Parasitology

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

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Research Article

Cite this article: Benovics M, Desdevises Y, Šanda R, Vukić J, Scheifler M, Doadrio I, Sousa-Santos C, Šimková A (2020). High diversity of fish ectoparasitic monogeneans (*Dactylogyrus*) in the Iberian Peninsula: a case of adaptive radiation? *Parasitology* **147**, 418–430. https://doi.org/10.1017/S0031182020000050

Received: 14 November 2019
Revised: 27 December 2019
Accepted: 7 January 2020
First published online: 22 January 2020

Key words:
Cyprinoidea; host-specificity; Iberian Peninsula; Monogenea; phylogeny; species delimitation

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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* parasitizing endemic *Luciobarbus* spp. only, and the second including all *Dactylogyrus* species of endemic leuciscids and four species of endemic cyprinids. Species delimitation analysis suggests a remarkable diversity and existence of a multitude of cryptic *Dactylogyrus* species parasitizing 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 colonized the Iberian Peninsula through multiple dispersion events.

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 and 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 ranges of different species rarely overlap (Doadrio, 1988; Gante *et al.*, 2015), suggesting that speciation is closely linked with the formation of river basins (Zardoya and Doadrio, 1998; Machordom and Doadrio, 2001; Doadrio *et al.*, 2002; Mesquita *et al.*, 2007; Casal-López *et al.*, 2017; Sousa-Santos *et al.*, 2019).

In contrast to the thorough previous and on-going research on Iberian cyprinoids, data on their helminth parasites are scarce (da Costa Eiras, 2016). In previous studies focused on freshwater fishes in different regions of the northern hemisphere (e.g. Mexico and the Balkans), it has been suggested that the biogeography of fish helminth parasites reflects the historical dispersion and current distribution of their hosts (e.g. Choudhury and Dick, 2001; Pérez-Ponce de León and Choudhury, 2005; Benovics *et al.*, 2018). However, very few studies have been carried out on cyprinoid monogeneans in the Iberian Peninsula, by far the most thorough being those of El Gharbi *et al.* (1992) and Šimková *et al.* (2017). The former study, describing seven species of *Dactylogyrus* from six cyprinid species (relying on morphological data only) suggested that the pattern of the geographical distribution of *Dactylogyrus* spp. follows the distribution of their cyprinid hosts, for which they are highly host-specific. The study by Šimková *et al.* (2017) focused on phylogenetic relationships between endemic *Dactylogyrus* from cyprinids in Iberia and *Dactylogyrus* from Central Europe and north-west Africa. The authors suggested multiple origins of endemic *Dactylogyrus* in the Iberian Peninsula as the presence of *Dactylogyrus* lineages in different *Luciobarbus* lineages was associated with specific dispersion events.

Gill monogeneans belonging to *Dactylogyrus* are currently the most species-diversified group within the Platyhelminthes [more than 900 nominal *Dactylogyrus* species, mostly described from morphology, are presently known according to the latest review by Gibson *et al.* (1996)]. *Dactylogyrus* species are strictly specific to cyprinoids and many *Dactylogyrus* species are specific to a single host species (Šimková *et al.*, 2006*b*). 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 specialized towards 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 sclerotized structures only. It has been suggested, therefore, that the shape of the sclerotized 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.*, 2006*b*; 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 hypothesized to be a mechanism to avoid hybridization (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 range, such as *Rutilus rutilus* and *Squalius cephalus*, harbour up to nine *Dactylogyrus* species (e.g. Šimková *et al.*, 2000; Seifertová *et al.*, 2008). In contrast, a maximum of five *Dactylogyrus* species per cyprinoid species have been reported from the southern European Peninsulas (Dupont and Lambert, 1986; El Gharbi *et al.*, 1992; Galli *et al.*, 2002, 2007; Benovics *et al.*, 2018).

In comparison to other European regions, cyprinoid monogenean communities have been underexplored in the Iberian Peninsula. Thus, the main objective of the present study was to investigate the diversity of *Dactylogyrus* spp. parasitizing endemic cyprinoids in this geographical region. A species delimitation method was applied to assess the species status of *Dactylogyrus* identified in this study based on genetic variability within and among each species, and to compare these results to species defined from morphology only. Moreover, the present study investigates the evolutionary history and phylogenetic relationships between endemic Iberian *Dactylogyrus* and *Dactylogyrus* from other Peri-Mediterranean regions, including cyprinoid species with a wide European distribution range, in order to (1) shed new light on cyprinoid phylogeography, (2) infer potential historical contacts between cyprinoids from different regions, and (3) evaluate the evolution of *Dactylogyrus* species diversity (using both morphology and species delimitation methods).

Material and methods

Parasite collection

Fish were collected over the years 2016 and 2017 from 17 localities in Portugal and Spain (Fig. 1). In total, 257 specimens representing 19 fish species were examined for the presence of *Dactylogyrus* parasites (Table 1). Fish were dissected following the standard protocol described by Ergens and Lom (1970). *Dactylogyrus* specimens were collected from the gills, mounted on slides and fixed in a mixture of glycerine and ammonium picrate (Malmberg, 1957) for further identification. Determination to species level was performed on the basis of the size and shape of the sclerotized parts of the attachment apparatus (anchor hooks, marginal hooks and connective bars of the haptor) and the reproductive organs (male copulatory organ and vaginal armament) following Pugachev *et al.* (2009). At least five specimens of each *Dactylogyrus* species from each host species examined were bisected using fine needles. One-half of the body (either the anterior part containing the reproductive organs or the posterior part with the attachment organ) was mounted on a slide and used for morphological identification. The other half was individually preserved in pure ethanol for subsequent DNA extraction.

DNA extraction, PCR and sequencing

DNA extraction was performed using the DNeasy Blood & Tissue Kit (Quiagen, Hilden, Germany) based on the standard protocol provided by the manufacturer. Two DNA regions were amplified. The partial gene coding 18S rRNA and complete ITS1 region was amplified using the primers S1 (forward, 5′-ATTCCGATAACGAACGAGACT-3′) and Lig5.8R (reverse, 5′-GATACTCGAGCCGAGTGATCC-3′) (Šimková *et al.*, 2003; Blasco-Costa *et al.*, 2012). Each amplification reaction was performed in a final volume of 20 μL, the reaction mixture comprising 1.5 U Taq polymerase (Fermentas), 1× buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.1 mg mL^{-1} BSA, 0.5 μM of each primer and 2

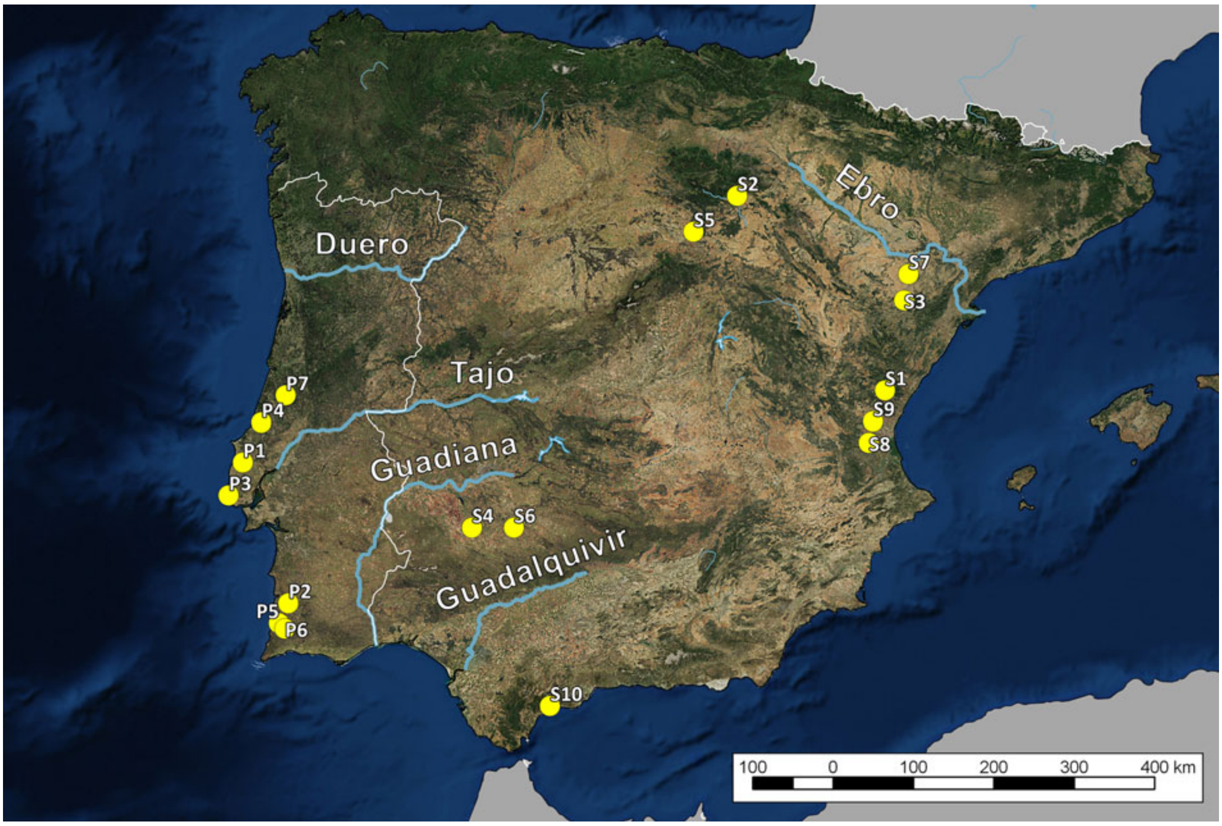

Fig. 1. Map of collection localities in the Iberian Peninsula. Collection localities are marked as yellow circles. The greatest Iberian rivers are highlighted in blue. The same codes for localities are used in Table 1 as locality IDs.

μL of pure DNA (20 ng μL^{-1}). PCR was carried out using the following steps: 3 min initial denaturation at 95 °C, followed by 40 cycles of 40 s at 94 °C, 30 s at 52 °C and 45 s at 72 °C, and 4 min of final elongation at 72°C. The second marker, a part of the gene coding 28S rRNA, was amplified using the primers C1 (forward, 5′-ACCCGCTGAATTTAAGCA-3′) and D2 (reverse, 5′-TGGTCCGTGTTTCAAGAC-3′) (Hassouna *et al.*, 1984), following the PCR protocol described in Šimková *et al.* (2006*a*). The PCR products were purified prior to sequencing using the ExoSAP-IT kit (Ecoli, Bratislava, Slovakia), following the standard protocol, and directly sequenced using the PCR primers and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing was carried out on an ABI 3130 Genetic Analyzer (Applied Biosystems). The newly generated sequences were deposited in GenBank (see Table 1 for accession numbers).

Phylogenetic and species delimitation analysis

Partial sequences coding 18S rRNA and 28S rRNA, and complete sequences of the ITS1 region were concatenated and aligned using the fast Fourier transform algorithm implemented in MAFFT (Katoh *et al.*, 2002) using the G-INS-i refinement method. Out of 71 DNA sequences used in the alignment, 35 were newly sequenced in this study. Sequences from 35 other *Dactylogyrus* species, used as representative species from different European regions, and sequences of *Ancyrocephalus percae*, used as an outgroup [phylogenetically closely related to *Dactylogyrus* according to Mendoza-Palmero *et al.* (2015)], were obtained from GenBank (see online Supplementary Table S1 for accession numbers). Gaps, hypervariable regions and ambiguously aligned regions were removed from the alignment using GBlocks v. 0.91 (Talavera and Castresana, 2007). The optimal DNA evolutionary model was selected separately for each part of the alignment corresponding to one of the three markers analysed (18S, ITS1, 28S) using the Bayesian information criterion in jModelTest v. 2.1.10 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012).

Maximum likelihood (ML) analysis was conducted in RAxML v. 8.2.11 (Stamatakis, 2006, 2014), applying the general time-reversible model (GTR; Lanave *et al.*, 1984) of nucleotide substitution. Internal node support was assessed by running 1000 bootstrap pseudoreplicates. Bayesian inference (BI) analysis was performed in MrBayes v. 3.2.6 (Ronquist *et al.*, 2012) using two parallel runs, each with four Markov chains (one cold and three heated) of 10^7 generations with trees sampled every 10^2 generations. The first 30% of trees were discarded as initial burn-in. Convergence was indicated by an average standard deviation of split 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 pairwise 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 the 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 a 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 the Poisson process (one describing speciation and

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

N = number of processed fish individuals from the respective locality, ID = code corresponding with localities marked in Fig. 1 and codes in following tables, numbers in columns 18S and 28S correspond to sequence accession numbers for the respective genetic markers in GenBank; 18S = sequences of partial gene coding 18S rRNA combined with complete ITS1 region, 28S = sequences or partial gene coding 28S rRNA. Sequence not used in the present study is marked by asterisk (*) Dashes represent localities where no *Dactylogyrus* parasites were collected and/or missing sequences.

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.

Results

Twenty-two *Dactylogyrus* species (identified using morphological characters, i.e. sclerotized 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. (five species on *L. guiraonis*, four species on *L. graellsii* and four species on *L. sclateri*). Both *Parachondrostoma* species, *Barbus haasi*, *Iberochondrostoma almacai* and *Phoxinus bigerri* were parasitized by a single *Dactylogyrus* species. Overall, *Dactylogyrus bocageii* exhibited the widest host range across the Iberian Peninsula, parasitizing four *Luciobarbus* species. Minor genetic variation was observed between *D. bocageii* collected from different hosts (p-distance $\leqslant$ 0.002 in the partial gene for 28S rRNA, p-distance $\leqslant$ 0.020 in the ITS1 region; Tables 2 and 3).

The final concatenated alignment of partial genes for 18S rRNA, 28S rRNA and the ITS1 region included 71 sequences and contained 1533 unambiguous nucleotide positions. The most suitable evolutionary models were TrNef + I + G, TPM2uf + G and GTR + I + G for the partial genes coding 18S rRNA, the ITS1 region and part of the gene for 28S rRNA, respectively. Both ML and BI analyses produced trees with congruent topologies varying only in some support values for individual nodes (Fig. 2). Phylogenetic analysis divided all taxa into three strongly supported clades.

The first group (Clade A; Fig. 2) included the majority of *Dactylogyrus* species from Europe, and especially the species parasitizing Leuciscidae. In addition, several *Dactylogyrus* species from *Barbus* and *Luciobarbus* (Cyprinidae) were also placed in this clade (i.e. *Dactylogyrus* of *Barbus* spp. and *Luciobarbus* spp. from Central Europe and the Balkans, and *D. balistae*, *D. legionensis*, *D. linstowoïdes* and *D. andalousiensis* of Iberian *Luciobarbus* spp.). *Dactylogyrus* from Iberian cyprinoids were divided into seven lineages within Clade A. *Dactylogyrus polylepidis* of *Achondrostoma arcasii* was in a well-supported sister position to the morphologically similar *D. vistulae*. *Dactylogyrus* from European cyprinids formed three well-supported groups within Clade A. *Dactylogyrus legionensis*, *D. balistae* and *D. linstowoïdes* were grouped in a sister position to common *Dactylogyrus* species from Central European *Barbus* spp. (*D. malleus*, *D. prespensis* and *D. petenyi*). The second group contained *D. andalousiensis* from two Iberian *Luciobarbus* species, and *D. omenti* from *Aulopyge huegelii* (Balkan endemic species). The third group contained *D. carpathicus* and *D. crivellius* (two common species of *Barbus* spp.) and two yet undescribed endemic *Dactylogyrus* species of endemic Balkan *Luciobarbus* species (*L. albanicus* and *L. graecus*). The phylogenetic position of *Dactylogyrus* sp. 1 from *S. aradensis* and *S. torgalensis* (morphologically identical but genetically slightly different; p-distance = 0.010) was not fully resolved and its sister position to *D. folkmanovae* was only supported by BI. The majority of Iberian *Dactylogyrus* species (*Dactylogyrus* sp. 2 to *Dactylogyrus* sp. 10) formed a well-defined phylogenetic lineage that also included *D. caucasicus*, *D. ergensi* and *D. tissensis*. The three latter species and the Iberian *Dactylogyrus* in this lineage all have the same or very similarly shaped male copulatory organs commonly

Table 2. Uncorrected pairwise genetic distances between individuals from clade B (Fig. 2) collected from different *Barbus* and *Luciobarbus* species in the Iberian Peninsula

No.	*Dactylogyrus* species	Host species	ID	1	2	3	4	5	6	7	8	9	10	11
1	*D. bocageii*	*L. bocagei*	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. lenkoranoides*	*B. haasi*	S3	0.016	0.016	0.017	0.015	0.017	X					
7	*D. lenkoranoides*	*L. graellsii*	S3	0.016	0.016	0.017	0.015	0.017	–	X				
8	*D. lenkoranoides*	*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

Distances are computed from the alignment of partial genes coding 28S rRNA. Identical sequences are marked by dashes (–). ID = code corresponding with localities marked in Fig. 1 and specified in Table 1.

Table 3. Uncorrected pairwise genetic distances between individuals from clade B (Fig. 2) collected from different *Barbus* and *Luciobarbus* species in the Iberian Peninsula

No.	*Dactylogyrus* species	Host species	ID	1	2	3	4	5	6	7	8	9	10	11
1	*D. bocageii*	*L. bocagei*	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. lenkoranoides*	*B. haasi*	S3	0.095	0.096	0.085	0.088	0.094	X					
7	*D. lenkoranoides*	*L. graellsii*	S3	0.095	0.096	0.085	0.088	0.094	-	X				
8	*D. lenkoranoides*	*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

Distances are computed from complete sequences of ITS1 region. Identical sequences are marked by dashes (-). ID = code corresponding with localities marked in Fig. 1 and specified in Table 1.

classified as 'ergensi' of the 'chondrostomi' type (see Pugachev *et al.*, 2009). Generalist *Dactylogyrus* species within Clade A (i.e. *D. legionensis*, *D. polylepidis*, *Dactylogyrus* sp. 1, *Dactylogyrus* sp. 7 and *Dactylogyrus* sp. 8) exhibited intraspecific genetic variability. The second major group (Clade B) comprised five *Dactylogyrus* species specific to Iberian *Luciobarbus*. Where intraspecific genetic variability was documented, all genetic variants formed well-supported clades (i.e. *D. bocageii*, *D. guadianensis* and *D. lenkoranoïdes*). The last strongly supported group (Clade C) encompassed *Dactylogyrus* species host specific to *Carassius* spp. and/or *Cyprinus carpio* distributed across the Europe and Asia.

In general, no pattern was observed in phylogenetic relatedness of individual *Dactylogyrus* species reflecting their geographic distribution. However, the phylogenetic relationships between genetic variants of single *Dactylogyrus* species (e.g. three genetic variants for *D. legionensis*, or *Dactylogyrus* sp. 7) were in congruence with the geographic distribution of their respective hosts (i.e. two genetic variants collected from hosts belonging to different species, but collected from geographically proximal localities, or the same river basin, were phylogenetically closer to each other, rather than to other genetic variants of the same *Dactylogyrus* species).

Genetic distances were computed between morphologically similar species from Clade B (Fig. 2). Three alignments of 12 sequences representing five *Dactylogyrus* species of group B were analysed to compare intra- and interspecific genetic variability calculated using genetic markers commonly used in monogeneans. The alignments comprised 486 nucleotide positions for the partial gene coding 18S rRNA combined, 716 nucleotide positions for the ITS1 segment and 807 nucleotide positions for the partial gene coding 28S rRNA. The lowest 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 pairwise nucleotide diversity varied from 0.002 to 0.010 (Table 4). Low pairwise interspecific diversity was also observed for the partial gene coding 28S rRNA (0.006–0.020); however, minor intraspecific genetic variability was observed in this gene (p-distance $\leqslant$0.002). Slight genetic distance in part of the gene for 28S rRNA was observed between different populations of *D. bocageii* (0.001–0.002) 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 pairwise 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 parasitizing 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 (Fig. 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, parasitizing 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

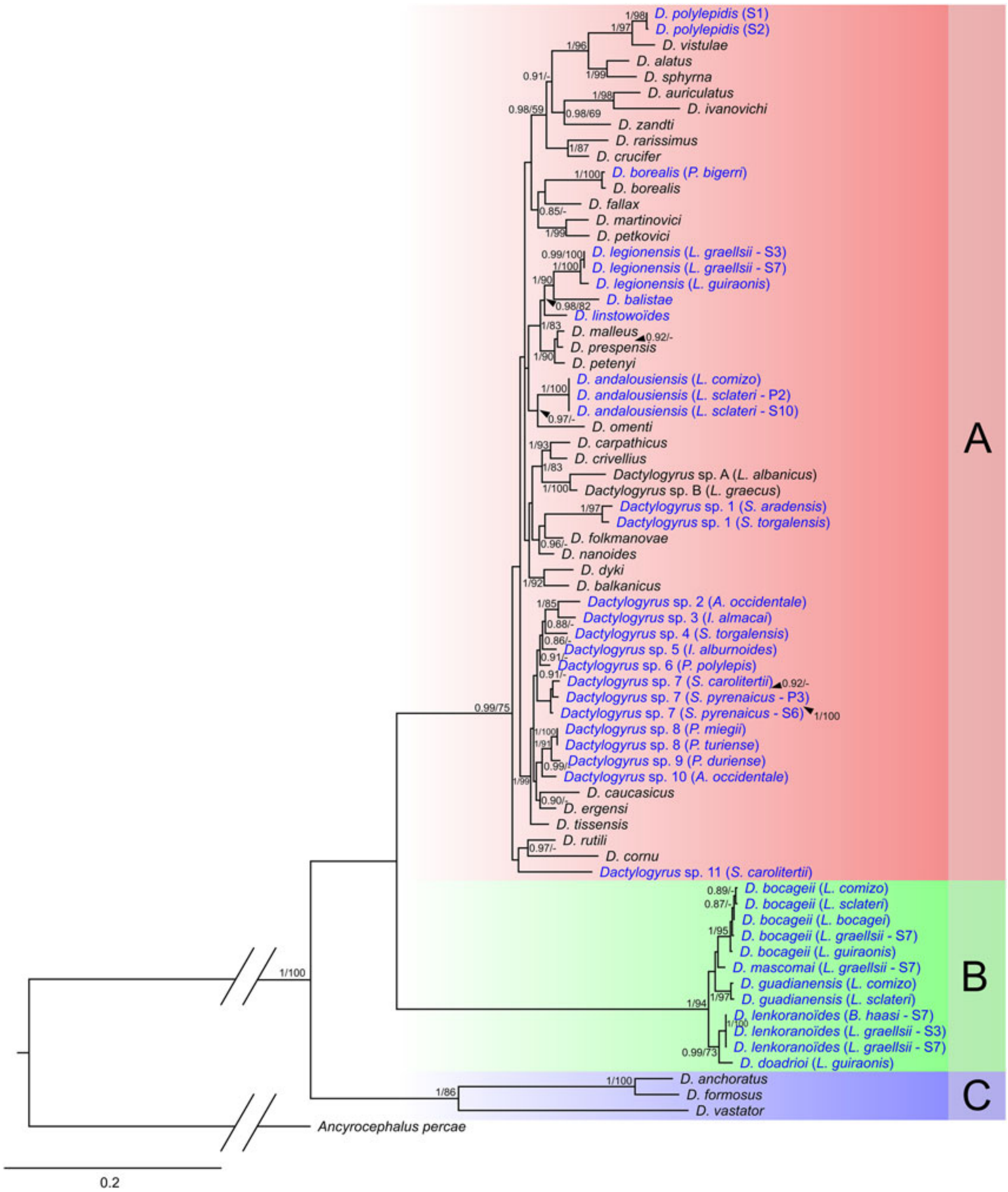

Fig. 2. Phylogenetic tree of 70 *Dactylogyrus* haplotypes reconstructed by Bayesian inference (BI). The tree is based on combined parts of genes coding 18S and 28S rRNA, and the complete ITS1 region. Values between branches indicate posterior probabilities from BI and bootstrap values from ML analysis. Values below 0.80 (BI) and 50 (ML) are shown as dashes (–). The letters A–C represent specific well-supported lineages, as described in the Results section.

P. bigerri in the Iberian Peninsula. bPTP analysis also suggested that *Parachondrostoma miegi* and *P. turiense* are both parasitized by a single *Dactylogyrus* species (*Dactylogyrus* sp. 8) that is morphologically similar and phylogenetically close to *Dactylogyrus* sp. 9, parasitizing *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.

Discussion

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

Table 4. Uncorrected pairwise genetic distances between individuals from clade B (Fig. 2) collected from different *Barbus* and *Luciobarbus* species in the Iberian Peninsula

No.	*Dactylogyrus* species	Host species	ID	1	2	3	4	5	6	7	8	9	10	11
1	*D. bocagei*	*L. bocagei*	P3	x										
2	*D. bocagei*	*L. comizo*	S6	–	x									
3	*D. bocagei*	*L. graellsii*	S7	–	–	x								
4	*D. bocagei*	*L. guiraonis*	S8	–	–	–	x							
5	*D. bocagei*	*L. sclateri*	P2	–	–	–	–	x						
6	*D. lenkoranoides*	*B. haasi*	S3	0.008	0.008	0.008	0.008	0.008	x					
7	*D. lenkoranoides*	*L. graellsii*	S3	0.008	0.008	0.008	0.008	0.008	–	x				
8	*D. lenkoranoides*	*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

Distances are computed from the alignment of partial genes coding 18S rRNA. Identical sequences are marked by dashes (-). ID = code corresponding with localities marked in Fig. 1 and specified in Table 1.

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 range (e.g. *R. rutilus*, *S. cephalus*). The same pattern was also observed in the Iberian Peninsula, where one to five *Dactylogyrus* species were found on a single cyprinoid host species. It should be noted, however, that parasite community composition may be strongly influenced by seasonal abiotic factors (e.g. González-Lanza and Alvarez-Pellitero, 1982; Lux, 1990; Appleby and Mo, 1997; Šimková *et al.*, 2001*b*; Poulin and Morand, 2004; Zhang *et al.*, 2015; Sinaré *et al.*, 2016). Until now, knowledge of *Dactylogyrus* diversity in southern European Mediterranean Peninsulas has been based on studies taking place in summer only (Benovics *et al.*, 2018, this study) as the *Dactylogyrus* diversity is expected to be highest during this period (Šimková *et al.*, 2001*b*).

In this study, a higher number of *Dactylogyrus* species was observed on *Luciobarbus* species. While the overall species richness on these fish was in accordance with the observations of El Gharbi *et al.* (1992), the species composition in the present study differed slightly from their data. In line with the study of El Gharbi *et al.* (1992), *D. bocageii* was the most common species (occurring on five *Luciobarbus* species), though its distribution range was wider, as proposed by Lambert and El Gharbi (1995), stretching *via* Zujar and Torgal rivers to the south-western part of the peninsula (south-west Iberian province; Filipe *et al.*, 2009). Interestingly, unlike other European regions, the only endemic representative of the genus *Barbus* in Iberia, *B. haasi*, harbours *Dactylogyrus* species typical of *Luciobarbus* spp. In the Balkans, endemic *Barbus* spp. are parasitized by common *Dactylogyrus* species for this fish genus (e.g. *D. dyki* and *D. crivellius*), while *Luciobarbus* spp. are parasitized by different, strictly host-specific species (Benovics *et al.*, 2017, 2018). In accordance with our own findings, El Gharbi *et al.* (1992) showed that *B. haasi* is a common host of *D. bocageii*, *D. mascomai* and *D. lenkoranoïdes*, while *D. dyki* and *D. carpathicus* (commonly distributed on European *Barbus* spp.) were only found in previous studies on *B. haasi* × *B. meridionalis* hybrids in the north-eastern part of the Peninsula. Nevertheless, Gutiérrez-Galindo and Lacasa-Millán (1999) also reported the latter two *Dactylogyrus* species from *B. haasi* in the River Llobregat (north-east Spain). However, the fish hosts from this study could potentially also be hybrids, as the presence of the *B. haasi* × *B. meridionalis* hybrids was previously documented in Llobregat basin (Machordom *et al.*, 1990). In contrast to the aforementioned studies, only *D. lenkoranoïdes* was collected from *B. haasi* in this study (Uldemo River; Ebro basin). This low parasite diversity may be linked with the seasonal fluctuation in parasite communities previously documented among Iberian *Dactylogyrus* [e.g. *D. legionensis* (González-Lanza and Alvarez-Pellitero, 1982) or *D. balistae* (Simón-Vicente, 1981)]. In addition to the common parasitization of Iberian *Barbus* by *Dactylogyrus* parasites typically recognized as specific to *Luciobarbus*, several cases of infection by *Dactylogyrus* species common for *Barbus* were also reported in Iberian *Luciobarbus* species. Gutiérrez-Galindo and Lacasa-Millán (2001) also reported that *L. graellsii* was parasitized by *D. dyki* and *D. extensus* (host-specific parasites of *Barbus* spp. and *C. carpio*, respectively). However, the presence of *D. dyki* on *Luciobarbus* spp. may result from non-detected instances of hybridization, as hybrids of cyprinoid species are usually parasitized by *Dactylogyrus* specific for each of the parental species (Šimková *et al.*, 2013; Krasnovyd *et al.*, 2017). Hybridization between Iberian *Luciobarbus* spp. (potentially also between *Luciobarbus* and *Barbus*; Gante *et al.*, 2015) appears to be quite common, especially between congeners living in sympatry (e.g. *Luciobarbus* spp.; Almodóvar *et al.*, 2008; Sousa-Santos *et al.*,

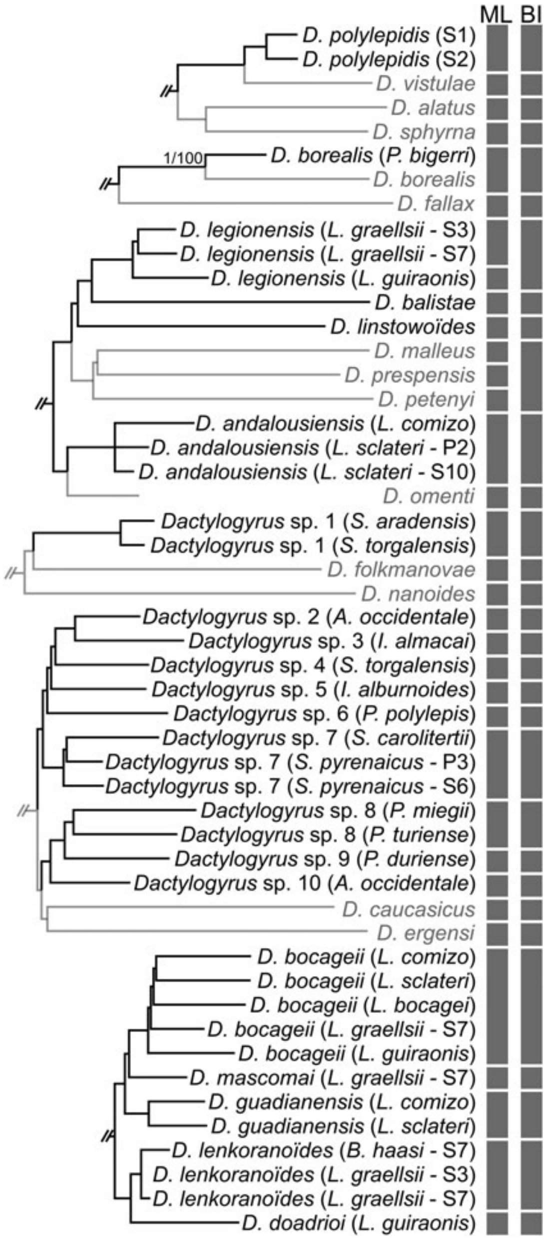

Fig. 3. Results of species bPTP delimitation analysis applied to clades comprising endemic *Dactylogyrus*. Brackets at the terminal branches indicate different species, as suggested by BI and ML analyses.

2018). Thus, host-switching is possible, most likely occurring between species from phylogenetically close genera (i.e. *Barbus* and *Luciobarbus*; Yang *et al.*, 2015) in north-eastern Iberian drainages where the distribution ranges of Central European barbels [e.g. *B. meridionalis*; see Kottelat and Freyhof (2007) for its distribution range] and Iberian barbels overlap.

Despite the presence of high numbers of endemic *Dactylogyrus* species in Iberia, *P. bigerri* was parasitized 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 *et al.*, 2000; Hewitt, 2011). Other common European *Dactylogyrus* species are absent from Iberia; for example, *D. vistulae*, which parasitizes 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, parasitizing 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 hypothesized 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.*, 2001*a*; 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 higher degree of host specificity, with most species harbouring at least one specific *Dactylogyrus* species. The majority of new species recorded are morphologically similar, with *Dactylogyrus* sp. 2 and *Dactylogyrus* sp. 10, for example, sharing the 'ergensi' type of male copulatory organ but differing in the shape and size of the haptoral hard parts. Phylogenetic analyses and species delimitation analyses supported their species identities, i.e. nine new species were recognized. Species delimitation has received much attention recently, and numerous methods have now been developed that help identify species by using molecular data in a rigorous framework alongside morphological examination (Carstens *et al.*, 2013; Zhang *et al.*, 2013; Grummer *et al.*, 2014). DNA-based delimitation methods have also been used to confirm or invalidate morphologically determined species, to identify cryptic species or highlight significant intraspecific genetic variability. The aforementioned diversity in haptoral part shape and size appears to be common in *Dactylogyrus* spp. and was previously hypothesized to be the result of adaptations to specific microhabitats (i.e. specific positions on fish gills; Šimková *et al.*, 2001*a*; Jarkovský *et al.*, 2004). Thus, minor morphological variabilities in the attachment organs may be observed in species with ongoing speciation parasitizing phylogenetically distant hosts, as is the case in the Iberian Peninsula.

Phylogeny of endemic Dactylogyrus

Phylogenetic reconstruction of *Dactylogyrus* parasitizing Iberian cyprinoids suggests that Iberian *Dactylogyrus* belong to two well-supported phylogenetic lineages (Clade A and Clade B; Fig. 2). One of these clades contains *Dactylogyrus* from endemic Cyprinidae only (representatives of five *Luciobarbus* species and *B. haasi*), while the second includes *Dactylogyrus* endemic to Iberian cyprinoids (both Cyprinidae and Leuciscidae) and *Dactylogyrus* parasitizing cyprinoids from other parts of Europe. This was previously reported by Šimková *et al.* (2017) following the analysis of phylogenetic relationships between *Dactylogyrus* from north-west Africa and those from the Iberian Peninsula, the authors suggesting multiple origins for *Dactylogyrus* from both Mediterranean areas in association with the historical biogeography of their cyprinid hosts. Clade B comprises *Dactylogyrus* species described by El Gharbi *et al.* (1992), using morphological characteristics of the haptor and reproductive organs. According to their study (also supported by our own morphometric data), all these species achieve a small body size and display remarkably similar morphological features (i.e. sclerotized parts of attachment and copulatory organs), in accordance with their phylogenetic proximity. Previously, their description was based on small differences in the shape and size of sclerotized parts only (e.g. spiralization of the male copulatory organ and the size of haptoral sclerites). However, as has been previously documented, such variability may be present within single species and is common in the different monogenean taxa (e.g. Rohde and Watson, 1985; Boeger and Kritsky, 1988; Vignon and Sasal, 2010), including *Dactylogyrus* (Rahmouni *et al.*, 2017). Nonetheless, the species status of each taxon in Clade B was supported by phylogenetic and species delimitation analyses, which was in concordance with their morphological determination. According to Šimková *et al.* (2017), Iberian *Dactylogyrus* species of this lineage are phylogenetically close to *Dactylogyrus* from north-west African *Carasobarbus fritschii*, suggesting different historical origins of *Dactylogyrus* in Clade B and Clade A. According to previous reports and the data presented here, each *Dactylogyrus* species within Clade B parasitizes several endemic *Luciobarbus* species. Considering the monophyletic origin of Iberian *Luciobarbus* (Yang *et al.*, 2015), its probable historical dispersion *via* northern Africa (Bianco, 1990; Doadrio, 1990; Zardoya and Doadrio, 1998), and the phylogenetic relatedness of *Dactylogyrus* from Clade B with north-west African *Dactylogyrus* (Šimková *et al.*, 2017), we may postulate that these species originated on the *Luciobarbus* ancestor, and may have host-switched in the past to endemic north-west African *Carasobarbus*, subsequently dispersing to the Iberian Peninsula during its historical connection with North Africa. The high number of morphologically similar species exhibiting a low molecular divergence (e.g. *D. bocageii*, *D. mascomai*, *D. guadianensis*, *D. lenkoranoïdes* and *D. doadrioi*) suggests subsequent rapid speciation, most likely linked with the radiation of *Luciobarbus* across individual river basins within the Iberian Peninsula (Doadrio, 1988; Zardoya and Doadrio, 1998; Doadrio *et al.*, 2002; Mesquita *et al.*, 2007; Gante *et al.*, 2015; Casal-López *et al.*, 2017). Addition of *Dactylogyrus* species from Asian *Capoeta* (phylogenetically sister group to Iberian *Luciobarbus*; Yang *et al.*, 2015) to phylogenetic reconstruction and assessing coevolutionary scenarios involving these parasites and their hosts may shed more light into the origin of the *Dactylogyrus* of Iberian *Luciobarbus* and finally resolve the phylogenetic relationships within this group of *Dactylogyrus*.

In contrast to *Dactylogyrus* from Clade B, the phylogenetic proximity of Iberian *Dactylogyrus* within Clade A to Central European and Balkan *Dactylogyrus* species supports their European origin. In accordance with the phylogeny proposed by Šimková *et al.* (2017), *Dactylogyrus* species from Iberian *Luciobarbus* form two well-supported lineages within Clade A, and cluster with *Dactylogyrus* from European *Barbus*. Two species within Clade A, *D. balistae* and *D. legionensis*, have a large body size, large haptoral sclerites and are missing the haptoral connective ventral bar (see El Gharbi *et al.*, 1992). These species form a well-supported clade in sister position with another Iberian species, *D. linstowoïdes*. This clade is closely related to *D. malleus*, *D. prespensis* and *D. petenyi*, all host-specific parasites to European *Barbus*. In contrast to *D. legionensis* and *D. balistae*, these three species have a small body size, similarly shaped small haptoral elements and a ventricular ventral bar (see Pugachev *et al.*, 2009). Based on the morphology, *D. linstowoïdes* represents the transient form between these two lineages, with the haptoral sclerites resembling *Dactylogyrus* of European *Barbus* and copulatory organs morphologically similar to Iberian species. Our results support a common 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* parasitizing *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* [hypothesized 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) have 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* 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 colonized 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)], parasitize leuciscids in river basins of the Atlantic slope [distribution according to Kottelat and 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 the two populations (Benovics *et al.*, 2018) supported the existence of two different *Alburnoides* species, as previously proposed by Stierandová *et al.* (2016).

In general, *Dactylogyrus* species diversity within the Iberian Peninsula appears to be associated with the historical dispersion of their cyprinoid hosts, with subsequent adaptive radiation following the peninsula's geographical isolation due to the elevation of the Pyrenees (Muñoz *et al.*, 1986; Puigdefàbregas *et al.*, 1992; Stange *et al.*, 2016). At least two historical origins can be inferred for Iberian *Dactylogyrus*, each associated with the different dispersion routes proposed for cyprinoids (Banarescu, 1989, 1992; Doadrio, 1990; Doadrio and Carmona, 2003; Perea *et al.*, 2010). Despite well-supported delineation between a multitude of endemic *Dactylogyrus* species, the phylogenetic relationships between *Dactylogyrus* species do not fully correspond to the phylogeny of their hosts, suggesting secondary contacts and host-switching between endemic Iberian cyprinoids.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020000050

Acknowledgements. We are grateful to Kateřina Čermáková, Jaroslav Červenka, Maria Lujza Červenka Kičinja and Tomáš Pakosta for their help with fish processing and parasite collection. Sampling was approved by the responsible governmental authorities in Portugal and in Spain (Andalusia, Extremadura, Aragon, Cataluña, Valencia and Castilla y Leon). Moreover, we are grateful to Ana Pereira, Jesus Hernandez and Pilar Risueño for their help with fieldwork. We kindly thank Kevin Roche for English revision of the final draft.

Financial support. This study was financially funded by the Czech Science Foundation (project number 15-1938S).

Conflict of interest. The authors declare that they have no conflict of interest.

Ethical standards. All applicable institutional, national and international guidelines for the care and use of animals were followed. This study was approved by the Animal Care and Use Committee of the Faculty of Science, Masaryk University in Brno (Czech Republic).

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