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1 **Cophylogenetic relationships between *Dactylogyrus* (Monogenea)**
2 **ectoparasites and endemic cyprinids of the north-eastern peri-**
3 **Mediterranean region**

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15
16 **Abstract**

17 The study of host-parasite coevolution is one of the cornerstones of evolutionary biology. The
18 majority of fish ectoparasites belonging to the genus *Dactylogyrus* (Monogenea) exhibit a high
19 degree of host specificity. Therefore, it is expected that their evolutionary history is primarily
20 linked with the evolutionary history of their cyprinid fish hosts and the historical formation of
21 the landmass. In the present study, we used a cophylogenetic approach to investigate
22 coevolutionary relationships between endemic Cyprinidae from selected regions in southern
23 Europe and their respective *Dactylogyrus* species. A total of 49 *Dactylogyrus* species including

24 endemic and non-endemic species were collected from 62 endemic cyprinid species in the
25 Balkan and Apennine Peninsulas. However, 21 morphologically identified *Dactylogyrus*
26 species exhibited different genetic variants (ranging from two to 28 variants per species) and
27 some of them were recognized as cryptic species on the basis of phylogenetic reconstruction.
28 Phylogenetic analyses revealed several lineages of endemic and non-endemic *Dactylogyrus*
29 species reflecting some morphological similarities or host affinities. Using distance-based and
30 event-based cophylogenetic methods, we found a significant coevolutionary signal between the
31 phylogenies of parasites and their hosts. In particular, statistically significant links were
32 revealed between *Dactylogyrus* species of Barbini and their hosts belonging to the genera
33 *Aulopyge*, *Barbus* and *Luciobarbus*. Additionally, a strong coevolutionary link was found
34 between the generalist parasite *D. vistulae* and its hosts, and between *Dactylogyrus* species of
35 *Pachychilon* and their hosts. Our cophylogenetic analyses suggest that host-switching played
36 an important role in the evolutionary history of *Dactylogyrus* parasitizing endemic cyprinids in
37 southern Europe. We propose that the high diversification of phylogenetically related cyprinid
38 species in the Mediterranean area is a process facilitating the host switching of specific parasites
39 among highly diverse congeneric cyprinids.

40

41 **Introduction**

42 Host-parasite coevolution plays an important role in the processes of parasite speciation and
43 represents one of the most fascinating topics in evolutionary biology (Poulin, 2007). If the host
44 specificity of the parasite is high (i.e. a parasite species restricted to a single host species or
45 very few closely-related host species) it is tempting to assume that the evolution of parasitic
46 organisms is associated with the evolution of their hosts (Ronquist, 1997). Hence, the
47 Fahrenholz rule (Stammer, 1957, Brooks & McLennan, 1993) states that parasite phylogeny
48 mirrors host phylogeny, and that cospeciation drives host-parasite coevolution. Congruent host-

49 parasite phylogenies has usually been inferred when the host-switching of parasites is
50 impossible or highly improbable, such as in the case of chewing lice and pocket gophers, where
51 parasite cospeciation likely resulted from an allopatric distribution of hosts and host-switching
52 was supported only in the case of physical contact between two gopher species (Hafner &
53 Nadler, 1988, Hafner et al., 1994, Page, 1996). However, the whole concept of the “Fahrenheit
54 rule” has been re-evaluated and several studies have suggested that cospeciation is not always
55 the predominant driver of parasite speciation during reciprocal host-parasite evolution. Host-
56 switching (Klassen, 1992) and parasite duplication, i.e. parasite speciation within a host lineage
57 (Johnson, Adams, Page & Clayton, 2003), play significant roles in parasite evolution, often
58 resulting in incongruent host and parasite phylogenies (e.g. Desdevises, Morand, Jousson &
59 Legendre, 2002, Šimková, Morand, Jobet, Gelnar & Verneau, 2004, Šimková, Serbielle,
60 Pariselle, Vanhove & Morand, 2013, Mendlová, Desdevises, Cívánová, Pariselle & Šimková,
61 2012). Despite the fact that frequent host-switching during the evolutionary history of parasite
62 taxa usually results in incongruent host-parasite phylogenies, a series of multiple host-switches
63 followed by parasite speciation can generate trees with similar topologies (de Vienne, Giraud
64 & Shykoff, 2007). Therefore, the independent estimation of the age of speciation events in host
65 and parasite trees should also be taken into account when interpreting the outputs of
66 cophylogenetic analyses.

67 *Dactylogyrus* Diesing, 1850 (Monogenea) are gill parasites generally exhibiting narrow host
68 specificity and high morphological variability with respect to their attachment organ (termed
69 haptor), putatively reflecting adaptations to their different host species or within-host
70 microhabitats (Gibson Timofeeva & Gerasev, 1996, Šimková, Desdevises, Gelnar & Morand,
71 2000, 2001, Šimková, Verneau, Gelnar & Morand, 2006b, Šimková & Morand, 2008). In
72 addition, *Dactylogyrus* currently represents the platyhelminth genus with the highest species
73 diversity (more than 900 described species according to Gibson et al., 1996), certainly largely

74 underestimated as new species have recently been described (e.g. Aydogdu, Molnár, Emre &
75 Emre, 2015, Nitta & Nagasawa, 2016, Benovics, Kičinjaová & Šimková, 2017, Rahmouni,
76 Řehulková, Pariselle, Rkhami & Šimková, 2017). This high species richness in *Dactylogyrus*
77 is associated with their narrow host specificity toward a single host species or closely-related
78 species, and with a high diversity of their host species – primarily freshwater fish in the family
79 Cyprinidae (or Cyprinodei considering recent phylogenetic studies, e.g. Schönhuth, Vukić,
80 Šanda, Yang & Mayden, 2018). Previous studies have suggested that each cyprinid species
81 harbour at least one *Dactylogyrus* species (Dupont & Lambert, 1986, Gibson et al., 1996,
82 Moravec, 2001, Galli, Stefani, Zaccara & Crosa, 2002). In regards to host specificity, Šimková
83 et al. (2006b) classified five groups of *Dactylogyrus* species ranging from strict specialists,
84 living on a single host species, to generalists parasitizing host species from different
85 phylogenetic lineages. The high host specificity of *Dactylogyrus* (and other monogeneans) is
86 linked with their direct life cycle, where the larva (oncomiracidium) actively searches for a
87 suitable (specific) host and attaches directly to the gills or surface. Oncomiracidia are sensitive
88 to chemical cues from hosts which can either initiate the hatching of oviparous species, attract
89 larvae, or initiate larva deciliation (Buchmann & Lindenstrøm, 2002). The recognition of these
90 signals most likely requires specific parasite adaptation (Buchmann, 1999, Whittington &
91 Kearn, 2011).

92 Their narrow host specificity and expected host-parasite coevolution make monogeneans
93 potential proxies for the study of the evolution and dispersion of their hosts. Previous studies
94 (on *Lamellodiscus* Johnston & Tiegs, 1922 parasitizing Sparidae (Desdevises et al., 2002),
95 *Gyrodactylus* von Nordmann, 1832 parasitizing Gobiidae (Huyse, Audenaert & Volckaert,
96 2003, Huyse & Volckaert, 2005, Huyse, Oeyen, Larmuseau & Volckaert, 2017), *Cichlidogyrus*
97 Paperna, 1960 and *Scutogyrus* Pariselle & Euzet, 1995 parasitizing Cichlidae (Mendlová et al.,
98 2012), and *Thaparocleidus* Jain, 1952 parasitizing Pangasiidae (Šimková et al., 2013))

99 suggested that cophylogenetic patterns between monogeneans and their hosts are complex,
100 involving less cospeciation than expected and a putatively high number of host switches,
101 duplications, and losses. Frequent host-switching in these systems may be expected because of
102 the active dispersion of the larvae and the capacity of adults to survive outside of the hosts for
103 a short period of time (Brooks & McLennan, 1991, Bakke Cable & Harris, 2007), potentially
104 allowing them to infect phylogenetically closely-related host species with similar ecological
105 requirements.

106 In spite of the large interest in host-specific monogeneans, few phylogenetic and/or
107 cophylogenetic studies have been performed for *Dactylogyrus*. In *Dactylogyrus* from central
108 European cyprinids, intrahost duplication was inferred as a more widespread diversification
109 process than host-switching (Šimková et al., 2004). Several coevolutionary scenarios were
110 proposed by Benovics et al. (2017), Benovics, Desdevises, Vukić, Šanda and Šimková (2018),
111 and Šimková, Benovics, Rahmouni and Vukić (2017) regarding *Dactylogyrus* and peri-
112 Mediterranean endemic cyprinids, the last one hypothesizing that Iberian cyprinids harbour
113 *Dactylogyrus* species originating from two different colonization events.

114 Southern European freshwater fauna is extremely rich in endemic cyprinid species (Kottelat &
115 Freyhof, 2007). For instance, the Balkan Peninsula is considered a hotspot of endemic
116 freshwater diversity and harbours 59 % of all European cyprinid species (Sušnik, Snoj, Wilson,
117 Mrdak & Weiss, 2007, Abell et al., 2008, Albrecht & Wilke, 2008, Schultheiss, Albrechts,
118 Bossneck & Wilke, 2008, Oikonomou, Leprieur & Leonardos, 2014), which have recently
119 become the common interest of ichthyologists (e.g. Marková et al., 2010, Gante, 2011, Perea,
120 Vukić, Šanda & Doadrio, 2016, Stierandová et al., 2016, Buj et al., 2017). According to Bianco
121 (1990), the Balkans are divided into 4 ichthyogeographical districts, each identified by the
122 presence of unique cyprinid species or lineages. One of them, the Padano-Venetian
123 ichthyogeographic district, partially covers the Balkan Peninsula and also includes the north-

124 eastern part of the Apennine Peninsula; therefore, several cyprinid species are shared between
125 these two geographical regions. In comparison to the species-rich Balkan Peninsula, only 14
126 endemic cyprinid species have been described from the Apennine Peninsula (Bianco, 1995).
127 Since most of this Peninsula was below the sea level during most of the Miocene era, it is
128 assumed that the Apennine ichthyofauna is of more recent origin in comparison to the
129 ichthyofauna in other southern European Peninsulas (Steininger & Rögl, 1984). The northern
130 part of the Apennine Peninsula is divided into two districts: the Tuscano-Latinum district which
131 corresponds to the distribution of *Squalius lucumonis*, and the Padano-Venetian district which
132 corresponds to the Po River basin during the last glacial maxima (Bianco, 1990). The native
133 Apennine ichthyofauna has been significantly influenced and threatened by the introduction of
134 numerous fish species (26 introduced species according to Bianco (1995)), which led to the
135 simultaneous introduction of their non-native parasite species (such as *Dactylogyrus*,
136 documented in Benovics et al., 2017). In general, Apennine cyprinids are phylogenetically more
137 related to Balkan cyprinids than to central European or Iberian species (Perea et al., 2010), as
138 cyprinid species from the northern part of the peri-Adriatic River system (confined to the Po
139 River basin in Italy and the Soča River basin in Slovenia) and recent Balkan species show a low
140 degree of molecular divergence (Sušnik, Snoj & Dovc, 2001, Perea et al., 2010). A possible
141 explanation is that during the last glacial maximum, the Po basin expanded and connected the
142 Italian and Balkan river systems, which led to the mixing of many primary native fish species
143 (Waelbroeck et al., 2002, Stefani, Galli, Crosa, Zaccara & Calamari, 2004).

144 Since cophylogenetic patterns and processes between peri-Mediterranean cyprinids and their
145 *Dactylogyrus* parasites are poorly known, we aimed to study the cophylogeny of these two
146 groups in selected southern European regions and to elucidate the historical dispersion of
147 endemic cyprinids using *Dactylogyrus* phylogeny. Therefore, the objectives of this study were
148 (1) to reconstruct the evolutionary histories of Balkan and Apennine endemic cyprinids and

149 their endemic *Dactylogyrus* in order to clarify the relationships between recent lineages, and
150 (2) to investigate the speciation patterns of host specific *Dactylogyrus* and to assess whether
151 parasite phylogeny is linked to host phylogeny and the historical formation of the landmass, or
152 rather to the recent distribution and introduction of non-native species into the investigated
153 regions.

154

155 **Material and Methods**

156 **Material collection and fixation**

157 Between 2014 and 2017, 76 cyprinid species were sampled from 56 localities across the Balkan
158 and Apennine Peninsulas (Table 1). A fin clip was obtained from all fish individuals and
159 preserved in 96 % ethanol. Fish were dissected using standard methods described by Ergens
160 and Lom (1970). *Dactylogyrus* parasites were collected from the gills and nasal cavity, mounted
161 on slides, and fixed using a mixture of glycerine and ammonium picrate (GAP, Malmberg
162 1957). Species determination was performed according to the size and shape of the sclerotized
163 hard parts of the haptor and the reproductive organs (male copulatory organ and vaginal
164 armament) using Pugachev, Gerasev, Gussev, Ergens & Khotenowski (2009). Identification at
165 the species level was performed using an Olympus BX51 microscope equipped with phase
166 contrast optics. Several representatives of each collected *Dactylogyrus* species were bisected
167 using fine needles. One half of the body (containing the reproductive organs) was mounted on
168 a slide, while the other was individually preserved in 96 % ethanol for further DNA extraction.

169

170 **DNA extraction, amplification, and sequencing**

171 Bisected *Dactylogyrus* individuals preserved in ethanol were dried using a vacuum centrifuge.
172 DNA extraction was performed following the standard protocol (DNeasy Blood & Tissue Kit,
173 Qiagen, Hilden, Germany). For molecular analyses, four genetic markers commonly applied

174 for monogeneans were used. Partial 18S rDNA, the entire ITS1 region, and partial 5.8S rDNA
175 were amplified using the primers S1 (forward, 5'-ATTCCGATAACGAACGAGACT-3') and
176 IR8 (reverse, 5'-GCTAGCTGCGTTCTTCATCGA-3'), which anneal to the 18S and 5.8S
177 rDNA regions respectively (Šimková, Plaisance, Matějusková, Morand & Verneau, 2003); PCR
178 followed the protocol optimized in Benovics et al. (2018). Partial 28S rDNA was amplified
179 using primers C1 (forward, 5'-ACCCGCTGAATTTAAGCA-3') and D2 (reverse, 5'-
180 TGGTCCGTGTTTCAAGAC-3') following Hassouna, Michot & Bachellerie (1984); PCR
181 followed the protocol optimized in Šimková, Matějusková and Cunningham (2006a). The PCR
182 products were checked on 1 % agarose gel and purified using the ExoSAP-IT kit (Ecoli,
183 Bratislava, SK) following the standard protocol. The purified products were directly sequenced
184 using the PCR primers and BigDye Terminator Cycle Sequencing kit (Applied Biosystems,
185 Foster City, CA). Sequencing was performed on an ABI 3130 Genetic Analyzer (Applied
186 Biosystems).

187 For fish DNA extraction, fin clips were removed from the ethanol and dried, and the
188 JETQUICK Tissue DNA Spin Kit (GENOMED) was applied following manufacturer's
189 instructions. The complete mtDNA cytochrome *b* gene was amplified using primers GluF
190 (forward, 5'-AACCACCGTTGTATTCAACTACAA-3') and ThrR (reverse, 5'-
191 ACCTCCGATCTTCGGATTACAAGACCG-3') according to Machordom and Doadrio
192 (2001a). The PCR reaction settings, amplification protocol, and PCR product purification
193 followed Šanda et al. (2008). The sequencing of the cyprinid cytochrome *b* gene was carried
194 out by the Macrogen Service Centre (Seoul, South Korea) using the PCR primers.

195 The new sequences for parasites and hosts obtained during this study were deposited in
196 GenBank (see Tables 1 and 2 for accession numbers). For *Dactylogyrus*, whole sequences
197 including the partial 18S rDNA and ITS1 regions were deposited in GenBank.

198

199 **Phylogenetic reconstruction**

200 DNA sequences of hosts and parasites were aligned using fast Fourier transform in MAFFT
201 (Kato, Misawa, Kuma & Miyata, 2002). The new sequences of *Dactylogyrus* (Table 2) were
202 trimmed to concur with the length of sequences from GenBank (see Table 2).

203 Gaps and ambiguously aligned regions were removed from the alignment of *Dactylogyrus*
204 sequences using GBlocks v. 0.91 (Talavera & Kastresana, 2007). The most appropriate DNA
205 evolutionary model was determined using the Bayesian information criterion (BIC) with
206 jModelTest 2.1.10 (Guindon & Gascuel, 2003, Darriba, Taboala, Doallo & Posada, 2012).
207 Phylogenetic trees were inferred by means of Bayesian inference (BI) and Maximum
208 Likelihood (ML) using MrBayes 3.2 (Ronquist et al., 2012) and RaxML v8.1.X (Stamatakis,
209 2014), respectively. BI trees were constructed using the Metropolis-coupled Markov chain
210 Monte Carlo algorithm, with 2 parallel runs of 1 cold and 3 hot chains, 10^7 generations, and
211 trees sampled every 100 generations. 30 % of all saved trees were discarded as burn-in after
212 checking that the standard deviation split frequency value fell below 0.01. Convergence was
213 assessed using Tracer v.1.6 (Rambaut, Drummond, Xie, Baele & Suchard, 2018). Posterior
214 probabilities (PP) were calculated as the frequency of samples recovering any particular clade.
215 The clade support for ML trees (bootstrap support, BS) was assessed by 1000 bootstrap
216 pseudoreplicates.

217 The phylogenetic reconstruction of 49 *Dactylogyrus* species was based on concatenated partial
218 18S and 28S rDNA sequences. The resulting phylogram was rooted by *Dactylogyrus* species
219 from *Carassius gibelio* (Bloch, 1782) and *Cyprinus carpio* L., following Šimková et al. (2004).
220 Data were treated as partitioned and the optimal evolutionary model was selected for each
221 marker individually, including the alpha parameter of the gamma distribution (G) accounting
222 for rate heterogeneity across sites and/or the proportion of invariable sites (I). The phylogenetic
223 reconstruction of 76 cyprinid species based on the complete cytochrome *b* gene was rooted

224 following Mayden et al. (2009), using the outgroup comprising four representatives of the
225 family Cobitidae (*Cobitis jadonaensis* Mustafić & Mrakovčić, 2008 (KP208162.1), *C. illyrica*
226 Freyhof & Stelbrink, 2007 (KJ487484.1), *C. narentana* Karaman, 1928 (KP208170.1) and *C.*
227 *elongata* Heckel & Kner, 1858 (EF672382.1)). Host sequence data were treated as codon
228 partitioned and optimal evolutionary models were selected independently for each position
229 within the codon, including both gamma distribution and the proportion of invariable sites.

230

231 **Cophylogenetic analyses**

232 The tanglegram connecting host and parasite phylogenetic trees via host-parasite associations
233 was built with TreeMap 3.0b (Charleston, 2012). From many existing methods to investigate
234 the congruence between parasite and host phylogenies (de Vienne et al., 2013), a distance-based
235 method and an event-based method were used in the present study. ParaFit (Legendre,
236 Desdevises & Bazin, 2002), implemented in CopyCat (Meier-Kolthoff, Auch, Huson & Göker,
237 2007), was used with patristic distances calculated for each host and parasite phylogeny, and
238 999 permutations to assess the statistical significance of global and individual coevolutionary
239 links. The event-based analysis was performed with Jane 4.0 (Conow, Fielder, Ovadia &
240 Libeskind-Hadas, 2010), which allows different costs to be set for each of the five
241 coevolutionary events (i.e. cospeciation, duplication, duplication followed by host switch, loss,
242 and failure to diverge where host speciation is not followed by parasite speciation). Eleven
243 models with different event cost schemes were applied, using 500 generations and a population
244 size of 50 as parameters of the genetic algorithm to assess the influence of each type of
245 evolutionary event. The Jane 4.0 default model, TreeMap default model (Charleston, 1998),
246 and TreeFitter default model (Ronquist, 1995) were included in our analyses following Deng
247 et al. (2013). Each of these default models assumes that cospeciation has the lowest cost (i.e. is
248 the most common evolutionary event). Several additional models were included in the

249 cophylogenetic analyses: TreeFitter models adjusted for host-switch and codivergence,
250 respectively; a model with equal weights for coevolutionary events following Mendlová et al.
251 (2012); and five models where each event is alternatively extremely penalized (cost set to 10,
252 following Deng et al., 2013). To statistically test whether the global reconstruction cost was
253 significantly lower than expected by chance, 500 randomizations were performed with the use
254 of random parasite trees.

255

256 **Results**

257 **Parasite phylogeny**

258 *Dactylogyrus* parasites were collected from 62 cyprinid species (Table 1). A total of 49
259 *Dactylogyrus* species (Table 2) were identified on the basis of morphological markers
260 (Pugachev et al., 2009). Genetic variability was observed among individuals of *Dactylogyrus*
261 species collected from multiple host species and, therefore, all genetic variants were included
262 in the final sequence alignment. The final 1,177 base-pair-long alignment of the 49 putative
263 *Dactylogyrus* species included 138 concatenated sequences of 18S rDNA combined with partial
264 28S rDNA. The following optimal evolutionary models were selected: TrNef+I for the 441 bp-
265 long partial 18S rDNA sequence alignment and TVM+I+G for the 736 bp-long partial 28S
266 rDNA sequence alignment. BI and ML analyses generated trees with identical topologies (the
267 BI tree is shown in Fig. 1). Morphological and molecular data suggested the presence of 10
268 potentially new species, labelled from *Dactylogyrus* sp. 1 to *Dactylogyrus* sp. 10. The
269 phylogenetic reconstruction divided *Dactylogyrus* species into 3 well-supported groups (A, B
270 and C in Fig. 1). The *D. rarissimus* group, which displayed a high level of intraspecific
271 variability (12 genetic variants), formed a sister group to these three large clades, but the
272 monophyly of *D. rarissimus* was not supported (PP = 0.49, BS = 51, respectively). The first
273 clade (group A, PP = 0.98, BS = 76) included *D. erhardovae*, *D. cabelleri* and *D. crucifer*.

274 These three species are common parasites of *Rutilus* spp. The second group (group B, PP = 1,
275 BS = 74) comprised the majority of *Dactylogyrus* species. Within this group, *Dactylogyrus*
276 species were divided into eight moderately to well-supported lineages. The monophyly of five
277 *Dactylogyrus* species was not supported. Different genetic variants of *D. ergensi* collected from
278 six *Chondrostoma* spp. clustered with *D. dirigerus* (a parasite of *Chondrostoma* spp.), *D.*
279 *caucasicus* and *D. tissensis* (both parasites of *Alburnoides* spp., lineage 1). All four
280 abovementioned species share a similar shape of male copulatory organ (see Pugachev et al.,
281 2009 for morphology). Each of the four species *D. balkanicus*, *D. dyki*, *D. folkmanovae*, and *D.*
282 *petenyi* contains morphologically similar but genetically different individuals (different genetic
283 forms of the given *Dactylogyrus* species parasitized different host species). However, such
284 different genetic forms of each abovementioned morphologically identified species did not
285 form monophyletic groups. The well-supported lineage 3 (PP = 1, BS = 100) comprised all
286 genetic variants of *D. dyki*, a common parasites of *Barbus* spp. in Europe, but also included
287 individuals of *D. balkanicus* resulting in the paraphyly of both species. Both *Dactylogyrus*
288 species from *Luciobarbus* (*Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 3) formed the well-
289 supported lineage 4. Two potentially new species collected from *C. knerii* and *S. tenellus*
290 (*Dactylogyrus* sp. 4 and *Dactylogyrus* sp. 5, respectively) clustered with *D. nanoides* from
291 *Squalius* spp. and *D. rysavyi*, a known parasite of *Alburnoides* spp. (but collected only from *A.*
292 *thessalicus* in this study). The phylogenetic proximity of the four abovementioned species
293 (lineage 5) was well supported by BI, but only weakly by ML (PP = 0.99, BS = 56). Lineage 6
294 exclusively comprised potentially new *Dactylogyrus* species collected from *Telestes* spp.
295 (*Dactylogyrus* sp. 6, *Dactylogyrus* sp. 7 and *Dactylogyrus* sp. 8). The monophyly of all *D.*
296 *petenyi* genetic variants was not supported (lineage 7). Lineage 8 within group B was formed
297 by *Dactylogyrus* species from *Pachychilon* spp. (PP = 1, BS = 95). The third well-supported
298 group (group C, PP = 1, BS = 91) included *D. alatus*, *D. sphyrna* and *D. vistulae*. Finally, all

299 28 genetic variants of *D. vistulae* collected from 25 cyprinid species from 7 genera formed a
300 well-supported clade (PP = 1, BS = 100).

301

302 **Host phylogeny**

303 The alignment of complete cytochrome *b* sequences was used for phylogenetic analyses of
304 cyprinid hosts. All investigated cyprinid species were included in the phylogenetic
305 reconstruction. Five species (*Barbus peloponnesius*, *B. prespensis*, *S. prespensis*, *S. squalus* and
306 *S. vardarensis*) showed interpopulation variability (each cyprinid species was reported in two
307 localities). Two haplotypes for each of these five species were included in the analyses. In
308 contrast, five species (*Alburnus neretvae*, *Chondrostoma vardarensis*, *Pachychilon pictum*,
309 *Pelasgus thesproticus* and *S. tenellus*) exhibited no interpopulation variability and, therefore,
310 only one haplotype from each of these species was included in the analyses. The final alignment
311 contained 85 sequences with 1140 unambiguous nucleotide positions. GTR+I+G was selected
312 as the best evolutionary model for each position within the codon. Both BI and ML analyses
313 yielded trees with congruent topologies and, therefore, only the phylogram resulting from BI
314 was used (Figure 2). Except for *Barbus* and *Telestes* (Bonaparte, 1837), all cyprinid genera
315 formed a well-supported monophyletic group (PP = 1, BS = 100). In general, phylogenetic
316 relationships between the respective leuciscin clades (genera) were in congruence with the
317 molecular phylogeny proposed by Perea et al. (2010) (e.g. *Telestes* formed a well-supported
318 monophyletic group with *Phoxinellus* Heckel, 1843 and *Chondrostoma* Agassiz, 1832;
319 *Delminichthys* Freyhof, Lieckfeldt, Bogutskaya, Pitra & Ludwig, 2006 and *Pelasgus* Kottelat
320 & Freyhof, 2007 formed a well-supported group, and the *Phoxinus* Rafinesque, 1820 clade
321 displayed a basal position to other leuciscins). The tribe Barbini formed a strongly supported
322 group in sister position to leuciscins. However, the monophyly of the genus *Barbus* was only

323 weakly supported by both analyses (PP = 0.68, BS = 56). According to the present dataset, *A.*
324 *huegelii* is phylogenetically closer to the clade comprising *Luciobarbus* spp.

325

326 **Cophylogeny**

327 BI phylogenetic reconstructions were used for cophylogenetic analyses (Figure 3). The
328 distance-based analysis using ParaFit yielded a highly significant ($P < 0.001$) overall
329 cophylogenetic structure. Out of 138 host-parasite individual links, 65 contributed significantly
330 to the global cophylogenetic structure ($P < 0.05$). Significant links ($P < 0.05$) were inferred
331 between the representatives of group C (*D. alatus*, *D. sphyrna* and *D. vistulae* or their genetic
332 variants, Figure 1) and their leusiscin host species, and between *Dactylogyrus* representatives
333 belonging to lineage 8 (*D. martinovici*, *D. petkovici* and *Dactylogyrus* sp. 10) and their
334 *Pachychilon* hosts. Highly significant individual links ($P < 0.001$) were found between
335 representatives of the genera *Barbus* and *Luciobarbus* and the monotypic *Aulopyge* and their
336 *Dactylogyrus* spp. (or genetic forms of these *Dactylogyrus*): “*D. balkanicus*”, *D. crivellius*, “*D.*
337 *dyki*”, “*D. petenyi*”, “*D. prespensis*” from *Barbus*, undescribed *Dactylogyrus* sp. 2 and
338 *Dactylogyrus* sp. 3 from *Luciobarbus* spp., and *D. omenti* from *A. huegelii*. Subsequent analysis
339 performed using the same number of permutations (999) and focused only on this group
340 supported the initial significant cophylogenetic structure ($P < 0.05$).

341 Applying different cost schemes, Jane produced reconstructions with similar proportions of
342 coevolutionary events (Table 3). Global costs using each scheme were all statistically
343 significant ($P < 0.01$). In general, it appears that *Dactylogyrus* speciation is primarily driven by
344 duplication followed by host-switching, which was an important component in 8 of the 11
345 models tested. The lowest total cost was produced by the host-switch-adjusted TreeFitter model.
346 The duplication-prohibited model and host-switch-prohibited model (also suggesting the
347 importance of host switching in the evolution of *Dactylogyrus*) resulted in a high number of

348 loss events and represented the scenarios with the highest total costs. Setting the duplication
349 cost to zero and equalizing the costs of the other events (codivergence adjusted TreeFitter
350 model) or extremely penalizing cospeciation cost (cospeciation prohibited model) resulted in a
351 higher occurrence of duplication events compared to cospeciation events in contrast to a
352 relatively low occurrence of duplication events within each of the other models. Additionally,
353 no losses were inferred in these models (models 4, 6 and also 9, Table 3). A high number of
354 cospeciations were inferred in models with the cospeciation cost set to zero or in models with
355 a high penalization of duplication, host switching, or failure to diverge. A low occurrence of
356 duplication events was found either when cospeciation was not penalized (TreeMap default
357 model), or when failure to diverge or duplication were highly penalized (FTD prohibitive model
358 and duplication prohibitive models, respectively). In the latter model, a remarkably high
359 number of losses were inferred (such as in the case of the host-switch prohibited model).

360 Applying the same cost schemes with the same number of generations and population size on
361 a selected subgroup of cyprinids belonging to the Barbini tribe and their respective specific
362 *Dactylogyrus* spp., between which a strong cophylogenetic signal was initially detected,
363 resulted in only five schemes yielding cophylogenetic scenarios with statistically significant
364 global costs (tested on 500 randomizations, Table 4). Three of these five models (schemes 1, 3
365 and 6) were set to expect duplication followed by host-switching as the least probable
366 coevolutionary event simulating the allopatric speciation of hosts where the host-switching of
367 parasites between new lineages is unlikely (an example of a cophylogenetic scenario from this
368 subsequent dataset is presented in Figure 4). Nevertheless, in the majority of scenarios,
369 duplication followed by host-switching was the most common coevolutionary event. This event
370 was omitted only in the case of its extremely high penalization, modelling the scenario where
371 physical contact between congeneric host species should be completely excluded. Equalizing
372 all event costs, or highly penalizing other coevolutionary events when compared to duplication

373 followed by host-switching resulted in the same proportions of coevolutionary events.
374 However, the results of all these cost schemes were not statistically significant.

375

376 **Discussion**

377 **Phylogeny of *Dactylogyrus***

378 Following the former phylogenetic study by Benovics et al. (2018) focussed on 53 *Dactylogyrus*
379 species parasitizing endemic cyprinids in the Balkans, this work is the first wide-ranging study
380 focusing on the cophylogenetic relationships between endemic cyprinids of the peri-
381 Mediterranean and their specific parasites. In the present study, a large dataset of 76 endemic
382 cyprinid species covering 95 % of the known cyprinid diversity of the whole north-eastern peri-
383 Mediterranean region (Balkan and Apennine Peninsulas) was used. A total of 49
384 morphologically identified *Dactylogyrus* species were recognized, representing 139 genetic
385 variants. In the majority of host-parasite associations, *Dactylogyrus* species were specific to a
386 single cyprinid species or to a group of congeneric cyprinids. For many *Dactylogyrus* species
387 parasitizing more cyprinid species, i.e. generalists, different genetic variants of morphologically
388 identical *Dactylogyrus* species were reported. In the majority of cases even these genetic
389 variants exhibited host specificity.

390 The phylogenetic position of *D. rarissimus* is in congruence with the findings of Benovics et
391 al. (2018), where this species represented a sister group to other *Dactylogyrus* from leuciscins.
392 However, the monophyly of this taxon was only weakly supported by ML analysis and
393 unsupported by BI. In contrast to the previous study by Benovics et al. (2018), our results
394 suggest the monophyly of three *Dactylogyrus* species common to *Rutilus* spp. (*D. caballeroi*,
395 *D. crucifer* and *D. erhardovae*, group A). The monophyly of the former two species was also
396 suggested by Šimková et al. (2004).

397 Group B, recognized from phylogenetic reconstruction, contained several well-to-moderately
398 supported clades. However, several *Dactylogyrus* species, formerly recognized on the basis of
399 morphology, were not phylogenetically supported as monophyletic. These species include *D.*
400 *ergensi*, *D. folkmanovae*, *D. dyki*, *D. balkanicus* and *D. petenyi*. The monophyly of *D. ergensi*
401 was not supported, as *D. caucasicus* collected from *Alburnoides* spp. was included in the well-
402 supported group comprising all *D. ergensi* individuals. However, two well supported groups
403 that follow the biogeographical distribution of cyprinid hosts were formed by *D. ergensi*
404 individuals (Figure 1). *Dactylogyrus ergensi* lineage 1, a sister group to *D. caucasicus*, included
405 individuals found on *Protochondrostoma genei*, *S. lucumonis* and *S. squalus*, all cyprinid
406 species native to the central/northern Adriatic and neighbouring Padano-Venetian
407 ichthyogeographic districts (Bianco, 1990). The other clade, *D. ergensi* lineage 3, contained the
408 genetic forms of *D. ergensi* collected from *C. ohridana* and *C. vardarensis*, both endemic to the
409 southern Balkans, specifically to the Albanian and north-eastern Aegean ichthyogeographic
410 districts (Kottelat & Freyhof, 2007). The present data suggest that *D. ergensi* encompasses
411 several species. In fact, the morphometric variability in the shape and size of the male
412 copulatory organ of *D. ergensi* from the *Chondrostoma* spp. in different regions of Europe was
413 reported in its original description by Gussev (1966). Later, Lambert (1977) proposed the
414 splitting of *D. ergensi* by separating *D. toxostomi* (parasitizing *C. toxostoma*), but its taxonomic
415 status was not considered valid since measurements of the sclerotized parts of the attachment
416 organ and male copulatory organ overlapped with *D. ergensi* individuals (Pugachev et al.,
417 2009). Therefore, on the basis of the present molecular data we can conclude that *D. ergensi*,
418 originally described as a parasite of *Chondrostoma* spp. (although its presence was also
419 documented on *Squalius* spp. in the Apennines), is in fact a species complex. Our results also
420 suggest that *D. caucasicus* evolved from *D. ergensi* by host switching to the phylogenetically
421 distant *Alburnoides* Jettles, 1861 species (Perea et al., 2010, Schönhuth et al., 2018), since

422 both of these *Dactylogyrus* species have a similar shape with respect to the male copulatory
423 organs (see Pugachev et al., 2009).

424 The previous phylogenetic reconstruction of *Dactylogyrus* performed by Šimková et al. (2004)
425 was focused on the species parasitizing central European cyprinids. Our study confirmed most
426 of the phylogenetic relationships between *Dactylogyrus* species previously suggested in their
427 study. For example, the sister species *D. minor* and *D. parvus* parasitizing *A. alburnus* L. in
428 Central Europe were also found on *A. scoranza* in the Balkans. *Dactylogyrus izjumovae*, *D.*
429 *difformis* and *D. difformoides*, all parasites of *Scardinius erythrophthalmus* L. in Central
430 Europe, formed a monophyletic group also reported in the phylogenetic reconstruction of
431 *Dactylogyrus* parasitizing endemic Balkan cyprinids, more specifically *S. plotizza* and *S.*
432 *dergle*. Congruency was also reported in the sister position of *D. prostaе* of the clade formed
433 by *Dactylogyrus* from *Scardinius* Bonaparte, 1837. The present results suggest that *D. nanoides*
434 is phylogenetically closer to the new *Dactylogyrus* species from *Chondrostoma knerii* and *S.*
435 *tenellus* (*Dactylogyrus* sp. 4 and sp. 5 respectively) and to *D. rysavi* rather than to *D.*
436 *folkmanovae* (as was shown in the phylogenetic reconstruction of *Dactylogyrus* parasitizing
437 Central European cyprinids by Šimková et al., 2004). However, *D. folkmanovae* collected from
438 seven *Squalius* species appears to be paraphyletic, as its representatives clustered with other
439 *Dactylogyrus* from leuciscins species including *D. prostaе* and *D. vranoviensis* parasitizing
440 *Squalius*, which also suggests the existence of a *D. folkmanovae* morphotype species complex.
441 The phylogenetic position of *D. borealis* is very interesting, as this species is host specific only
442 of representatives of the genus *Phoxinellus* in the Balkans and Central Europe. According to
443 Šimková et al. (2004) *D. borealis* is phylogenetically proximal to *Dactylogyrus amphibothrium*
444 Wagener, 1857 and *Dactylogyrus hemiamphibothrium* Ergens, 1956, both parasitizing
445 *Gymnocephalus cernuus* L. (Percidae) in the Czech Republic. However, considering only
446 *Dactylogyrus* of cyprinids (more specifically only leuciscins in our study), *D. borealis* clusters

447 together with *Dactylogyrus* spp. of *Pachychilon* Steindachner, 1882 (*Dactylogyrus* lineage 8),
448 which is endemic in the Balkans and represents the ancestral cyprinid lineage in this region.
449 The high molecular diversity among *Dactylogyrus* individuals collected from three *Telestes*
450 species (*T. karsticus*, *T. muticellus* and *T. metohiensis*) suggests the existence of three new
451 *Dactylogyrus* species (*Dactylogyrus* sp. 6, sp. 7 and sp. 8 respectively, representing
452 *Dactylogyrus* lineage 6). Extrapolating from the branch lengths and molecular similarity, we
453 can postulate that these species are of recent origin, diverging probably by cospeciation with
454 the *Telestes* genus (see phylogeny in Ketmaier et al., 2004, Perea et al., 2010, Schönhuth et al.,
455 2018). On the basis of the shape and size of sclerotized elements of the haptor and copulatory
456 organs, these three potentially new species greatly resemble *D. nanus* and *D. suecicus*,
457 belonging together with *D. rutili* to the clade which is sister to the clade including three new
458 *Dactylogyrus* species parasitizing *Telestes*. *Dactylogyrus nanus*, *D. rutili* and *D. suecicus* are
459 common parasites of *Rutilus*, the cyprinid species which is phylogenetically closely related to
460 *Telestes* (Perea et al., 2010, Schönhuth et al., 2018, and also supported by our results, see
461 below).

462 The group C, also recognized in previous phylogenetic studies (Šimková et al., 2004, Benovics
463 et al., 2018), was strongly supported in the present study. It comprises *D. alatus*, *D. sphyrna*
464 and *D. vistulae*, which all possess large haptoral anchor hooks ('sphyrna' morphotype) and miss
465 a ventral connective bar except for *D. alatus*, which has a thin 'phoxini' type ventral connective
466 bar (Pugachev et al., 2009). Šimková et al. (2004) also suggested that *Dactylogyrus similis*
467 Wagener, 1909, morphologically close to *D. sphyrna* and *D. vistulae*, is included in this group,
468 but this species was not found on endemic cyprinids of the north-eastern peri-Mediterranean
469 region. While *D. alatus* and *D. sphyrna* were collected from two *Alburnus* Rafinesque, 1820
470 and three *Rutilus* Rafinesque, 1820 species, *D. vistulae* used a wide range of host species
471 representing different genera and exhibiting a wide biogeographical distribution. The basal

472 position of *D. vistulae* individuals from *C. vardarensis* and its sister position to individuals from
473 *A. thessalicus* suggest that this species originated from the north-western Aegan
474 ichthyogeographic district and further dispersed through host switching onto geographically
475 adjacent cyprinid lineages (extrapolating from the results of cophylogenetic analyses). To
476 investigate the true origin of *D. vistulae* we suggest that the representatives from Central
477 European cyprinids (e.g. *Squalius cephalus* L. or *Chondrostoma nasus* L.), in which molecular
478 variability was also observed (Šimková et al., 2004), should be included in future studies, based
479 on population genetic markers to be developed.

480

481 **Phylogeny of Cyprinidae**

482 The phylogenetic reconstruction of the north-eastern peri-Mediterranean cyprinids obtained in
483 this study is in general agreement with the molecular phylogeny proposed by Perea et al. (2010)
484 and Schönhuth et al. (2018). The only incongruences concern the relationship between
485 *Alburnoides* and *Tropidophoxinellus* Stephanidis, 1971, sister groups in Schönhuth et al.
486 (2018). Our study supports the phylogenetic proximity of *Alburnus*, *Scardinius* and
487 *Tropidophoxinellus*, which was previously hypothesized (e.g. Brito, Briolay, Galtier, Bouvet &
488 Coelho, 1997, Briolay, Galtier, Brito & Bouvet, 1998, Zardoya & Doadrio, 1999, Perea et al.
489 2010). Interestingly, all three genera harbour *Dactylogyrus* from different evolutionary
490 lineages. While *Alburnus* spp. are parasitized by *D. alatus*, *D. minor*, *D. parvus* and *D.*
491 *rarissimus* (the last is a common species on *Rutilus* spp. and *Telestes* spp. and rare on *Pelagius*
492 spp.), *Scardinius* and *Tropidophoxinellus* harbour host-specific *Dactylogyrus* spp. (*D.*
493 *difformis*, *D. difformoides*, *D. izjumovae* and *Dactylogyrus* sp. 9). The phylogenetic
494 relationships within the *Alburnoides* clade follow the biogeographical distribution of
495 *Alburnoides* species: a clade formed by *A. ohridanus*, *A. prespensis*, *A. devolli* and *A.*
496 *fangfangae* comprises species distributed in the Albanian ichthyogeographical district (Kottelat

497 & Freyhof, 2007), and a second clade is formed by *A. strymonicus* and *A. thessalicus* from the
498 Aegan district. The position of *A. economoui* was unresolved; however, Stierandová et al.
499 (2016) and Schönhuth et al. (2018) suggested that this species is closely related to the
500 ‘Albanian’ group within the *Alburnoides* clade. A similar pattern was observed for
501 *Chondrostoma* spp., where two groups were recognized: the first comprises central Adriatic
502 species (*C. knerii* and *C. phoxinus*), and the second is formed by species from the southern
503 Balkans (*C. ohridana* and *C. vardarensis*). We could not resolve the phylogenetic position of
504 *Protochondrostoma genei*, the only representative of this monotypic genus, which is distributed
505 strictly in the Apennine Peninsula. According to Perea et al. (2010), this species should be in a
506 sister position to four other Balkan, Apennine and Iberian genera recently described from
507 *Chondrostoma* s.l. (*Achondrosotma*, *Iberochondrostoma*, *Parachondrostoma* and
508 *Pseudochondrostoma*).

509 The genus status of *Pachychilon* was highly controversial in the past and its representatives
510 shifted between various genera until this genus was established and species placed within
511 *Pachychilon* confirmed using osteological data (Soric, 1992). The genus *Pachychilon* was also
512 later supported by molecular data (Zardoya, Economidis & Doadrio, 1999). According to Levy,
513 Doadrio, and Almada (2009), this taxon represents one of the oldest lineages in the Balkans,
514 which diverged from other leuciscins approximately 43 Mya. Kottelat and Freyhof (2007)
515 suggested that *Pachychilon* contains only two species distributed in the western Balkans, and
516 that the distribution range of *Pachychilon* species is delimited by the area of the Albanian
517 ichthyogeographic district (Bianco, 1990). The phylogenetic position of the *Pachychilon* clade
518 and its high degree of endemism is likely reflected in its specific parasite fauna. In total, six
519 *Dactylogyrus* species were found on *Pachychilon* spp., each exhibiting a narrow host specificity
520 and belonging to three phylogenetic lineages (Benovics et al., 2018, present results).

521 The present study only weakly supports the monophyly of *Barbus*. The relationships within the
522 *Barbus* clade are in contrast to those found by Yang et al. (2015), being, in general, more
523 congruent with the phylogeny proposed by Gante et al. (2011). However, it is important to take
524 into account that both studies included different sets of species. The phylogenetic position of
525 *A. huegelii* appears uncertain in different studies, including this one. Yang et al. (2015)
526 suggested that *A. huegelii* occupied a sister position to the *Barbus* lineage, whilst Gante et al.
527 (2011) supported that this species is in basal position relatively to to a group comprising *Barbus*
528 and *Luciobarbus* species. In the present study, *A. huegelii* was phylogenetically closer to the
529 *Luciobarbus* clade, although its position was only moderately supported. The relationships
530 among *Barbus* and *Luciobarbus* species should be more deeply investigated, by including
531 additional *Luciobarbus* representatives as well as African and Middle Eastern *Capoeta*, in order
532 to attempt to resolve the phylogenetic uncertainty between these clades.

533

534 **Cophylogenetic host-parasite relationships**

535 In spite of their direct life cycle and narrow host specificity, previous cophylogenetic studies of
536 monogeneans and their fish hosts suggested that cospeciation is a rare event, much less common
537 than host switching and intra-host speciation (e.g. Desdevises et al., 2002, Zietara & Lumme,
538 2002, Huyse et al., 2003, Šimková et al., 2004, 2013, Mendlová et al., 2012, Messu Mandeng
539 et al., 2015).

540 It has been hypothesised that during evolutionary time monogeneans developed very
541 specialized haptors specifically to attach to (generally one) well-defined host species (Sasal,
542 Trouvé, Müller-Graf & Morand, 1999, Šimková et al., 2001, Jarkovský, Morand, Šimková &
543 Gelnar, 2004). For example, Šimková et al. (2001) found a positive correlation between the size
544 of *Dactylogyrus* anchor hooks and the size of their host species. Such highly adapted attachment
545 organs would make the switch to a different host species very difficult, and even unlikely (but

546 that may depend on the intraspecific variability of the sclerified pieces in this organ, see Kaci-
547 Chaouch et al. 2008). However, some *Dactylogyrus* species, such as *D. vistulae*, parasitize
548 phylogenetically distant hosts, from small-sized (e.g. *Alburnoides* spp. or *Phoxinellus* spp.) to
549 large-sized species (e.g. *Chondrostoma* spp., *Squalius* spp. or *Telestes* spp.), displaying only
550 minor morphological variability in their haptoral sclerites (Benovics, unpublished data). This
551 species clusters among the largest *Dactylogyrus* species (see Pugachev et al., 2009 for
552 morphology), exhibiting also large anchor hooks, which suggests that monogenean species
553 developing large attachment structures as an adaptation to large-sized hosts can host-switch to
554 smaller-size hosts.

555 According to our results, host-switching clearly appears to be the main coevolutionary event
556 inferred from the cophylogenetic reconstructions of *Dactylogyrus* and their hosts, followed by
557 cospeciation (Table 3). Host-switches likely result here from the sympatric distribution of
558 phylogenetically distant cyprinid species linked to the historical shift of the landmass and/or
559 from the more recent human induced introduction of non-native cyprinid species into the
560 Balkans and Apennines. In the present study, intra-host speciation (i.e. duplication) is suggested
561 to be a rather rare coevolutionary event. This is in contrast to previous cophylogenetic studies
562 on dactylogyrids, where intra-host duplication was the most commonly inferred coevolutionary
563 event (e.g. *Dactylogyrus* by Šimková et al., 2004, *Cichlidogyrus* and *Scutogyrus* on cichlids by
564 Mendlová et al., 2012, or *Thaparocleidus* on pangasiids by Šimková et al., 2013). This may be
565 explained by the fact that these studies included either a limited number of host species from
566 the investigated area or a high number of representatives from phylogenetically distant host
567 species where host-switching was highly improbable, in contrast to our study where highly
568 diversified groups of phylogenetically close and/or sympatric cyprinid species were included.
569 This suggest that host-switching is the primary cause of speciation in *Dactylogyrus*, followed
570 by intra-host speciation only if host-switching is not possible due to geographical isolation or

571 phylogenetic divergence (then presenting too large differences in parasites' microhabitat)
572 among fish species living in sympatry.

573 In the present study, a statistically significant overall cophylogenetic structure was inferred
574 among *Dactylogyrus* and their Cyprinidae hosts. The significant global fit computed with
575 ParaFit relies on 47 % significant individual host-parasite links. Among these individual
576 associations, the most significant were found between cyprinids of the Barbini tribe and their
577 *Dactylogyrus* spp. All these *Dactylogyrus* species are genus-specific and their phylogenetic
578 relationships followed the evolutionary history of barbels. However, this *Dactylogyrus* group
579 is potentially subjected to cospeciation, as suggested in testing different cost schemes and
580 reconstructing scenarios from phylogenetic trees topologies and the divergences of lineages.
581 Cophylogenetic analyses considering only fish in Barbini and their *Dactylogyrus* species
582 confirmed this significant cophylogenetic structure and suggested scenarios strongly implying
583 duplication events in the evolutionary history of *Dactylogyrus* from Barbini. This intimate
584 coevolutionary history between 'barbels' and their specific *Dactylogyrus* lineages could be
585 related to the fact that Barbini belong to the ancestral Cyprinidae lineage (Machordom &
586 Doadrio, 2001b, Yang et al., 2015). We can hypothesize that during this long evolutionary
587 period several *Dactylogyrus* species (i.e. *D. balkanicus*, *D. dyki*, *D. crivellius*) specialized on
588 barbels, as supported by their specific distribution on European *Barbus* and the strong
589 cophylogenetic structure between *Dactylogyrus* and Barbini in the Balkan and Apennine
590 Peninsulas (Figure 4). However, two species, *D. petenyi* and *D. prespensis* (representatives of
591 *Dactylogyrus* lineage 7 in our phylogenetic reconstruction), likely colonized their host via a
592 recent host-switching from phylogenetically distant cyprinid taxa, followed by fast speciation
593 on endemic barbels.

594 A strong cophylogenetic signal was also inferred between *D. alatus* and *D. sphyrna*, each with
595 their respective hosts. In central Europe, these two species parasitize hosts from two or more

596 cyprinid genera (Moravec, 2001), while in southern European peninsulas they use only
597 *Alburnus* spp. and *Rutilus* spp., respectively. Frequent host-switching in the evolutionary
598 history of these *Dactylogyrus* species, inferred by the event-based analyses in Jane, suggest that
599 these species originally parasitized *Alburnus* and *Rutilus*, and subsequently switched to other
600 cyprinid genera in central Europe, where the number of phylogenetically related (congeneric)
601 cyprinid species living in sympatry is lower when compared to the diversity of congeneric
602 endemic cyprinids in southern Europe (Kottelat & Freyhof, 2007).

603 The cophylogenetic history of *Pachychilon* and their *Dactylogyrus* parasites reconstructed in
604 this study is noteworthy. Despite the fact that all *Dactylogyrus* species are genus or species-
605 specific, they do not form a monophyletic group. Three of the six *Dactylogyrus* species from
606 *Pachychilon* spp. found in this study formed a clade within group B (lineage 8), and a strong
607 cophylogenetic signal was observed exclusively between these species and their representative
608 *Pachychilon* hosts. This suggests that *D. petkovici* and the common ancestor of *D. martinovici*
609 and *Dactylogyrus* sp. 10 originated from an intra-host duplication during the evolutionary
610 history of *Pachychilon*, and that *Dactylogyrus* sp. 10 with *D. martinovici* originated from
611 cospeciation during the divergence of *Pachychilon* species. Additionally, *D. rosickyi* is
612 phylogenetically close to *Dactylogyrus* species from *Barbus* spp., which suggests a more recent
613 host switch of parasites between these phylogenetically distant cyprinid taxa. *Dactylogyrus*
614 *rosickyi* was collected only from *P. pictum* in the Aoos River (north-western Greece, a tributary
615 of the Adriatic Sea), where the occurrence of *Barbus* species (*B. prespensis*) was also
616 documented, and this *Dactylogyrus* species was not present on *P. pictum* in Lake Ohrid. *D.*
617 *rosickyi* was originally described by Ergens (1970) from Lake Skadar, which is a part of the
618 ancient Dessaretas Lake system (Albrecht & Wilke, 2008). This system potentially represent
619 the area of *D. rosickyi* origin where took place the initial transfer between ancestral *Barbus*
620 lineages and *Pachychilon* spp.

621 **Figure 1. Phylogenetic tree of 139 haplotypes from 49 *Dactylogyrus* species collected in the**
622 **Balkan and Apennine Peninsulas reconstructed by Bayesian inference (BI).** The tree is
623 based on concatenated partial 18S rDNA and partial 28S rDNA sequences. Values among
624 branches indicate posterior probabilities from BI and bootstrap values from ML analyses.
625 Values below 0.80 (BI) and 50 (ML) are shown as dashes. Branch lengths represent the number
626 of substitutions per site. Letters in boxes (A-C) and numbers in the coloured areas (1-8)
627 represent specific and well supported lineages described in the Results section. Numbers of
628 genetic variants within each collapsed group are shown in brackets.

629

630 **Figure 2. Phylogenetic tree of 85 haplotypes belonging to 76 endemic cyprinid species from**
631 **the Balkan and Apennine Peninsulas, reconstructed by Bayesian inference (BI).** The tree
632 is based on 1140 bp long complete cytochrome *b* sequences and rooted using four
633 representatives of the family Cobitidae. Values among branches indicate posterior probabilities
634 from BI and bootstrap values from ML analyses. Values below 0.60 (BI) and 50 (ML) are
635 shown as dashes. Branch lengths represent the number of substitutions per site. Coloured areas
636 represent clades comprising individual genera.

637

638 **Figure 3. Tanglegram showing the associations between Cyprinidae (left) and their**
639 ***Dactylogyrus* parasites (right).** Phylogenetic trees were reconstructed by Bayesian inference
640 (Figures 1 and 2). Coloured lines represent statistically significant links computed with ParaFit
641 (green $p < 0.05$; red $p < 0.001$). Each bracket represents the haplotypes belonging to one
642 *Dactylogyrus* species. Cyprinid taxa without *Dactylogyrus* are shown in grey.

643

644 **Figure 4. One of the optimal cophylogenetic scenario between representatives of the ribe**
645 **Barbini and their specific *Dactylogyrus* species constructed with Jane 4.0 (11 cospeciations,**

646 1 duplication, 18 duplications followed by host switch, 4 losses and 0 failure to diverge). Black
647 branches represent the host phylogeny and blue branches represent the parasite phylogeny.

648

649 **Table 1. List of cyprinid species including localities of their collection and accession**
650 **numbers for complete cytochrome *b* sequences deposited in GenBank.** LocID = codes used
651 in all tables and figures for specific localities, N = number of processed fish individuals, NP =
652 number of collected *Dactylogyrus* species. Cyprinid species without *Dactylogyrus* are shown
653 by cross symbol (†). New sequences obtained in this study are shown by asterisks (*). Dashes
654 represent sequences not used in the analyses.

655

656 **Table 2. List of all collected *Dactylogyrus* species and their cyprinid hosts.** Codes
657 representing collection localities and GenBank accession numbers are included. New sequences
658 obtained in this study are shown by asterisks (*).

659

660 **Table 3. Outputs of cophylogenetic analyses calculated using 11 models with different cost**
661 **schemes.** Total costs represent the sum of inferred numbers of each evolutionary event
662 multiplied by their respective costs. P-values were computed using 500 random reconstructions.

663

664 **Table 4. Outputs of cophylogenetic analyses calculated using 11 models with different cost**
665 **schemes applied to subset of cyprinids from the tribe Barbini and their respective**
666 ***Dactylogyrus* species.** Total costs represent the sum of inferred numbers of each evolutionary
667 event multiplied by their respective costs. P-values were computed using 500 random
668 reconstructions, n.s. – no significant scenario.

669

670

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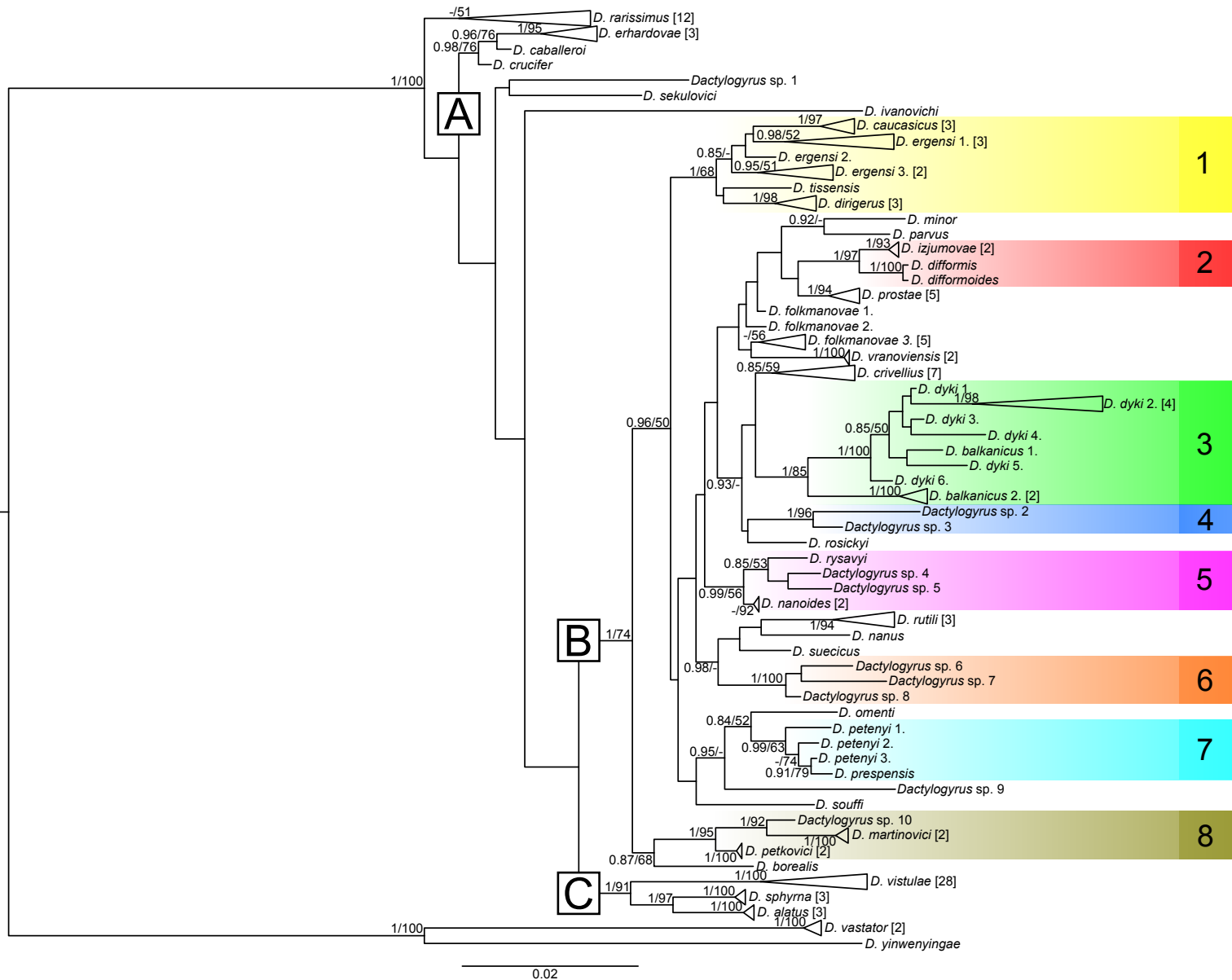
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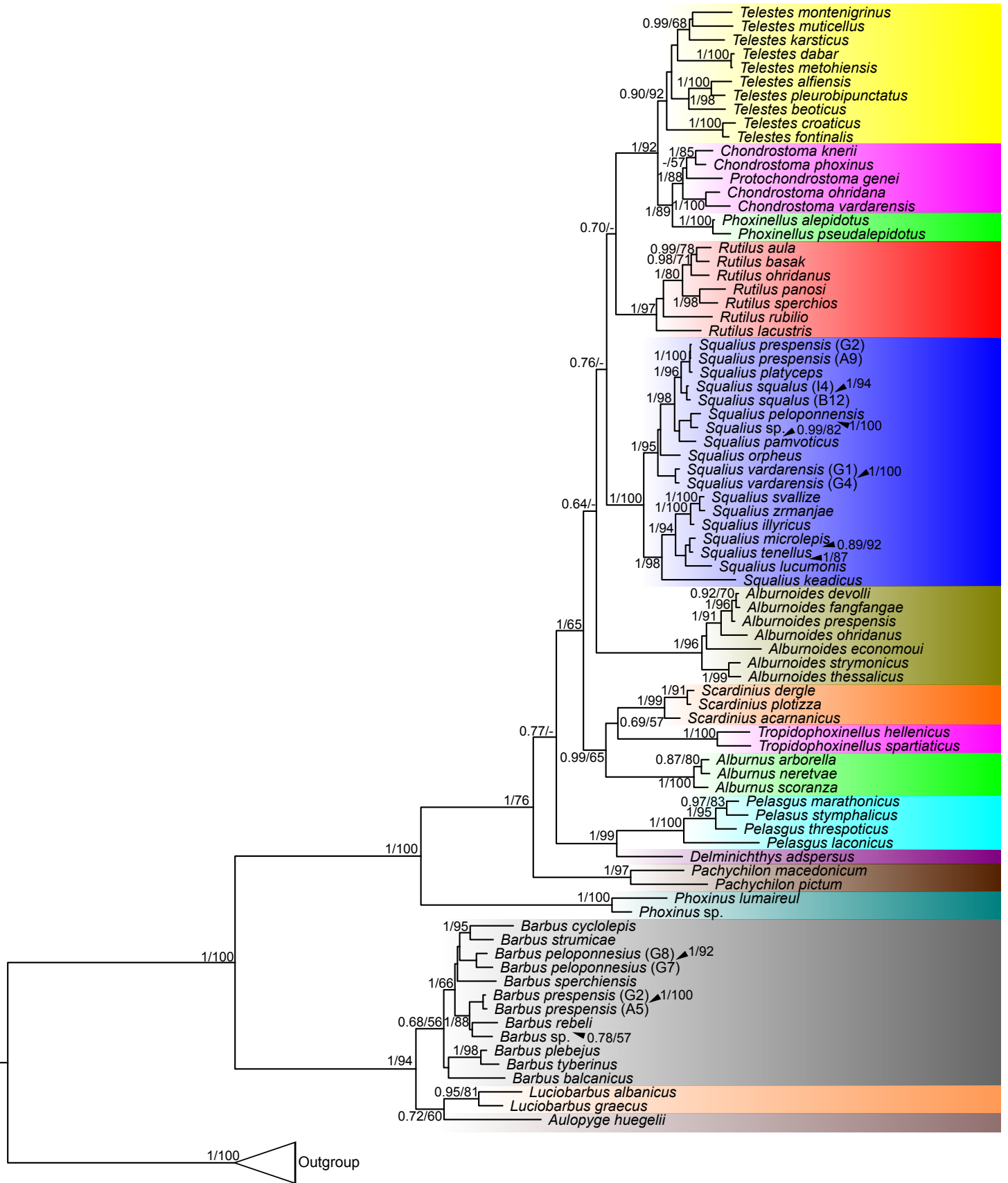
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○ Duplication

⤵ Duplication and host-switch

⋮ Loss

