High diversity of fish ectoparasitic monogeneans (Dactylogyrus) in the Iberian Peninsula: a case of adaptive radiation?

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2   **Iberian Peninsula: a case of adaptive radiation?**

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Abstract

The epicontinental fauna of the Iberian Peninsula is strongly influenced by its geographical history. As the possibilities for dispersion of organisms into and from this region were (and still are) limited, the local fauna consists almost exclusively of endemic species. Almost all Iberian freshwater fishes of the families Leuciscidae and Cyprinidae are endemic and on-going research on these taxa continually uncovers new species. Nevertheless, information on their host-specific parasites remains scarce. In this study, we investigate the diversity and phylogenetic relationships in monogeneans of the genus *Dactylogyrus* (gill ectoparasites specific to cyprinoid fish) in the Iberian Peninsula. Twenty-two species were collected and identified from 19 host species belonging to Cyprinidae and Leuciscidae. A high degree of endemism was observed, with 21 *Dactylogyrus* species reported from Iberia only and a single species, *D. borealis*, also reported from other European regions. Phylogenetic analysis split the endemic Iberian *Dactylogyrus* into two well-supported clades, the first encompassing *Dactylogyrus* parasitising endemic *Luciobarbus* spp. only, and the second including all *Dactylogyrus* species of endemic leuciscids and four species of endemic cyprinid. Species delimitation analysis suggests a remarkable diversity and existence of a multitude of cryptic *Dactylogyrus* species parasitising endemic leuciscids (*Squalius* spp. and representatives of *Chondrostoma* s.l.). These results suggest a rapid adaptive radiation of *Dactylogyrus* in this geographically isolated region, closely associated with their cyprinoid hosts. Moreover, phylogenetic analysis supports that *Dactylogyrus* parasites colonised the Iberian Peninsula through multiple dispersion events.

Keywords

Monogenea; phylogeny; host-specificity; species delimitation; Iberian Peninsula; Cyprinoidea

1   Introduction

The Iberian Peninsula has a remarkable biological diversity, harbouring more than 50% of European animal and plant species (Médail and Quézel, 1997, Martín et al., 2000, Williams et al., 2000, Araújo et al., 2007, Cardoso, 2008, Rueda et al., 2010, López-López et al., 2011, Penado et al., 2016) and approximately 31% of all European endemic vertebrate and plant species (Williams et al., 2000). This high species diversity is linked with several climatic and geological changes occurring over the region since the Cenozoic period (Hsü et al., 1973, Rosenbaum et al., 2001), when putative migration routes periodically emerged and disappeared. However, the main factor influencing the degree of endemism is most likely geographical isolation resulting from the elevation of the Pyrenees in the north-east combined with the generally mountainous topography of the peninsula, which provided a multitude of refuges during glacial periods (Gante et al., 2009, Hewitt, 2011).

While the species diversity of Iberian freshwater ichthyofauna is relatively low in comparison to other European regions (Kottelat and Freyhof, 2007), the majority of species are endemic. The Peninsula hosts representatives of just a few native freshwater fish groups, with most species belonging to the Cyprinidae and Leuciscidae families (order Cyprinoidea; following the classification proposed by Schönhuth et al., 2018). The Leuciscidae (previously considered as Leuciscinae within Cyprinidae; Ketmaier et al., 2004, Levy et al., 2009, Perea et al., 2010, Imoto et al., 2013) are represented by the monotypic genus *Anaecypris*, the genera *Phoxinus*, *Iberocypris* and *Squalius*, and by four recently erected genera belonging to *Chondrostoma sensu lato*: *Achondrostoma, Iberochondrostoma, Parachondrostoma* and *Pseudochondrostoma* (Kottelat and Freyhof, 2007, Robalo et al., 2007, Schönhuth et al., 2018). In contrast to the leuciscids, cyprinids are represented by just two genera: *Barbus* and *Luciobarbus* (Kottelat & Freyhof, 2007, Gante, 2011, Gante et al., 2015). The distribution of a given cyprinoid species is usually confined to a specific ichthyogeographic province and the

88 ranges of different species rarely overlap (Doadrio, 1998, Gante et al., 2015), suggesting that

89 speciation is closely linked with the formation of river basins (Zardoya and Doadrio, 1998,

90 Machordom and Doadrio, 2001, Doadrio et al., 2002, Mesquita et al., 2007, Casal-López et

91 al., 2017, Sousa-Santos et al., 2019).

92 In contrast to the thorough previous and on-going research on Iberian cyprinoids, data on their

93 helminth parasites are scarce (da Costa Eiras, 2016). In previous studies focussed on

94 freshwater fishes in different regions of the northern hemisphere (e.g. Mexico and the

95 Balkans), it has been suggested that the biogeography of fish helminth parasites reflects the

96 historical dispersion and current distribution of their hosts (e.g. Choudhury and Dick, 2001,

97 Pérez-Ponce de León et al., 2005, Benovics et al., 2018). However, very few studies have

98 been carried out on cyprinoid monogeneans in the Iberian Peninsula, by far the most thorough

99 being those of el Gharbi et al. (1992) and Šimková et al. (2017). The former study, describing

100 seven species of *Dactylogyrus* from six cyprinid species (relying on morphological data only)

101 suggested that the pattern of geographical distribution of *Dactylogyrus* spp. follows the

102 distribution of their cyprinid hosts, for which they are highly host-specific. The study by

103 Šimková et al. (2017) focussed on phylogenetic relationships between endemic *Dactylogyrus*

104 from cyprinids in Iberia and *Dactylogyrus* from Central Europe and north-west Africa. The

105 authors suggested multiple origins of endemic *Dactylogyrus* in the Iberian Peninsula as the

106 presence of *Dactylogyrus* lineages in different *Luciobarbus* lineages was associated with

107 specific dispersion events.

108 Gill monogeneans belonging to *Dactylogyrus* are currently the most species-diversified group

109 within the Platyhelminthes (more than 900 nominal *Dactylogyrus* species, mostly described

110 from morphology, are presently known according to the latest review by Gibson et al., 1996).

111 *Dactylogyrus* species are strictly specific to cyprinoids and many *Dactylogyrus* species are

112 specific to a single host species (Šimková et al., 2006b). However, the degree of host

specificity across *Dactylogyrus* species differs and, in some cases, host specificity is likely to reflect the ecology and recent distribution of their hosts (Benovics et al., 2018). *Dactylogyrus* species with a narrow host range are most common in regions with a high number of endemic host species. In Europe, such regions include the Balkan Peninsula, where a multitude of strictly host-specific endemic *Dactylogyrus* species has been documented (Dupont and Lambert, 1986, Benovics et al., 2017, 2018), and the Iberian Peninsula, where many *Dactylogyrus* endemic species have been documented for *Luciobarbus* (el Gharbi et al., 1992). It has been suggested that such a high degree of endemism in *Dactylogyrus* is the result of co-speciation with their hosts over long evolutionary periods in geographically isolated regions (Dupont, 1989). Over time, the *Dactylogyrus* parasites have developed an attachment organ (haptor) that is highly specialised toward their host (Šimková et al., 2000, Jarkovský et al., 2004, Šimková and Morand, 2008). As such, the shape and size of monogenean haptoral sclerites are considered to be species specific and represent suitable morphological characters for species determination. Nevertheless, some species exhibit haptoral sclerites that are very similar in shape and size (see Pugachev et al., 2009); thus, species identification is often difficult from the observation of haptoral sclerotised structures only. It has been suggested, therefore, that the shape of the sclerotised parts of copulatory organs are more suitable for the identification of monogeneans to species level due to their putative faster evolutionary rate (Pouyaud et al., 2006, Šimková et al., 2006b, Vignon et al., 2011, Mendlová et al., 2012, Mandeng et al., 2015, Benovics et al., 2017). Rapid morphological diversification in the monogenean copulatory organs is hypothesised to be a mechanism to avoid hybridisation (Rohde 1989), which is especially likely for *Dactylogyrus* species living on the same hosts in overlapping microhabitats (Šimková et al., 2002, Šimková and Morand, 2008).

Compared with Central Europe, *Dactylogyrus* communities in the southern European Peninsulas generally appear to be species poor. Cyprinoids with a wide European distribution

138 range, such as *Rutilus rutilus* and *Squalius cephalus*, harbour up to nine *Dactylogyrus* species

139 (e.g. Šimková et al., 2000, Seifertová et al., 2008). In contrast, a maximum of five

140 *Dactylogyrus* species per cyprinoid species have been reported from the southern European

141 Peninsulas (Dupont and Lambert, 1986, el Gharbi et al., 1992, Galli et al., 2002, 2007,

142 Benovics et al., 2018).

143 In comparison to other European regions, cyprinoid monogenean communities have been

144 underexplored in the Iberian Peninsula. Thus, the main objective of the present study was to

145 investigate the diversity of *Dactylogyrus* spp. parasitising endemic cyprinoids in this

146 geographical region. A species delimitation method was applied to assess the species status of

147 *Dactylogyrus* identified in this study based on genetic variability within and among each

148 species, and to compare these results to species defined from morphology only. Moreover, the

149 present study investigates the evolutionary history and phylogenetic relationships between

150 endemic Iberian *Dactylogyrus* and *Dactylogyrus* from other Peri-Mediterranean regions,

151 including cyprinoid species with a wide European distribution range, in order to (1) shed new

152 light on cyprinoid phylogeography, (2) infer potential historical contacts between cyprinoids

153 from different regions, and (3) evaluate the evolution of *Dactylogyrus* species diversity (using

154 both morphology and species delimitation methods).

155 **2   Material and Methods**

156 **2.1   Parasite collection**

157 Fish were collected over years 2016 and 2017 from 17 localities in Portugal and Spain (Figure

158 1). In total, 257 specimens representing 19 fish species were examined for presence of

159 *Dactylogyrus* parasites (Table 1). Fish were dissected following the standard protocol

160 described by Ergens and Lom (1970). *Dactylogyrus* specimens were collected from the gills,

161 mounted on slides and fixed in a mixture of glycerine and ammonium picrate (GAP;

162 Malmberg, 1957) for further identification. Determination to species level was performed on

163　the basis of the size and shape of the sclerotised parts of the attachment apparatus (anchor

164　hooks, marginal hooks and connective bars of the haptor) and the reproductive organs (male

165　copulatory organ and vaginal armament) following Pugachev et al. (2009). At least five

166　specimens of each *Dactylogyrus* species from each host species examined were bisected using

167　fine needles. One-half of the body (either the anterior part containing the reproductive organs

168　or the posterior part with the attachment organ) was mounted on a slide and used for

169　morphological identification. The other half was individually preserved in pure ethanol for

170　subsequent DNA extraction.

171　**2.2　DNA extraction, PCR and sequencing**

172　DNA extraction was performed using the DNeasy Blood & Tissue Kit (Quiagen, Hilden,

173　Germany) based on the standard protocol provided by the manufacturer. Two DNA regions

174　were amplified. The partial gene coding 18S rRNA and complete ITS1 region was amplified

175　using the primers S1 (forward, 5'-ATTCCGATAACGAACGAGACT-3') and Lig5.8R

176　(reverse, 5'-GATACTCGAGCCGAGTGATCC -3') (Šimková et al., 2003, Blasco-Costa et

177　al., 2012). Each amplification reaction was performed in a final volume of 20 μl, the reaction

178　mixture comprising 1.5 U Taq polymerase (Fermentas), 1x buffer, 1.5 mM MgCl2, 0.2 mM of

179　dNTPs, 0.1 mg/ml BSA, 0.5 μM of each primer and 2 μl of pure DNA (20 ng/μl). PCR was

180　carried out using the following steps: 3 min initial denaturation at 95 °C, followed by 40

181　cycles of 40 seconds at 94 °C, 30 seconds at 52 °C and 45 seconds at 72 °C, and 4 minutes of

182　final elongation at 72 °C. The second marker, a part of the gene coding 28S rRNA, was

183　amplified using the primers C1 (forward, 5'-ACCCGCTGAATTTAAGCA-3') and D2

184　(reverse, 5'-TGGTCCGTGTTTCAAGAC-3') (Hassouna et al., 1984), following the PCR

185　protocol described in Šimková et al. (2006a). The PCR products were purified prior to

186　sequencing using the ExoSAP-IT kit (Ecoli, Bratislava, Slovakia), following the standard

187　protocol, and directly sequenced using the PCR primers and the BigDye Terminator Cycle

188   Sequencing kit (Applied Biosystems, Foster City, CA). Sequencing was carried out on an ABI

189   3130 Genetic Analyzer (Applied Biosystems). The newly generated sequences were deposited

190   in GenBank (see Table 1 for accession numbers).

191   **2.3   Phylogenetic and species delimitation analysis**

192   Partial sequences coding 18S rRNA and 28S rRNA, and complete sequences of the ITS1

193   region were concatenated and aligned using the fast Fourier transform algorithm implemented

194   in MAFFT (Katoh et al., 2002) using the G-INS-i refinement method. Out of 71 DNA

195   sequences used in the alignment, 35 were newly sequenced in this study. Sequences from 35

196   other *Dactylogyrus* species, used as representative species from different European regions,

197   and sequences of *Ancyrocephalus percae*, used as an outgroup (phylogenetically closely

198   related to *Dactylogyrus* according to Mendoza-Palmero et al., 2015), were obtained from

199   GenBank (see Table S1 in supplementary material for accession numbers). Gaps,

200   hypervariable regions and ambiguously aligned regions were removed from the alignment

201   using GBlocks v. 0.91 (Talavera and Castresana, 2007). The optimal DNA evolutionary

202   model was selected separately for each part of the alignment corresponding to one of the three

203   markers analysed (18S, ITS1, 28S) using the Bayesian information criterion in jModelTest v.

204   2.1.10 (Guindon and Gascuel, 2003, Darriba et al., 2012).

205   Maximum likelihood (ML) analysis was conducted in RAxML v. 8.2.11 (Stamatakis, 2006,

206   2014), applying the general time-reversible model (GTR; Lanave et al., 1984) of nucleotide

207   substitution. Internal node support was assessed by running 1000 bootstrap pseudoreplicates.

208   Bayesian inference (BI) analysis was performed in MrBayes v. 3.2.6 (Ronquist et al., 2012)

209   using two parallel runs, each with four Markov chains (one cold and three heated) of 10^7

210   generations with trees sampled every 10^2 generations. The first 30% of trees were discarded

211   as initial burn-in. Convergence was indicated by an average standard deviation of split

212   frequencies per parallel run of < 0.01, subsequently checked using Tracer v. 1.7.1 (Rambaut et

al., 2018). Posterior probabilities were calculated as the frequency of samples recovering particular clades.

To investigate genetic diversity in the commonly used genetic markers between well-defined endemic *Dactylogyrus* species, uncorrected pair-wise genetic distances (*p*-distances) were computed for 12 selected taxa in MEGA X (Kumar et al., 2018). Three sequence alignments were used: the partial gene coding 18S rRNA, the complete ITS1 region and the partial gene coding 28S rRNA. All positions containing gaps and missing data were removed from final computations.

The Bayesian implemented Poisson Tree Processes model (bPTP; Zhang et al., 2013) was applied to the phylogram resulting from BI in order to infer putative species of Iberian *Dactylogyrus*. The bPTP method only requires a phylogenetic tree as its input and uses branch lengths to estimate the mean expected number of substitutions per site between two branching events. Within species, branching events will be frequent whereas they will be rarer between species. The model implements two independent classes of Poisson process (one describing speciation and the other describing coalescent processes) and searches for transition points between interspecific and intraspecific branching events. Potential species clusters are then determined by identifying the clades or single lineages that originate after these transition points. The computation was run for 5×10^5 generations with the first 30% of trees discarded as initial burn-in. The distant outgroup taxon was removed from the final analysis to improve delimitation in the results.

3   Results

Twenty-two *Dactylogyrus* species (identified using morphological characters, i.e. sclerotised parts of the haptor and reproductive organs) were collected from endemic Iberian cyprinoid species (Table 1). From one to five *Dactylogyrus* species were recorded per host species, with highest species richness found on *Luciobarbus* spp. (5 species on *L. guiraonis*, 4 species on *L.*

238 *graellsii*, and 4 species on *L. sclateri*). Both *Parachondrostoma* species, *Barbus haasi*,

239 *Iberochondrostoma almacai* and *Phoxinus bigerri* were parasitised by a single *Dactylogyrus*

240 species. Overall, *Dactylogyrus bocageii* exhibited the widest host range across the Iberian

241 Peninsula, parasitising four *Luciobarbus* species. Minor genetic variation was observed

242 between *D. bocageii* collected from different hosts (p-distance ≤ 0.002 in the partial gene for

243 28S rRNA, p-distance ≤ 0.020 in the ITS1 region; Tables 2 and 3).

244 The final concatenated alignment of partial genes for 18S rRNA, 28S rRNA and the ITS1

245 region included 71 sequences contained 1533 unambiguous nucleotide positions. The most

246 suitable evolutionary models were TrNef+I+G, TPM2uf+G and GTR+I+G for the partial

247 genes coding 18S rRNA, the ITS1 region and part of the gene for 28S rRNA, respectively.

248 Both ML and BI analyses produced trees with congruent topologies varying only in some

249 support values for individual nodes (Figure 2). Phylogenetic analysis divided all taxa into

250 three strongly supported clades.

251 The first group (Clade A; Figure 2) included the majority of *Dactylogyrus* species from

252 Europe, and especially the species parasitising Leuciscidae. In addition, several *Dactylogyrus*

253 species from *Barbus* and *Luciobarbus* (Cyprinidae) were also placed in this clade (i.e.

254 *Dactylogyrus* of *Barbus* spp. and *Luciobarbus* spp. from Central Europe and the Balkans, and

255 *D. balistae, D. legionensis, D. linstowoïdes* and *D. andalousiensis* of Iberian *Luciobarbus*

256 spp.). *Dactylogyrus* from Iberian cyprinoids were divided into seven lineages within Clade A.

257 *Dactylogyrus polylepidis* of *Achondrostoma arcasii* was in a well-supported sister position to

258 the morphologically similar *D. vistulae. Dactylogyrus* from European cyprinids formed three

259 well-supported groups within Clade A. *Dactylogyrus legionensis*, *D. balistae* and *D.*

260 *linstowoïdes* were grouped in a sister position to common *Dactylogyrus* species from Central

261 European *Barbus* spp. (*D. malleus*, *D. prespensis* and *D. petenyi*). The second group

262 contained *D. andalousiensis* from two Iberian *Luciobarbus* species, and *D. omenti* from

11

263 *Aulopyge huegelii* (Balkan endemic species). The third group contained *D. carpathicus* and *D.*

264 *crivellius* (two common species of *Barbus* spp.) and two yet undescribed endemic

265 *Dactylogyrus* species of endemic Balkan *Luciobarbus* species (*L. albanicus* and *L. graecus*).

266 The phylogenetic position of *Dactylogyrus* sp. 1 from *S. aradensis* and *S. torgalensis*

267 (morphologically identical but genetically slightly different; *p*-distance = 0.010) was not fully

268 resolved and its sister position to *D. folkmanovae* was only supported by BI. The majority of

269 Iberian *Dactylogyrus* species (*Dactylogyrus* sp. 2 to *Dactylogyrus* sp. 10) formed a well-

270 defined phylogenetic lineage that also included *D. caucasicus, D. ergensi* and *D. tissensis*.

271 The three latter species and the Iberian *Dactylogyrus* in this lineage all have the same or very

272 similarly shaped male copulatory organs commonly classified as 'ergensi' of the

273 'chondrostomi' type (see Pugachev et al., 2009). Generalist *Dactylogyrus* species within

274 Clade A (i.e. *D. legionensis¸ D. polylepidis*, *Dactylogyrus* sp. 1, *Dactylogyrus* sp. 7 and

275 *Dactylogyrus* sp. 8) exhibited intraspecific genetic variability. The second major group (Clade

276 B) comprised five *Dactylogyrus* species specific to Iberian *Luciobarbus*. Where intraspecific

277 genetic variability was documented, all genetic variants formed well supported clades (i.e. *D.*

278 *bocageii, D. guadianensis* and *D. lenkoranoïdes*). The last strongly supported group (Clade C)

279 encompassed *Dactylogyrus* species host specific to *Carassius* spp. and/or *Cyprinus carpio*

280 distributed across the Europe and Asia.

281 Genetic distances were computed between morphologically similar species from Clade B

282 (Figure 2). Three alignments of 12 sequences representing five *Dactylogyrus* species of group

283 B were analysed to compare intra- and interspecific genetic variability calculated using

284 genetic markers commonly used in monogeneans. The alignments comprised 486 nucleotide

285 positions for the partial gene coding 18S rRNA combined, 716 nucleotide positions for the

286 ITS1 segment and 807 nucleotide positions for the partial gene coding 28S rRNA. The lowest

287 genetic variability was observed for the partial gene coding 18S rRNA. No intraspecific/inter-

population genetic variability was observed (*p*-distance = 0.000) and interspecific pair-wise

nucleotide diversity varied from 0.002 to 0.010 (Table 4). Low pair-wise interspecific

diversity was also observed for the partial gene coding 28S rRNA (0.006 to 0.020); however,

minor intraspecific genetic variability was observed in this gene (*p*-distance ≤ 0.002). Slight

genetic distance in part of the gene for 28S rRNA was observed between different populations

of *D. bocageii* (0.001 to 0.002) and between individuals from different populations of *D.*

guadianensis (*p*-distance = 0.001). The highest genetic diversity was observed in the ITS1

region, in which intraspecific distances varied from 0.000 (*D. lenkoranoïdes*) to 0.020 (*D.*

bocageii). The pair-wise interspecific diversity in the ITS1 region varied from 0.031 between

D. doadrioi and *D. guadianensis* to 0.135 between *D. doadrioi* and *D. mascomai*.

The species status of *Dactylogyrus* collected from endemic Iberian cyprinoids was

investigated using the bPTP method, with the addition of *Dactylogyrus* species parasitising

cyprinoids in other parts of Europe used as a reference of previously delimited species

(Benovics et al., 2018). The results of the bPTP analysis were largely consistent with the

species previously described on the basis of morphology (Figure 3), though the ML solution

suggested a higher species diversity. Based on ML results, *D. legionensis* encompasses two

species, each being host-specific (one to *L. graellsii* and the other to *L. guiraonis*), as well as

Dactylogyrus sp. 1 (*S. aradensis* and *S. torgalensis*). Both BI and ML supported solutions,

obtained from bPTP analysis, suggested a generalist status for *D. andalousiensis, D. bocageii,*

D. lenkoranoïdes and *D. guadianensis* (i.e. there were no host-specific parasites within these

delimited species). A potentially new species, *Dactylogyrus* sp. 7, was also supported by the

species delimitation analysis as a generalist, parasitising both *S. carolitertii* and *S. pyrenaicus*.

This analysis also suggested that *D. borealis,* determined using morphological characters, is a

common parasite of *Phoxinus* spp. in other parts of Europe and is also found on *P. bigerri* in

the Iberian Peninsula. bPTP analysis also suggested that *Parachondrostoma miegi* and *P.*

13

turiense are both parasitised by a single *Dactylogyrus* species (*Dactylogyrus* sp. 8) that is morphologically similar and phylogenetically close to *Dactylogyrus* sp. 9, parasitising *P. duriense*. Finally, species delimitation analysis supported the discovery of at least 11 unknown *Dactylogyrus* species in the Iberian Peninsula, as all other Iberian genetic variants were identified as individual host-specific species.

4   Discussion

4.1   Parasite diversity and distribution

The Iberian Peninsula harbours a high diversity of cyprinoids that have been the subject of extensive research; nevertheless, the species diversity of their host-specific parasites is still underexplored, especially in areas with a high diversity of endemic cyprinoids. Following previous research on the *Dactylogyrus* (or Monogenea in general) of Iberian cyprinids (el Gharbi et al., 1992, Lacasa-Millán and Gutiérrez-Galindo, 1995, Gutiérrez-Galindo and Lacasa-Millán, 2001), this study is the first to investigate the overall diversity of Iberian *Dactylogyrus*, including molecular data for both cyprinoid fish and their host-specific *Dactylogyrus*.

The present study revealed the presence of several potentially new *Dactylogyrus* species to science, all of which were well supported by the bPTP species delimitation method. This strongly suggests that endemic Iberian cyprinoid species harbour an endemic *Dactylogyrus* fauna, as previously suggested for Iberian *Luciobarbus* species by el Gharbi et al. (1992). In contrast to the Balkan and Apennine Peninsulas (Dupont and Lambert, 1986, Dupont and Crivelli, 1988, Dupont, 1989, Galli et al., 2002, 2007, Benovics et al., 2018), Iberian *Dactylogyrus* spp. appear to exhibit a higher degree of host specificity as the majority of *Dactylogyrus* species from Leuciscidae were restricted to a single host species. Benovics et al. (2018) proposed that southern European endemic cyprinoids harbour species-poor *Dactylogyrus* communities compared with European cyprinoids with a wide distribution

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338 range (e.g. *Rutilus rutilus, Squalius cephalus*). The same pattern was also observed in the

339 Iberian Peninsula, where one to five *Dactylogyrus* species were found on a single cyprinoid

340 host species. It should be noted, however, that parasite community composition may be

341 strongly influenced by seasonal abiotic factors (e.g. González-Lanza and Alvarez-Pellitero,

342 1982, Lux, 1990, Appleby and Mo, 1997, Šimková et al., 2001b, Poulin and Morand, 2004,

343 Zhang et al., 2015, Sinaré et al., 2016). Until now, knowledge of *Dactylogyrus* diversity in

344 southern European Mediterranean Peninsulas has been based on studies taking place in

345 summer only (Benovics et al, 2018, this study) as the *Dactylogyrus* diversity is expected to be

346 highest during this period (Šimková et al., 2001b).

347 In this study, a higher number of *Dactylogyrus* species was observed on *Luciobarbus* species.

348 While the overall species richness on these fish was in accordance with the observations of el

349 Gharbi et al. (1992), the species composition in the present study differed slightly from their

350 data. In line with the study of el Gharbi et al. (1992), *D. bocageii* was the most common

351 species (occurring on five *Luciobarbus* species), though its distribution range was wider, as

352 proposed by Lambert and el Gharbi (1995), stretching via Zujar and Torgal rivers to the

353 south-western part of the peninsula (south-west Iberian province, Filipe et al., 2009).

354 Interestingly, unlike other European regions, the only endemic representative of the genus

355 *Barbus* in Iberia, *Barbus haasi*, harbours *Dactylogyrus* species typical of *Luciobarbus* spp.. In

356 the Balkans, endemic *Barbus* spp. are parasitised by common *Dactylogyrus* species for this

357 fish genus (e.g. *D. dyki* and *D. crivellius*), whilst *Luciobarbus* spp. are parasitised by different,

358 strictly host-specific species (Benovics et al., 2017, 2018). In accordance with our own

359 findings, El Gharbi et al. (1992) showed that *B. haasi* is a common host of *D. bocageii, D.*

360 *mascomai* and *D. lenkoranoïdes*, whilst *D. dyki* and *D. carpathicus* (commonly distributed on

361 European *Barbus* spp.) were only found in previous studies on *B. haasi* x *B. meridionalis*

362 hybrids in the north-eastern part of the Peninsula. Nevertheless, Gutiérrez-Galindo and

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363   Lacasa-Millán (1999) also reported the latter two *Dactylogyrus* species from *B. haasi* in the

364   River Llobregat (north-east Spain). However, the fish hosts from this study could potentially

365   also be hybrids, as presence of the *B. haasi* x *B. meridionalis* hybrids was previously

366   documented in Llobregat basin (Machordom et al., 1990). In contrast to the aforementioned

367   studies, only *D. lenkoranoïdes* was collected from *B. haasi* in this study (Uldemo River; Ebro

368   basin). This low parasite diversity may be linked with the seasonal fluctuation in parasite

369   communities previously documented among Iberian *Dactylogyrus* (e.g. *D. legionensis*

370   (González-Lanza and Alvarez-Pellitero, 1982) or *D. balistae* (Simón-Vicente, 1981)). In

371   addition to the common parasitisation of Iberian *Barbus* by *Dactylogyrus* parasites typically

372   recognised as specific to *Luciobarbus*, several cases of infection by *Dactylogyrus* species

373   common for *Barbus* were also reported in Iberian *Luciobarbus* species. Gutiérrez-Galindo and

374   Lacasa-Millán (2001) also reported that *L. graellsii* was parasitised by *D. dyki* and *D. extensus*

375   (host specific parasites of *Barbus* spp. and *Cyprinus carpio*, respectively). However, the

376   presence of *D. dyki* on *Luciobarbus* spp. may result from non-detected instances of

377   hybridisation, as hybrids of cyprinoid species are usually parasitised by *Dactylogyrus* specific

378   for each of the parental species (Šimková et al., 2013, Krasnovyd et al. 2017). Hybridisation

379   between Iberian *Luciobarbus* spp. (potentially also between *Luciobarbus* and *Barbus*; Gante

380   et al., 2015) appears to be quite common, especially between congeners living in sympatry

381   (e.g. *Luciobarbus* spp.; Almodóvar et al., 2008, Sousa-Santos et al., 2018). Thus, host-

382   switching is possible, most likely occurring between species from phylogenetically close

383   genera (i.e. *Barbus* and *Luciobarbus*; Yang et al., 2015) in north-eastern Iberian drainages

384   where the distribution ranges of Central European barbels (e.g. *B. meridionalis*; see Kottelat

385   and Freyhof, 2007 for its distribution range) and Iberian barbels overlap.

386   Despite the presence of high numbers of endemic *Dactylogyrus* species in Iberia, *P. bigerri*

387   was parasitised by *D. borealis*, a common species on European *Phoxinus* spp. (Moravec,

2001, Šimková et al., 2004, Benovics et al., 2018). The presence of this common European *Dactylogyrus* species is in contrast to the expected high degree of endemism in south European peninsulas (Williams, 2000, Hewitt, 2011). Other common European *Dactylogyrus* species are absent from Iberia; for example, *D. vistulae*, which parasitises the highest number of cyprinoid species across Europe, is absent from Iberia, and only the closely related *D. polylepidis* is found on Iberian cyprinoids. These findings suggest that either (1) *D. borealis* was only recently introduced into the Iberian Peninsula with another *Phoxinus* species coming from different European areas (see Corral-Lou et al., 2019), or (2) *D. borealis* represent an extremely slowly evolving species, meaning that the Iberian lineage would be morphologically and genetically similar to *D. borealis* from other European areas. In the present study, *D. polylepidis*, originally described from *Pseudochondrostoma polylepis* (Alvarez-Pellitero et al., 1981), was found for the first time on three host species (all members of the Leuciscidae). The wider host range recorded for *D. polylepidis* indicates that this species represents a true generalist parasite, probably endemic to this region. In contrast to *D. polylepidis*, the morphologically similar and phylogenetically closely related *D. vistulae* is a typical generalist in Europe (except Iberia) and Asia, parasitising a multitude of cyprinoid species and genera (Moravec, 2001, Benovics et al., 2018). *Dactylogyrus polylepidis* and *D. vistulae* share remarkably similar morphological traits, including an enlarged seventh pair of marginal hooks, large anchor hooks and a similar size and shape of the copulatory organs (see Pugachev et al., 2009). It has previously been hypothesised that large attachment structures (or structures with variable size and shape) in monogeneans increases the probability of switching to fish species of different body sizes, which is in accordance with the low degree of host specificity observed in *D. vistulae* (e.g. Šimková et al., 2001a, Benovics et al., 2018) and *D. polylepidis* (this study). Compared to endemic cyprinids, endemic leuciscids harbour species poor *Dactylogyrus* communities, though leuciscid *Dactylogyrus* species exhibit a

413 higher degree of host specificity, with most species harbouring at least one specific

414 *Dactylogyrus* species. The majority of new species recorded are morphologically similar, with

415 *Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 10, for example, sharing the 'ergensi' type of male

416 copulatory organ but differing in the shape and size of the haptoral hard parts. Phylogenetic

417 analyses and species delimitation analyses supported their species identities, i.e. nine new

418 species were recognized. Species delimitation has received much attention recently, and

419 numerous methods have now been developed that help identify species by using molecular

420 data in a rigorous framework alongside morphological examination (Carstens et al., 2013,

421 Zhang et al., 2013, Grummer et al., 2014). DNA-based delimitation methods have also been

422 used to confirm or invalidate morphologically determined species, to identify cryptic species

423 or highlight significant intraspecific genetic variability. The aforementioned diversity in

424 haptoral part shape and size appears to be common in *Dactylogyrus* spp. and was previously

425 hypothesised to be the result of adaptations to specific microhabitats (i.e. specific positions on

426 fish gills; Šimková et al., 2001a, Jarkovský et al., 2004). Thus, minor morphological

427 variabilities in the attachment organs may be observed in species with ongoing speciation

428 parasitising phylogenetically distant hosts, as is the case in the Iberian Peninsula.

429 **4.2   Phylogeny of endemic *Dactylogyrus***

430 Phylogenetic reconstruction of *Dactylogyrus* parasitising Iberian cyprinoids suggests that

431 Iberian *Dactylogyrus* belong to two well-supported phylogenetic lineages (Clade A and Clade

432 B; Figure 2). One of these clades contains *Dactylogyrus* from endemic Cyprinidae only

433 (representatives of five *Luciobarbus* species and *Barbus haasi*), while the second includes

434 *Dactylogyrus* endemic to Iberian cyprinoids (both Cyprinidae and Leuciscidae) and

435 *Dactylogyrus* parasitising cyprinoids from other parts of Europe. This was previously reported

436 by Šimková et al. (2017) following analysis of phylogenetic relationships between

437 *Dactylogyrus* from north-west Africa and those from the Iberian Peninsula, the authors

438 suggesting multiple origins for *Dactylogyrus* from both Mediterranean areas in association

439 with the historical biogeography of their cyprinid hosts. Clade B comprises *Dactylogyrus*

440 species described by el Gharbi et al. (1992), using morphological characteristics of the haptor

441 and reproductive organs. According to their study (also supported by our own morphometric

442 data), all these species achieve a small body size and display remarkably similar

443 morphological features (i.e. sclerotised parts of attachment and copulatory organs), in

444 accordance with their phylogenetic proximity. Previously, their description was based on

445 small differences in the shape and size of sclerotised parts only (e.g. spiralisation of the male

446 copulatory organ and size of haptoral sclerites). However, as has been previously

447 documented, such variability may be present within single species and is common in the

448 different monogenean taxa (e.g. Rohde and Watson, 1985, Boeger and Kritsky, 1988, Vignon

449 and Sasal, 2010), including *Dactylogyrus* (Rahmouni et al., 2017). Nonetheless, the species

450 status of each taxon in Clade B was supported by phylogenetic and species delimitation

451 analyses, which was in concordance with their morphological determination. According to

452 Šimková et al. (2017), Iberian *Dactylogyrus* species of this lineage are phylogenetically close

453 to *Dactylogyrus* from north-west African *Carasobarbus fritschii*, suggesting different

454 historical origins of *Dactylogyrus* in Clade B and Clade A. According to previous reports and

455 the data presented here, each *Dactylogyrus* species within Clade B parasitises several endemic

456 *Luciobarbus* species. Considering the monophyletic origin of Iberian *Luciobarbus* (Yang et

457 al., 2015), its probable historical dispersion via northern Africa (Bianco, 1990, Doadrio, 1990,

458 Zardoya and Doadrio 1998), and the phylogenetic relatedness of *Dactylogyrus* from Clade B

459 with north-west African *Dactylogyrus* (Šimková et al., 2017), we may postulate that these

460 species originated on the *Luciobarbus* ancestor, and may have host-switched in the past to

461 endemic north-west African *Carasobarbus*, subsequently dispersing to the Iberian Peninsula

462 during its historical connection with North Africa. The high number of morphologically

463   similar species exhibiting a low molecular divergence (e.g. *D. bocageii, D. mascomai, D.*

464   *guadianensis, D. lenkoranoïdes* and *D. doadrioi*) suggests subsequent rapid speciation, most

465   likely linked with the radiation of *Luciobarbus* across individual river basins within the

466   Iberian Peninsula (Doadrio, 1998, Zardoya and Doadrio, 1998, Doadrio et al., 2002, Mesquita

467   et al., 2007, Gante et al., 2015, Casal-López et al., 2017). Addition of *Dactylogyrus* species

468   from Asian *Capoeta* (phylogenetically sister group to Iberian *Luciobarbus*; Yang et al., 2015)

469   to phylogenetic reconstruction and assessing coevolutionary scenarios involving these

470   parasites and their hosts may shed more light into origin of the *Dactylogyrus* of Iberian

471   *Luciobarbus* and finally resolve the phylogenetic relationships within this group of

472   *Dactylogyrus*.

473   In contrast to *Dactylogyrus* from Clade B, the phylogenetic proximity of Iberian *Dactylogyrus*

474   within Clade A to Central European and Balkan *Dactylogyrus* species supports their European

475   origin. In accordance with the phylogeny proposed by Šimková et al. (2017), *Dactylogyrus*

476   species from Iberian *Luciobarbus* form two well-supported lineages within Clade A, and

477   cluster with *Dactylogyrus* from European *Barbus*. Two species within Clade A, *D. balistae*

478   and *D. legionensis*, have large body-size, large haptoral sclerites and are missing the haptoral

479   connective ventral bar (see el Gharbi et al., 1992). These species form a well-supported clade

480   in sister position with another Iberian species, *D. linstowoïdes*. This clade is closely related to

481   *D. malleus, D. prespensis* and *D. petenyi*, all host-specific parasites to European *Barbus*. In

482   contrast to *D. legionensis* and *D. balistae*, these three species have small body size, similarly

483   shaped small haptoral elements and a ventricular ventral bar (see Pugachev et al., 2009).

484   Based on morphology, *D. linstowoïdes* represents the transient form between these two

485   lineages, with the haptoral sclerites resembling *Dactylogyrus* of European *Barbus* and

486   copulatory organs morphologically similar to Iberian species. Our results support a common

487   origin for these species, with *D. balistae, D. legionensis* and *D. linstowoïdes* possibly

evolving in Iberia from a common ancestor and thereafter switching to *Luciobarbus*, following which *D. balistae* and *D. legionensis* secondarily lost their haptoral connective ventral bar.

In this study, Leuciscids generally harboured poorer *Dactylogyrus* species communities than cyprinids. However, due to the higher species richness of this fish family in the Iberian Peninsula, a remarkably high species diversity was observed among their *Dactylogyrus* parasites, and specifically among *Dactylogyrus* parasitising *Squalius* spp. and the genera erected from *Chondrostoma* s.l.. Almost each genetic variant was supported as a species by the species delimitation analysis. *Dactylogyrus* from Iberian leuciscids formed three major phylogenetic lineages. The first comprised *Dactylogyrus* sp. 1 only, collected from two endemic *Squalius* species, *S. torgalensis* and *S. aradensis*. Previous molecular phylogenetic studies suggested that these sister species have a basal position to other representatives of *Squalius* in Iberia (Sanjur et al., 2003, Waap et al., 2011, Perea et al., 2016, Sousa-Santos et al., 2019). The distribution of *S. torgalensis* and *S. aradensis* is limited to the south-western extremity of the Iberian Peninsula, and the same distribution range was found for *Dactylogyrus* sp. 1. Extrapolating from the phylogenetic reconstruction, *Dactylogyrus* sp. 1 is phylogenetically close to common *Dactylogyrus* species from European *Squalius* spp., i.e. *D. folkmanovae* and *D. nanoides* (hypothesised to be genus specific according to Šimková et al., 2004 and Benovics et al., 2018), and probably represents an ancestral *Dactylogyrus* lineage that has coevolved in Iberia with its endemic *Squalius* hosts.

The majority of endemic leuciscid *Dactylogyrus* formed a well-supported clade, with *D. caucasicus* from *Alburnoides* spp. and *D. ergensi* from *Chondrostoma* spp. in sister position. Benovics et al. (2018) has previously suggested that *D. caucasicus* originated from the ancestor of *D. ergensi* by host-switching to *Alburnoides*. The species delimitation analysis suggested the existence of nine potentially new species (*Dactylogyrus* sp. 2 to *Dactylogyrus*

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sp. 10) phylogenetically related to *D. ergensi* (the species with the widest distribution range across Europe), which may indicate that endemic *Dactylogyrus* sp. 2 to *Dactylogyrus* sp. 10 also share a common ancestor with *D. ergensi*. As suggested by Robalo et al. (2007), the ancestor of *Chondrostoma* s.l. could have dispersed into Iberia prior to the Messinian period, when the host-specific ancestral *Dactylogyrus* species associated with these hosts most likely colonised Iberia. Our data suggest that the rapid radiation of *Chondrostoma*-related species promoted the speciation of their host-specific *Dactylogyrus*. Even if parasite phylogeny is not fully congruent with that of their hosts, all Iberian *Dactylogyrus* species, excluding *Dactylogyrus* sp. 8 (collected from *Parachondrostoma* species only distributed in rivers of the Mediterranean slope (Kottelat and Freyhof, 2007)), parasitise leuciscids in river basins of the Atlantic slope (distribution according to Kottelat & Freyhof, 2007, Robalo et al., 2007, Sousa-Santos et al., 2019). Considering that the distribution of cyprinoid species in Iberia is almost non-overlapping, the incongruence between host and parasite phylogenies could be the result of secondary contacts between fish host species, as recently documented in some Iberian rivers (e.g. Doadrio, 2001, Sousa-Santos et al., 2019). *Dactylogyrus* sp. 7, for example, was collected from two separate species, *S. pyrenaicus* and *S. carolitertii*. Sousa-Santos et al. (2019) and Waap et al. (2011) suggested that *S. pyrenaicus* consists of two different species, each associated with different river basins. Previous multilocus phylogenetic analyses (Sousa-Santos et al., 2019) have supported that *S. pyrenaicus* is paraphyletic, as genetic variants of this species from the Tagus and Colares basins were both grouped with *S. carolitertii*. Exactly the same pattern was observed among genetic variants of *Dactylogyrus* sp. 7, with individuals collected from *S. pyrenaicus* being in paraphyly and individuals from the River Colares grouped with individuals from *S. carolitertii*. A similar situation has also been observed in *Dactylogyrus* spp. from the Balkans, where the phylogenetic positions of two populations of *D. vistulae* within the *D. vistulae* clade (i.e. paraphyly) and molecular dissimilarity between

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538 the two populations (Benovics et al., 2018) supported the existence of two different

539 *Alburnoides* species, as previously proposed by Stierandová et al. (2016).

540 In general, *Dactylogyrus* species diversity within the Iberian Peninsula appears to be

541 associated with the historical dispersion of their cyprinoid hosts, with subsequent adaptive

542 radiation following the peninsula's geographical isolation due to elevation of the Pyrenees

543 (Munoz et al., 1986, Puigdefàbregas et al., 1992; Stange et al., 2016). At least two historical

544 origins can be inferred for Iberian *Dactylogyrus*, each associated with the different dispersion

545 routes proposed for cyprinoids (Banarescu, 1989, 1992, Doadrio, 1990, Doadrio & Carmona,

546 2003, Perea et al., 2010). Despite well-supported delineation between a multitude of endemic

547 *Dactylogyrus* species, the phylogenetic relationships between *Dactylogyrus* species do not

548 fully correspond to the phylogeny of their hosts, suggesting secondary contacts and host-

549 switching between endemic Iberian cyprinoids.

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551

552 **Figure 1.** *Map of collection localities in the Iberian Peninsula*. The same codes are used in

553 Table 1 as locality IDs.

554

555 **Figure 2.** *Phylogenetic tree of 70 Dactylogyrus haplotypes reconstructed by Bayesian*

556 *inference (BI)*. The tree is based on combined parts of genes coding 18S and 28S rRNA, and

557 the complete ITS1 region. Values between branches indicate posterior probabilities from BI

558 and bootstrap values from ML analysis. Values below 0.80 (BI) and 50 (ML) are shown as

559 dashes (-). The letters A–C represent specific well-supported lineages, as described in the

560 Results section.

561

562 **Figure 3.** *Results of species bPTP delimitation analysis applied to clades comprising endemic*

563 *Dactylogyrus*. Brackets at the terminal branches indicate different species, as suggested by BI

564 and ML analyses.

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874

876 **Table 1.** *List of cyprinoid species including localities of their collection and list of collected*

877 *Dactylogyrus species from respective hosts.*

Host species	N	ID	Locality	*Dactylogyrus* species	18S	28S
Achondrostoma arcasii	15	S1	Chico River, flow of Palancia	*D. polylepidis*	MN365664	MN338198
	10	S2	Tera River	*D. polylepidis*	MN365665	MN338199
Achondrostoma occidentale	13	P1	Alcabrichel	*Dactylogyrus* sp. 2	MN365666	MN338200
				Dactylogyrus sp. 10	MN365667	MN338201
Barbus haasi	4	S3	Beceite, Uldemo River	*D. lenkoranoïdes*	MN365668	MN338202
Iberochondrostoma almacai	19	P2	Torgal River, Mira basin	*Dactylogyrus* sp. 3	MN365669	MN338203
Iberocypris alburnoides	12	S4	Near Llera, Retin River	*Dactylogyrus* sp. 5	MN365670	MN338204
Luciobarbus bocagei	6	P3	Colares (Portugal)	*D. balistae*	KY629344	MN338205
				D. bocageii	MN365671	KY629347
	10	S5	Ucero River (Spain)	*D. mascomai*	no seq	MN338206
Luciobarbus comizo	5	S6	Peraleda de Zancejo, Zujar River	*D. andalousiensis*	MN365672	MN338207
				D. bocageii	MN365673	MN338208
				D. guadianensis	MN365674	MN338209
Luciobarbus graellsii	1	S3	Beceite, Uldemo River	*D. legionensis*	MN365678	MN338210
				D. lenkoranoïdes	MN365676	MN338211
	5	S7	upstream Mella, tributary of Materraña	*D. bocageii*	MN365675	MN338212
				D. lenkoranoïdes	MN365677	MN338213
				D. legionensis	MN365679	MN338214
				D. mascomai	MN365680	MN338215
Luciobarbus guiraonis	6	S8	Magro River	*D. bocageii*	MN365681	MN338216
				D. legionensis	KY629330	KY629350
				D. doadrioi	MN365682	KY629346
	4	S9	Turia River	*D. linstowoïdes*	KY629329	KY629349
				D. mascomai	-	KY629348*
Luciobarbus sclateri	5	P2	Torgal River, Mira basin	*D. andalousiensis*	KY629331	KY629351
				D. bocageii	MN365684	MN338218
	10	S10	Benehavis, Guadalmina River	*D. andalousiensis*	MN365683	MN338217
				D. guadianensis	MN365685	MN338219
Parachondrostoma miegii	12	S3	Beceite, Uldemo River	*Dactylogyrus* sp. 8	MN365686	MN338220
Parachondrostoma turiense	18	S9	Turia River	*Dactylogyrus* sp. 8	MN365687	MN338221
Phoxinus bigerri	12	S5	Ucero River	*D. borealis*	MN365688	MN338222
Pseudochondrostoma duriense	9	S5	Ucero River	*Dactylogyrus* sp. 9	MN365689	MN338223
				D. polylepidis	no seq	no seq
Pseudochondrostoma polylepis	10	P4	Alcoa, Fervenca	*Dactylogyrus* sp. 6	MN365690	MN338224
	15	P3	Colares	-	-	-
Squalius aradensis	5	P5	Seixe	*Dactylogyrus* sp. 1	MN365691	MN338225
	6	P6	tributary of Seixe	-	-	-
Squalius carolitertii	15	P7	Arunca, Mondego basin (Vermoil)	*Dactylogyrus* sp. 7	MN365692	MN338226
				Dactylogyrus sp. 11	MN365693	MN338227
				D. polylepidis	-	-
Squalius pyrenaicus	5	P3	Colares	*Dactylogyrus* sp. 7	MN365694	MN338228
	5	S6	Peraleda de Zancejo, Zujar River	*Dactylogyrus* sp. 7	MN365695	MN338229
Squalius torgalensis	10	P2	Torgal River, Mira basin	*Dactylogyrus* sp. 1	MN365696	MN338230
				Dactylogyrus sp. 4	MN365697	MN338231

878 N = number of processed fish individuals from respective locality, ID = code corresponding with localities

879 marked in Figure 1 and codes in following tables, numbers in columns 18S and 28S correspond to sequence

880 accession numbers for the respective genetic markers in GenBank; 18S = sequences of partial gene coding 18S

881 rRNA combined with complete ITS1 region, 28S = sequences or partial gene coding 28S rRNA. Sequence not

882 used in the present study is marked by asterisk (*). Dashes represent localities where no *Dactylogyrus* parasites

883 were collected and/or missing sequences.

885 **Table 2.** *Uncorrected pair-wise genetic distances between individuals from clade B (Figure*

886 *2) collected from different Barbus and Luciobarbus species in Iberian Peninsula.*

No.	*Dactylogyrus* species	Host species	ID	1	2	3	4	5	6	7	8	9	10	11
1	*D. bocageii*	*L. bocageii*	P3	X										
2	*D. bocageii*	*L. comizo*	S6	-	X									
3	*D. bocageii*	*L. graellsii*	S7	0.001	0.001	X								
4	*D. bocageii*	*L. guiraonis*	S8	0.001	0.001	0.002	X							
5	*D. bocageii*	*L. sclateri*	P2	0.001	0.001	0.002	0.002	X						
6	*D. lenkoranoïdes*	*B. haasi*	S3	0.016	0.016	0.017	0.015	0.017	X					
7	*D. lenkoranoïdes*	*L. graellsii*	S3	0.016	0.016	0.017	0.015	0.017	-	X				
8	*D. lenkoranoïdes*	*L. graellsii*	S7	0.016	0.016	0.017	0.015	0.017	-	-	X			
9	*D. guadianensis*	*L. comizo*	S6	0.019	0.019	0.020	0.017	0.020	0.015	0.015	0.015	X		
10	*D. guadianensis*	*L. sclateri*	S10	0.017	0.017	0.019	0.016	0.019	0.014	0.014	0.014	0.001	X	
11	*D. doadrioi*	*L. guiraonis*	S8	0.017	0.017	0.019	0.016	0.019	0.006	0.006	0.006	0.016	0.015	X
12	*D. mascomai*	*L. graellsii*	S7	0.010	0.010	0.011	0.009	0.011	0.009	0.009	0.009	0.011	0.010	0.010

887 Distances are computed from the alignment of partial genes coding 28S rRNA. Identical sequences are marked

888 by dashes (-). ID = code corresponding with localities marked in Figure 1 and specified in Table 1.

889 **Table 3.** *Uncorrected pair-wise genetic distances between individuals from clade B (Figure*

890 *2) collected from different Barbus and Luciobarbus species in Iberian Peninsula.*

No.	*Dactylogyrus* species	Host species	ID	1	2	3	4	5	6	7	8	9	10	11
1	*D. bocageii*	*L. bocageii*	P3	X										
2	*D. bocageii*	*L. comizo*	S6	0.004	X									
3	*D. bocageii*	*L. graellsii*	S7	0.015	0.020	X								
4	*D. bocageii*	*L. guiraonis*	S8	0.018	0.020	0.003	X							
5	*D. bocageii*	*L. sclateri*	P2	0.001	0.003	0.017	0.020	X						
6	*D. lenkoranoïdes*	*B. haasi*	S3	0.095	0.096	0.085	0.088	0.094	X					
7	*D. lenkoranoïdes*	*L. graellsii*	S3	0.095	0.096	0.085	0.088	0.094	-	X				
8	*D. lenkoranoïdes*	*L. graellsii*	S7	0.095	0.096	0.085	0.088	0.094	-	-	X			
9	*D. guadianensis*	*L. comizo*	S6	0.078	0.080	0.070	0.073	0.077	0.094	0.094	0.094	X		
10	*D. guadianensis*	*L. sclateri*	S10	0.078	0.082	0.071	0.074	0.080	0.096	0.096	0.096	0.008	X	
11	*D. doadrioi*	*L. guiraonis*	S8	0.109	0.110	0.096	0.099	0.108	0.031	0.031	0.031	0.101	0.105	X
12	*D. mascomai*	*L. graellsii*	S7	0.109	0.110	0.099	0.101	0.108	0.131	0.131	0.131	0.119	0.123	0.135

891 Distances are computed from complete sequences of ITS1 region. Identical sequences are marked by dashes (-).

892 ID = code corresponding with localities marked in Figure 1 and specified in Table 1.
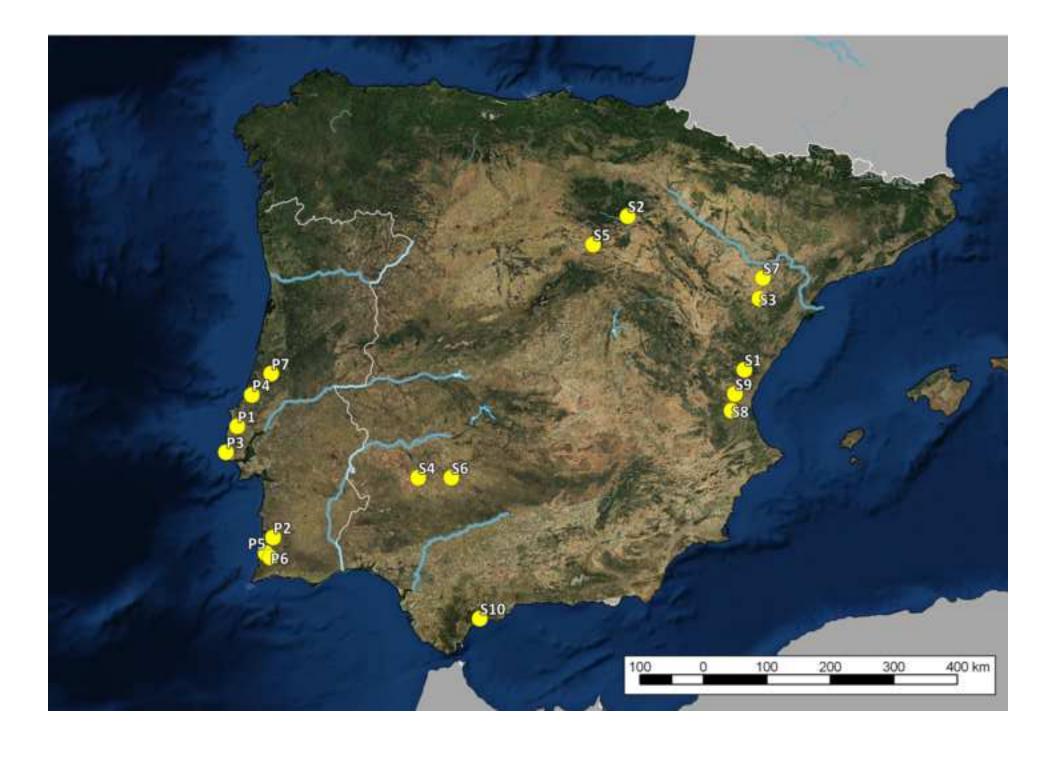
893 **Table 4.** *Uncorrected pair-wise genetic distances between individuals from clade B (Figure*

894 *2) collected from different Barbus and Luciobarbus species in Iberian Peninsula.*

No.	*Dactylogyrus* species	Host species	ID	1	2	3	4	5	6	7	8	9	10	11
1	*D. bocageii*	*L. bocageii*	P3	X										
2	*D. bocageii*	*L. comizo*	S6	-	X									
3	*D. bocageii*	*L. graellsii*	S7	-	-	X								
4	*D. bocageii*	*L. guiraonis*	S8	-	-	-	X							
5	*D. bocageii*	*L. sclateri*	P2	-	-	-	-	X						
6	*D. lenkoranoïdes*	*B. haasi*	S3	0.008	0.008	0.008	0.008	0.008	X					
7	*D. lenkoranoïdes*	*L. graellsii*	S3	0.008	0.008	0.008	0.008	0.008	-	X				
8	*D. lenkoranoïdes*	*L. graellsii*	S7	0.008	0.008	0.008	0.008	0.008	-	-	X			
9	*D. guadianensis*	*L. comizo*	S6	0.002	0.002	0.002	0.002	0.002	0.010	0.010	0.010	X		
10	*D. guadianensis*	*L. sclateri*	S10	0.002	0.002	0.002	0.002	0.002	0.010	0.010	0.010	-	X	
11	*D. doadrioi*	*L. guiraonis*	S8	0.008	0.008	0.008	0.008	0.008	0.004	0.004	0.004	0.010	0.010	X
12	*D. mascomai*	*L. graellsii*	S7	-	-	-	-	-	0.008	0.008	0.008	0.002	0.002	0.008

895 Distances are computed from the alignment of partial genes coding 18S rRNA. Identical sequences are marked

896 by dashes (-). ID = code corresponding with localities marked in Figure 1 and specified in Table 1.

Figure 1

Figure 2

Figure 3

ML BI

D. polylepidis (S1)
D. polylepidis (S2)
D. vistulae
D. alatus
D. sphyrna

1/100
D. borealis (P. bigerri)
D. borealis
D. fallax

D. legionensis (L. graellsii - S3)
D. legionensis (L. graellsii - S7)
D. legionensis (L. guiraonis)
D. balistae
D. linstowoïdes
D. malleus
D. prespensis
D. petenyi

D. andalousiensis (L. comizo)
D. andalousiensis (L. sclateri - P2)
D. andalousiensis (L. sclateri - S10)
D. omenti

Dactylogyrus sp. 1 (S. aradensis)
Dactylogyrus sp. 1 (S. torgalensis)
D. folkmanovae
D. nanoides

Dactylogyrus sp. 2 (A. occidentale)
Dactylogyrus sp. 3 (I. almacai)
Dactylogyrus sp. 4 (S. torgalensis)
Dactylogyrus sp. 5 (I. alburnoides)
Dactylogyrus sp. 6 (P. polylepis)
Dactylogyrus sp. 7 (S. carolitertii)
Dactylogyrus sp. 7 (S. pyrenaicus - P3)
Dactylogyrus sp. 7 (S. pyrenaicus - S6)
Dactylogyrus sp. 8 (P. miegii)
Dactylogyrus sp. 8 (P. turiense)
Dactylogyrus sp. 9 (P. duriense)
Dactylogyrus sp. 10 (A. occidentale)
D. caucasicus
D. ergensi

D. bocageii (L. comizo)
D. bocageii (L. sclateri)
D. bocageii (L. bocagei)
D. bocageii (L. graellsii - S7)
D. bocageii (L. guiraonis)
D. mascomai (L. graellsii - S7)
D. guadianensis (L. comizo)
D. guadianensis (L. sclateri)
D. lenkoranoïdes (B. haasi - S7)
D. lenkoranoïdes (L. graellsii - S3)
D. lenkoranoïdes (L. graellsii - S7)
D. doadrioi (L. guiraonis)