The DAPC analysis identified three separate genetic clusters: the first comprising
335 Spanish and two Italian farm populations; the second comprising the Italian wild population;
336 and the third cluster comprising the Italian Adriatic farmed population and both the Croatian
337 and Greek wild and farmed populations (Figure 2A). Interestingly, when the analyses were run
338 for all wild vs all farmed populations, no genetic differentiation was inferred (Figure 2B).

339 Similarly, the admixture patterns largely reflect the clusters identified by the DAPC and
340 confirm an optimal division into three clusters. A notable separation exists between the mixed
341 populations of Spanish and Italian farms (Italy west coast) and those of Italy east coast
342 (farmed), Greece and Croatia, with the division marked by the wild population in Italy (Italy
343 east coast). The latter is the only truly wild parasite population, in contrast to others that infect
344 wild, but farm-associated fish. Admixture is particularly evident in the wild and farmed
345 Croatian and Greek populations (Figure 2C).

346 Finally, the dendrogram produced by fineRADstructure analysis corroborated the
347 number of clusters and association of populations to a specific cluster as indicated by the
348 genetic structural analysis (DAPC and Admixture) mentioned above (Figure 3). The first
349 cluster (Spain farmed, Italy farmed 1 and 2) and the third cluster (Italy farmed 3, Croatia and
350 Greece wild and farmed) showed a relatively high level of shared ancestry within themselves
351 and a relatively low level of ancestry when compared to each other (i.*e*., cluster 1 vs cluster 3).
352 In contrast, the second cluster (Italian wild) showed a clear divergence from the others, but was
353 more closely related to the genotypes of cluster 3 (Croatian and Greek wild and farmed) (Figure

354 3). This is slightly in contrast with DAPC analyses (Figure 2A), but consistent with
355 ADMIXTURE clustering results (Figure 2C).
356
357 *3.4. Isolation by Distance (IBD)*
358 Mantel test revealed a significant positive correlation between the pairwise genetic
359 distances (F_{ST}) and geographic distances (r=0.31, p=0.031), indicating that distance-based
360 isolation may have a significant effect on shaping the genetic differentiation of *S. chrysophrii*
361 populations (Figure 4). In addition, a species tree obtained by Treemix analysis revealed three
362 primary genetic groups, aligning with the results from the population genetic structure study.
363 The parasite populations in Greece and Croatia (both farmed and wild), including the Italian
364 farmed 3 population, displayed a close genetic linkage, which is distinct from the other parasite
365 groups. Similarly, the parasite populations from Spain and Italy farmed 1 and 2 are grouped
366 together. Interestingly, the tree is best supported by a model that incorporates two migration
367 events: both directed from the Italian wild population towards the other two genetic clusters,
368 but with different migration weights and drift parameters (Figure 5).
369
370 *3.5. Host range*
371 *Sparicotyle chrysophrii* was isolated from sparid species other then GSB collected from the
372 farming site in Adriatic and Greece: annular seabream (*D. annularis*), axillary seabream (*P.*
373 *acarne*), blotched picarel (*S. maena*), bogue (*B. boops*), and common pandora (*P. erythrinus*).
374
375 **4. Discussion**
376 Transmission of *S. chrysophrii* between wild and reared GSB is recognised as an important
377 factor in the epidemiology of sparicotylosis (Arechavala-Lopez et al., 2013) and is supported
378 by molecular evidence from sequencing of conventional mitochondrial and nuclear markers

379 (Mladineo and Maršić Lučić, 2007; Mladineo et al., 2009; Farjallah et al., 2023).
380 Nevertheless, this is the first time that next-generation sequencing has been used to discover
381 hundreds of single nucleotide polymorphisms of *S. chrysophrii* and to genotype specimens
382 over a large geographic range to infer their genetic structure. First, our data confirm that there
383 is a high gene flow between polyopisthocotyleans from wild and farmed fish at the
384 geographic (country) level, *i.e.*, transmission of the parasite between wild and farmed hosts is
385 successful, resulting in lack of clearly observed genetic structure. Secondly, when all
386 Mediterranean-wide wild genotypes have been pooled and compared with all farmed
387 genotypes, the genomic structure remains elusive, attributing a panmictic character to *S.*
388 *chrysophrii*.

389 Observed heterozygosity was lower than expected throughout all *S. chrysophrii*
390 populations, suggesting a possibility of inbreeding (Schmidt et al., 2021), consistent with its
391 hermaphroditic reproduction. Inbreeding can also result from a succession of several
392 generations on a single host specimen, for which the data for *S. chrysophry* are lacking, but
393 which represents a trade-off between the "secured" continuity of a population and a genetic
394 bottleneck (Huyse et al., 2003). The farmed Spanish *S. chrysophrii* population exhibited the
395 highest number of private alleles, which may indicate low gene flow from other populations
396 and suggest some degree of genetic differentiation. This pattern could result from limited
397 migration or unique selection pressures within the aquaculture environment (Shults et al.,
398 2023). The Tajima's D values across populations were low and not significant, suggesting
399 that these populations are not experiencing recent demographic expansions or contractions
400 detectable by this test. The apparent genetic stability in the farmed Spanish population since
401 its establishment in 1986 (Mladineo et al., 2023) may reflect the closed nature of aquaculture
402 systems and minimal introduction of new genetic material.

403 The comparison among populations in the Mediterranean region resulted in a distinct
404 pattern of genetic structure conditioned by geographical distance. Namely, there are three
405 distinct genetic clusters distributed from the geographical area referred as "West" (consisting
406 of farmed Spanish and Italian west coast *S. chrysophrii* populations), to the intermediate
407 genetic cluster consisting only of a single wild Italian east coast population, to the genetic
408 cluster "East" (both wild and farmed Croatian and Greek populations, and Italian farmed east
409 coast population). Many mechanisms account for genetic differentiation between populations,
410 *e.g.*, vicariance processes as consequence of historical barriers, ocean currents, habitat
411 discontinuities, local adaptation, larval behavior, isolation by distance, and other limitations
412 of the dispersal capabilities (Schunter et al., 2011). In the case of *S. chrysophrii*, the physical
413 barrier of the Apennine Peninsula between the western and eastern clusters appears to prevent
414 the admixture of polyopisthocotylean genotypes from the Italian west and east coasts (at least
415 in the case of farmed genotypes), allowing the formation of a discrete intermediate genetic
416 pool consisting only of Italian wild population of *S. chrysophrii*. The latter is the only truly
417 wild population, and not farm-associated, indicating the local genetic variation shaped
418 probably by isolation by distance. However, as no specimens from the southern areas of Italy
419 were collected for the study, their status remains to be ascertained in the future.
420 When evaluating the three genetic clusters, farmed Greek *S. chrysophrii* were genetically
421 close to the Croatian farmed parasites, and together formed the so-called the East cluster.
422 Considering the large geographical distance between the sampling sites on the eastern
423 Adriatic and the Aegean coast (approximately 2,000 km of coastline) and the absence of
424 densely integrated aquaculture facilities on the aforementioned coast, the admixture of
425 genotypes suggests it might have resulted from human intervention, through the accidental
426 transportation of polyopisthocotylean eggs or infected fish. However, it is also plausible that
427 the eggs were dispersed from Greek sites in the Aegean Sea, facilitated by major dynamical

428 and oceanographic processes within the Adriatic-Ionian basin. Namely, circulation in the
429 Ionian Sea involves cyclonic or anticyclonic circular current regimes in its northern parts that
430 result in warm and salty Levantine Intermediate Water flowing toward the Adriatic Sea. From
431 there, it mixes with the Eastern Adriatic Current, which flows upward along the eastern
432 Adriatic coast in the surface and intermediate layers (Vilibić et al., 2016) and passes by
433 Croatian aquaculture sites. However, as in the case of the physical barrier between the eastern
434 and western Italian *S. chrysophrii* populations, a future collection of specimens along the
435 coast of the Ionian Sea would provide a more robust overview of the East cluster.
436 The "intermediate" genetic cluster formed only by the Italian wild *S. chrysophrii*, is close to
437 the East cluster, but still represents an independent pool of genotypes. Interestingly, these
438 genotypes appear to have been the source of two migration events: an intense gene flow event
439 towards the more distant West cluster (Spain farmed and Italy farmed 1 and 2) and a second,
440 less intense event of gene flow towards the closer East cluster (Croatian and Greek wild and
441 farmed, including the Italian farmed 3), which may indicate human-assisted transmission.
442 The central and East clusters show a similar drift (*i.e.*, evolutionary change) from the
443 ancestral *S. chrysophrii* population, presumably representing the source of infection for the
444 West cluster.
445 This high dispersal capacity is also supported by the lack of genetic structure observed
446 within the West genetic cluster made of *S. chrysophrii* genotypes from Spanish and Italian
447 farmed fish, the latter being located on the west coast of the peninsula (*i.e.*, farms from the
448 Ligurian and Tyrrhenian Seas). Farjallah et al. (2023) have recently evaluated the populations
449 of *S. chrysophrii* inhabiting the area from the Spanish Mediterranean coast to Sardinia, which
450 can be considered a subgroup of the West cluster described here. Using a mitochondrial and a
451 nuclear marker, the authors also found no genetic structure. The geographical distance
452 measured along the coast between the most distant Spanish and Italian farms is almost twice

453 as large as the distance between Greek and Croatian farms. The surface circulation in the
454 Balearic Sea is strong throughout the year, resulting from the Catalan Front on the slope of
455 the continental shelf and the Balearic Front, on the slope of the Balearic Islands shelf (La
456 Violette et al., 1990). In the northern area, the water moves counterclockwise from the
457 Tyrrhenian Sea towards the Balearic Islands, which could indicate that the origin of the far
458 West genotypes is from the Tyrrhenian gene pool. The Spanish wild genotypes, which were
459 excluded from the analyzes due to their low-quality, may have supported or rejected the
460 hypothesis of a *S. chrysophrii* panmixia in this geographical area.
461 Transmission of the polyopisthocotylean in nature is achieved via eggs and free-living
462 oncomiracidia, which are typically dispersed passively by water currents or by active
463 swimming. Therefore, the swimming behavior and persistence of the free-living stages are
464 essential for the success of parasite transmission (Bush et al., 2001; Marcogliese, 2005). In
465 the case of *S. chrysophrii*, the swimming ability of oncomiracidia decreases with the increase
466 in seawater temperature and the time lapsed post-hatching. On average, oncomiracidia only
467 live for 12 hours (Repullés-Albelda et al., 2012) and succeed in swimming vertically (*i.e.*,
468 their most successful swimming direction) for only 6-8 hours, which is only prolonged in
469 darkness (Villar-Torres et al., 2018). Considering that their speed increases at lower water
470 temperatures (from 1.9 mm/s to 2.9 mm/s at 26 and 18 ºC, respectively) (Villar-Torres et al.,
471 2023a) and that approximately up to 51% of oncomiracidia infect their host within the ideal
472 temperature limits of 18-22 ºC (Villar-Torres et al. 2023b), the chance of finding a sparid host
473 in the wild is limited compared to the number of encounters within the aquaculture system.
474 However, Villar-Torres et al. (2023) suggested that the longer period of upward swimming at
475 18 ºC, and faster swimming between 14 and 18 ºC might allow for longer multidirectional
476 dispersal of larvae in the wild.

477 When active dispersal of oncomiracidia ceases, dispersal may be supported by passive drift
478 in currents (Kearn, 1981). The Mediterranean-wide dispersal of *S. chrysophrii* likely relies
479 more on passive dispersal of thick-shelled eggs with positive buoyancy rather than short-lived
480 oncomiracidia. Hypothetically, the eggs could also be accidentally shifted via ballast waters,
481 although specific data for marine helminths is missing (Volodymyr et al., 2011). The discrete
482 structuring of three genetic clusters geographically distributed across the path of dominant
483 seawater masses supports the concept that marine species can have strong genetic population
484 structuring even at very small scales, in contrast to the earlier hypothesis of open,
485 homogeneous, and interconnected seas (Selkoe and Toonen, 2011). However, for the tangible
486 evidence of empirical structuring of *S. chrysophrii* throughout the Mediterranean, sampling
487 should focus on the areas not covered here, in particular the southern area of the Apennine
488 Peninsula and the Middle East, for which data on parasite loads in fish are not available.
489 Finally, host and parasite evolutionary pathways are interlinked in a process of continuous
490 selection of adaptations and counter-adaptations, where the former evades or manipulates
491 host immune response, and the latter tolerates or fights the infection (Buckingham and
492 Ashby, 2022). Panmictic distribution of the polyopisthocotylean therefore may mirror the
493 genetic distribution of the host. Namely, the pattern of genetic differentiation of the wild
494 gilthead seabream in relation to geographic distribution in Aegean and Ionian Seas, and along
495 the Italian coast suggests a lack of strong population structure, potentially linked to
496 widespread of larval stages or aquaculture escapees (Franchini et al., 2012; Gkagkavouzis et
497 al., 2019). Interestingly, despite the evidence for connectivity and gene flow, those studies
498 identified the same level of population structure, highlighting the complexity of factors
499 influencing population dynamic. It remains further to explore, the level of coevolutionary
500 congruence between genetic differentiation of *S. chrysophrii* and the gilthead seabream in the
501 Mediterranean.

502 An important misconception about the life history of *S. chrysophrii* is that it is generally
503 considered to be host-specific, which is a trait that usually limits the spread of the parasite but
504 allows more efficient exploitation of the host due to a tight parasite-host coevolution (Sasal et
505 al., 1999). Specialization on a single host type could lead to local extinction of the parasite and
506 reduce the likelihood of successful establishment in new regions (Bush and Kennedy, 1999).
507 Only a few previous studies have evidenced that the species is present in other Spariadae, such
508 as the bogue and sharpsnout seabream (Mladineo et al., 2009; Sánchez-García et al 2015),
509 while here the annular seabream, axillary seabream, blotched picarel, and common pandora
510 have also been identified as hosts. This indicates that the actual host range is likely
511 underestimated due to limited sampling efforts (Mladineo et al., 2023), but understanding the
512 extent of the former is important for better framing of the polyopisthocotylean genetic
513 structure.
514

515 5. Conclusions

516 *Sparicotyle chrysophrii* has a panmictic distribution in the Mediterranean basin but forms three
517 discrete genetic pools or clusters, driven by geographic constraints. Gene flow within these
518 three clusters is at least facilitated by the dispersal of polyopisthocotylean eggs via the
519 circulation of dominant currents and fronts, but anthropogenic influences should also be
520 considered. Gene flow between parasites inhabiting wild and farmed GSB stocks is
521 pronounced, but other sparids, such as the annular seabream, axillary seabream, blotched
522 picarel, bogue, and common pandora, could also play a role in the transmission. This
523 polyopisthocotylean should not be considered a host-specific, but rather a family-specific
524 parasite.
525

526 Author contribution statement

Ivona Mladineo: conceptualization, investigation, statistical analysis, writing and editing.

Jerko Hrabar: sample collection and analyses, review, and editing.

Zeljka Trumbic: sample collection and analyses, review, and editing.

Somaye Rasouli-Dogaheh: statistical analysis, writing and editing.

Paola Beraldo: sample collection and analyses, review, and editing.

Donatella Volpatti: sample collection and analyses, review, and editing.

George Rigos: sample collection and analyses, review, and editing.

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Ariadna Sitjà-Bobadilla: funding acquisition and coordination, sample collection, writing and editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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563

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836 **Tables**

837 **Table 1**: Comparative genetic metrics across populations of the polyopisthocotylean *Sparicotyle chrysophrii* isolated from wild (W) and farmed

838 (F, F1-F3) gilthead seabream (*Sparus aurata*) from Spain, Italy, Croatia, and Greece. N: number of collected parasites, Nx: number of sequences

839 used in analyses after filtering of low-quality reads; Pi: nucleotide diversity; He: expected heterozygosity; Ho: observed heterozygosity; MLH:

840 multi-locus heterozygosity (MLH); TD: Tajima's D; Hom: observed homozygosity; K: frequency of private alleles; F: inbreeding coefficient.

841 Statistics were not reported for populations with fewer than 10 individuals.

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Populations	Lat/ lon	N	Nx	Pi	He	Ho	MLH	TD	Hom	K	F
Spain F	39°59'02.7"N 0°08'09.3"E	78	40	0.031	0.0398	0.027	0.027	-0.1358	0.095	0.213	0.1132
Italy F1	44°4'23.57"N 9°50'28.33"E	24	17	0.0289	0.036	0.025	0.025	-0.1279	0.046	0.192	0.04581
Italy F2	41°13'48.24"N 13°35'56.83"E	10	6	-	-	-	-	-	-	-	--
Italy F3	45°45'56.64"N 13°35'28.87"E	24	17	0.028	0.037	0.024	0.024	-0.1527	0.138	0.183	0.1215
Italy W	45°42'1.57"N 13°15'27.97"E	22	14	0.027	0.038	0.028	0.028	-0.1424	0.026	0.183	0.0213
Croatia F	44°01'57.7"N 15°13'28.5"E	54	20	0.044	0.039	0.029	0.029	-0.0975	0.045	0.186	0.0667
Croatia W	44°01'57.7"N 15°13'28.5"E	39	3	-	-	-	-	-	-	-	-
Greece F	38° 32' 00" N, 21° 05' 00"E	70	54	0.030	0.041	0.032	0.032	-0.0893	0.042	0.195	0.0721

Greece W	38° 32' 00" N, 21° 05' 00" E	9	2	-	-	-	-	-	-	-	-

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843 **Table 2:** Pairwise F_{ST} values among nine pre-defined populations of the polyopisthocotylean
844 *Sparicotyle chrysophrii* infecting wild sparids (W) and farmed (F) gilthead seabream *Sparus*
845 *aurata*. F_{ST} values were calculated only for populations with more than 10 individuals.
846 Numbers in bold indicate statistically significant differences in genetic structure ($P<0.05$).

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	Croatia F	Greece F	Italy F1	Italy F3	Italy W	Spain F
Croatia F	0	0	0	0	0	0
Greece F	0.018	0	0	0	0	0
Italy F1	**0.066**	**0.076**	0	0	0	0
Italy F3	0.008	0.022	0.054	0	0	0
Italy W	**0.034**	**0.041**	**0.032**	0.042	0	0
Spain F	**0.071**	**0.076**	0.002	0.059	0.037	0

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856 **Figures**

857 **Figure 1:** Map of sampling locations of fish infected by polyopisthocotylean *Sparicotyle*
858 *chrysophrii* in the Mediterranean. The triangles represent sampling sites of the farmed
859 gilthead seabream (*Sparus aurata*), and circles represent sampling sites of the wild sparids.

860

861 **Figure 2:** Summary of genetic differentiation among predefined populations of *Sparicotyle*
862 *chrysophrii* infecting wild sparids and farmed gilthead seabream (*Sparus aurata*) in the
863 Mediterranean: A) Scatterplot showing the first two principal components of the
864 Discriminant Analyses of Principal Components (DAPC) performed on *S. chrysophrii*

865 nuclear ddRAD SNPs as prior clusters (explaining 32% and 2.1% of variance) depicted in
866 different colors; B) Scatterplot generated using *S. chrysophrii* populations grouped as wild
867 and farmed; C) Group membership probability for each individual of *S. chrysophrii* derived
868 using Admixture analysis and visualized in a STRUCTURE-like plot. Each vertical stacked
869 column (100%) indicates an individual representing the proportions of ancestry in K
870 constructed ancestral populations. The color gradient shows the degree of difference among
871 individuals.
872
873 **Figure 3:** Clustered fineRADstructure coancestry matrix based on nuclear ddRAD data.
874 Predicted populations of *Sparicotyle chrysophrii* (Spain farmed, Italy farmed 1, 2, 3, Italy wild,
875 Croatia farmed, Croatia wild, Greece farmed, Greece wild) are collectively grouped within the
876 corresponding dendrogram. Red, purple, and dark purple colors in the matrix indicate a higher
877 proportion of loci with shared coancestry.
878
879 **Figure 4:** Isolation by distance (IBD) scatterplot shows the results of the Mantel test between
880 the geographic distance and the genetic distance (F_{ST}) based on SNPs among populations of
881 *Sparicotyle chrysophrii* from wild sparids and farmed gilthead seabream (*Sparus aurata*). Two-
882 dimensional kernel estimation of the correlation of the genetic and geographic distances are
883 shown ($r=0.31$, $p < 0.05$); line shows the correlation trend, and colors represent the relative
884 density of points: blue low density, yellow medium density, and orange to red high density.
885
886 **Figure 5:** Maximum likelihood population tree inferred by TreeMix constructed based on
887 ddRAD-generated unlinked SNPs of *Sparicotyle chrysophrii* isolated from wild sparids and
888 farmed gilthead seabream (*Sparus aurata*). Branch lengths are proportional to the evolutionary
889 change (the drift parameter), and terminal nodes are labeled by population codes. The two

890 migration events are depicted as heatmap-colored arrows: light yellow for a lower, and red for
891 a higher migration weight, *i.e.*, the percentage of ancestry received from the source population.
892
893 Supplementary material
894 Supplementary Table 1: Data of sampled *Sparicotyle chrysophrii* in the study, including
895 sampling date, country, extraction date from the fish gills, coordinates of the sampling site,
896 fish origin (wild or farmed) and fish species, fish total length, fish total weight, parasite id
897 number, fish id number, total number of parasites, number of adult/ juvenile/ oncomiracidia
898 parasites stages per 1st, 2nd, 3rd and 4th gill arch left (L) and right (R).
899 Supplementary Figure 1: Optimization of the -M, -m, and -N parameters of de novo assembly
900 of RAD-seq data of *Sparicotyle chrysophrii* that recorded the number of assembled loci,
901 polymorphic loci, and SNPs generated for each run.
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